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Gene regulation of prostaglandin and steroid hormone biosynthesis in equine preovulatory follicles

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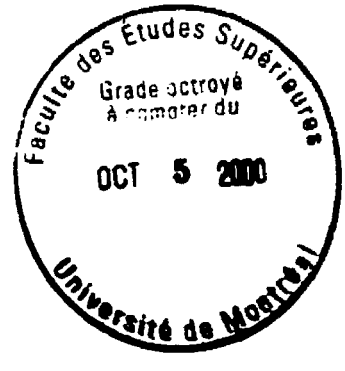

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Gene regulation of prostaglandin and steroid hormone biosynthesis in equine preovulatory follicles

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SOMMAIRE

La montée préovulatoire d'hormone luténisante est le signal physiologique qui déclenche deux processus reproducteurs cruciaux: l'ovulation et la lutéinisation.

L'ovulation a été comparée à une réaction inflammatoire. En effet, ces deux processus présentent des similarités histologiques et fonctionnelles, dont la présence de prostaglandines qui agissent comme médiateurs moléculaires. La prostaglandine G/H synthétase-2 (PGHS-2) catalyse la première étape limitante de la biosynthèse de toutes les prostanoïdes à partir de l'acide arachidonique. Le premier article présenté dans cette thèse, dont l'objectif est d'élucider le rôle de la PGHS-2 dans le processus ovulatoire équin, porte sur l'isolement du transcrit primaire à partir d'une génothèque d'ADNc folliculaire. La caractérisation de la régulation de l'ARNm de la PGHS-2 a été réalisée à l'aide d'analyses de type Northern effectuées sur des follicules préovulatoires isolés à différents moments après l'administration d'une dose ovulatoire d'hCG. Les résultats ont démontré qu'il y avait induction transitoire de l'ARNm de la PGHS-2 à partir de la 30e heure post-hCG dans les cellules de la granulosa uniquement. Cette induction tardive de la PGHS-2 équine a donc lieu environ 10 h avant l'ovulation, un délai identique à celui qui a déjà été rapporté chez la vache et la ratte, dont le processus ovulatoire diffère pourtant au regard de la durée (respectivement 39-42 h, 28 h et 12-14 h chez la jument, la vache et la ratte). Globalement, ces données suggèrent donc que l'induction de la PGHS-2 est un élément déterminant de l'horloge ovulatoire chez les mammifères.

L'élucidation des mécanismes fondamentaux qui régissent la durée du processus ovulatoire chez les mammifères nécessitera des études comparatives de la régulation transcriptionnelle ovarienne de la PGHS-2 chez différentes espèces. La durée du processus ovulatoire équin et l'important retard de l'induction de la PGHS-2 qui l'accompagne font de cette espèce un modèle approprié à l'étude de la régulation transcriptionnelle de la PGHS-2 chez les espèces à long processus ovulatoire. Le gène complet a été isolé à partir d'une génothèque et caractérisé par analyses de séquences et d'extensions d'amorces afin de mettre au point les outils nécessaires à ces études.

La lutéinisation conduit à la différenciation terminale des cellules de la paroi folliculaire en cellules lutéales et s'accompagne d'un changement de la synthèse hormonale ovarienne prédominante, qui passe de l'œstradiol-17β à la progestérone. On sait que ce phénomène exige la restructuration de la machinerie stéroïdogène, mais les mécanismes moléculaires en jeu sont encore incomplètement élucidés, particulièrement chez les grandes espèces mono-ovulatoires. Une étape limitante initiale de la régulation de la stéroïdogénèse est le transport des molécules de cholestérol du cytoplasme à la membrane mitochondrienne interne. Bien qu'il ait été démontré qu'il dépend de l'action de la "steroidogenic acute regulatory protein" (StAR), la nature exacte du rôle de cette dernière demeure nébuleuse. Un article annexé à cette thèse (Annexe I) porte sur l'isolement à partir d'une génothèque d'ADNc folliculaire de deux transcrits de la StAR, qui ne diffèrent que par la longueur de leur régions 3' non-traduites. Il a ainsi été possible d'étudier la régulation de la StAR équine dans des follicules préovulatoires isolés à différents moments après l'administration

d'une dose ovulatoire de hCG. Les analyses de type Northern effectuées sur des échantillons de paroi folliculaire intacte (thèque interne et granulosa) n'ont révélé aucun changement significatif dans les niveaux d'ARNm de la StAR. Par contre, les analyses effectuées sur des cellules isolées de la granulosa ont permis de démontrer l'existence d'une induction de la StAR 30 h post-hCG associée à une augmentation des concentrations intrafolliculaires de progestérone. Contrairement aux cellules de la granulosa, les cellules de la thèque interne présentaient des niveaux élévés d'ARNm de la StAR jusqu'à 33 h post-hCG, niveaux qui chutaient par la suite. Le résultat net de ces processus est une redirection graduelle de l'expression de la StAR de la thèque interne vers les cellules de la granulosa. Ce phénomène serait lié à la supposée dégénérescence de la thèque interne durant le processus ovulatoire chez la jument, chez qui le corps jaune (CL) serait formé uniquement de cellules dérivées de la granulosa.

Le cholestérol peut être converti en progestérone par l'action successive du cytochrome P450 "cholesterol side-chain cleavage" (P450scc) et de la 3β-hydroxystéroïdo-déshydrogénase/Δ5-Δ4 isomérase (3β-HSD). Le deuxième article fait état du clonage des transcrits de la P450scc et de la 3β-HSD équines réalisé dans le but de vérifier les effets de l'hormone hCG sur les niveaux d'ARNm des enzymes de la synthèse de la progestérone dans les follicules préovulatoires. Des follicules préovulatoires isolés à différents moments après l'administration d'hCG ont été soumis à des analyses de type Northern. Dans les préparations de cellules isolées, la thèque interne s'est avérée le site prédominant d'expression de la P450scc dans les follicules avant l'administration d'hCG. Par contre, le niveau de trancrits a diminué dans la

thèque interne entre 33 et 36 h et a augmenté dans les cellules de la granulosa à 39 h, de façon à rendre ces dernières le siège principal d'expression de la P450scc à la fin du processus ovulatoire. Les niveaux d'expression les plus élevés ont été détectés dans le CL. Cette redirection de l'expression de la P450scc à l'intérieur de la paroi folliculaire (de la thèque vers la granulosa) est similaire à ce qui a déjà été observé pour la StAR et pourrait donc aussi être liée à une possible dégénérescence de la thèque interne. Contrairement aux autres espèces, chez les équins l'expression de la 3β-HSD s'est avérée limitée aux cellules de la granulosa et les niveaux de transcrits sont demeurés stables durant le processus ovulatoire. L'augmentation de la biosynthèse de la progestérone induite par la LH serait donc liée à la redirection de la StAR et de la P450scc de la thèque interne en dégénérescence aux cellules lutéinisantes de la granulosa.

La progestérone peut être convertie en œstrogènes par l'action successive du cytochrome P450 17α-hydroxylase (P45017α) et du cytochrome P450 aromatase (P450arom). Le troisième article présenté dans le cadre de cette thèse décrit l'isolement à partir d'une génothèque d'ADNc de transcrits équins de la P45017α et de la P450arom pour étudier la régulation des enzymes équines de la synthèse des œstrogènes durant le processus ovulatoire. Le clonage moléculaire de la P450arom a produit deux ADNc distincts, l'un dérivé du promoteur 1f et codant pour une protéine de 503 acides aminé, l'autre dérivé du promoteur II et codant pour une protéine tronquée. Des follicules préovulatoires isolés à différents moments après l'administration de hCG ont été soumis à des analyses de type Northern. La régulation de l'ARNm de la P450arom s'est avérée biphasique: les niveaux les plus élevés se retrouvaient dans les cellules de la

granulosa à 0 h post-hCG, diminuaient de façon significative durant le processus ovulatoire à 12 et 24 h, pour ensuite augmenter à nouveau 30 à 39 post-hCG et se maintenir dans le CL. Des sondes spécifiques employées pour détecter les variantes d'ARNm de la P450arom ont révélé que l'utilisation du promoteur II se traduisait par la production des transcrits dans le follicules à 0 h post-hCG et dans le CL, tandis que le promoteur 1f était utilisé exclusivement durant le processus ovulatoire. Ceci représente un nouveau phénomène d'échange de promoteurs d'aromatase, un processus qui a aussi lieu notamment dans la cancérogénèse du sein et qui est responsable d'une surproduction d'œstrogène par les tissus cancéreux. Le follicule préovulatoire équin constitue donc un nouveau système modèle applicable à l'étude des mécanismes régissant l'échange de promoteurs d'aromatase. Les niveaux d'ARNm de la P45017α étaient élevés dans la thèque interne à 0 h, puis diminuaient de façon significative dans les follicules à 36 h et 39 h post-hCG et dans le CL, un phénomène vraisemblablement lié à la possible dégénérescence de la thèque interne. L'ensemble de ces données fournit des indices importantes sur la nature des mécanismes en jeu dans la chute de la biosynthèse des œstrogènes par l'hormone hCG dans les follicules préovulatoires au cours du processus de lutéinisation. Afin de produire les outils requis pour approfondir les études sur la régulation de la P450arom et de l'échange des promoteurs, les promoteurs II et 1f ont été clonés à partir d'une génothèque. Les sites de démarrage de la transcription ont alors été identifiés par l'analyse d'extensions d'amorces. L'analyse de séquences permit de révéler certains éléments régulateurs potentiels.

Le récepteur nucléaire orphelin "steroidogenic factor-1" (SF-1) a la capacité de se lier à des élément régulateurs présents dans les promoteurs de divers hydroxylases stéroïdiennes et cette propriété est à l'origine de son isolement. Depuis, il a été démontré que le SF-1 assurait la régulation transcriptionnelle de tous les gènes stéroïdogènes étudiés dans les articles 2 et 3 et dans l'Annexe I. Cette uniformité contraste avec la variabilité des processus régulateurs de l'ARNm observé chez ces gènes et chez les différentes populations cellulaires. Dans le quatrième article, il est question du clonage du SF-1 équin et de la caractérisation de la régulation de son transcrit dans la thèque interne et les cellules de la granulosa après l'administration de l'hCG. Les liens éventuels entre la régulation des ARNm de la SF-1 et celle des différent gènes stéroïdogènes y sont également évoqués. Le clonage moléculaire du SF-1 a aussi permis l'isolement fortuit d'un ADNc codant pour le NR5A2, un récepteur nucléaire orphelin proche parent, dont l'isolement à partir des tissus gonadiques n'avait jamais été rapporté. Les transcrits de SF-1 et de NR5A2 ont été isolés en fragments à l'aide d'une variété de techniques de clonage. L'analyse des séquences a démontré qu'ils codent pour des protéines à plus de 60% similaires dont les domaines de liaison à l'ADN sont presqu'identiques, ce qui s'accorde avec les spécificités de liaison à l'ADN identiques du SF-1 et du NR5A2 humains. Des analyses de types Northern et RT-PCR effectués sur différents tissus ont démontré un lien entre l'expression du SF-1 et des gènes stéroïdogènes dans la plupart des cas. Une exception: le CL, où les niveaux d'ARNm de SF-1 ont chuté de façon marquée par rapport aux niveaux retrouvés dans les follicules préovulatoires et ce malgré une hausse des niveaux

d'expression de la StAR et de la P450scc. La présence d'une forte expression de la 3β-HSD et de la P450arom dans les cellules de la granulosa a d'abord semblé irréconciliable avec les bas niveaux d'ARNm de SF-1. Cependant, la forte expression du NR5A2 dans ces tissus, qui surpasse de loin celle du SF-1, pourrait indiquer que le NR5A2 joue un rôle dans la régulation des gènes stéroïdogènes dans les cellules de la granulosa et le CL. Des analyses de type RT-PCR/Southern d'ARN folliculaire isolé à différents moments après l'administration d'hCG ont permis d'étudier la régulation des ARNm du SF-1 et du NR5A2 durant le processus ovulatoire. Comme c'est le cas chez la ratte en ce qui a trait au SF-1, des diminutions des niveaux d'ARNm de SF-1 et de NR5A2 jusqu'au tiers de leurs valeurs initiales ont été atteintes à 30 h post-hCG dans les cellules de la granulosa. Ces processus de régulation de l'ARNm présentent peu de ressemblances avec ceux qui sont observés chez les gènes stéroïdogènes. Il existerait donc des mécanismes spécifiques de chaque gène qui agiraient en aval de la régulation de l'ARNm du SF-1/NR5A2 pour gérer leur commande transcriptionnelle dans ce type cellulaire. Les niveaux d'ARNm du SF-1 d'abord élevés dans la thèque interne ont par la suite chuté de façon significative 36 h post-hCG, parallèlement, on peut le supposer, à la dégénérescence de ce tissu. Contrairement à la situation observée dans les cellules de la granulosa, la régulation de l'ARNm du SF-1 dans la thèque interne ressemblait fortement à celle de la StAR, de la P450scc et de la P45017α; il y aurait donc un lien direct avec leur régulation transcriptionnelle. Le NR5A2, qui est exprimé dans les cellules de la granulosa, ne le serait pas dans la thèque interne.

Globalement, ces études permettent de proposer un modèle de la commande de la stéroïdogénèse ovarienne chez la jument. La distribution tissulaire de l'expression des gènes stéroïdogènes dans les follicules préovulatoires équines démontre la prédominance de la voie Δ5 pour la biosynthèse de l'œstradiol-17β. Une fois le processus ovulatoire déclenché, on observe un déclin rapide de l'expression de la P450arom par l'intermédiaire du promoteur II et, par conséquent, une diminution relative de l'importance de cette voie métabolique. La biosynthèse de la progestérone est alors accélérée par la redirection de l'expression des gènes de la synthèse de la progestérone de la thèque interne en dégénérescence vers les cellules lutéinisantes originant des cellules de la granulosa. Le déclin de l'expression des gènes stéroïdogènes induit par l'hCG dans la thèque interne se déroule simultanément avec la perte de transcrits du SF-1, suggèrant ainsi un mécanisme simple et dépendant de la SF-1 pour assurer leur régulation transcriptionnelle. La régulation des gènes stéroïdogènes dans les cellules de la granulosa et le CL semble bien plus complexe et pourrait dépendre en partie du NR5A2. Des études supplémentaires seront nécessaires pour arriver à démontrer le rôle du NR5A2 dans la transactivation des gènes stéroïdogènes ovariens.

SUMMARY

The preovulatory surge of luteinizing hormone is the physiological trigger for two key reproductive processes: ovulation and luteinization.

Ovulation has been likened to an inflammatory reaction in that both processes share similarities at the histological and functional levels, such as their dependance upon prostaglandins as molecular mediators. Prostaglandin G/H synthase-2 (PGHS-2) catalyzes the first rate-limiting step in the biosynthesis of all prostanoids from arachidonic acid. To elucidate the role of PGHS-2 in the equine ovulatory process, the first article presented in this thesis reports the isolation of its primary transcript from a follicular cDNA library. Determination of the regulation of PGHS-2 mRNA was accomplished using Northern blots prepared from preovulatory follicles isolated on a time-course after administration of an ovulatory dose of hCG. Results showed a transient induction of PGHS-2 mRNA starting 30 h post-hCG which occurs selectively in granulosa cells. This late induction of equine PGHS-2 occured approximately 10 h prior to ovulation, which is the same timespan that has been previously reported in cows and rats despite the very different overall lengths of their ovulatory processes (28 h and 12-14 h, respectively). Collectively, these data therefore suggest that PGHS-2 induction is a determinant of the mammalian ovulatory clock. The fundamental mechanisms underlying the length of the ovulatory process in mammals could therefore be revealed by comparative studies of the ovarian transcriptional regulation of PGHS-2 in different species. The length of the equine ovulatory process, with the accompanying extended

delay before PGHS-2 induction, indicates that the mare represents an important model to study the transcriptional regulation of PGHS-2 in species with long ovulatory processes. To generate the tools to commence these studies, the complete gene was isolated from a genomic library and characterized by sequence and primer extension analyses.

Luteinization is a hormonally induced differentiation process of the steroidogenic cells of the follicle wall that is accompanied by a shift in the predominant ovarian hormonal product from estradiol-17β to progesterone. While this clearly requires the restructuring of the cellular steroidogenic machinery, the molecular basis for it remains incompletely elucidated, particularly in large monoovulatory species. An initial rate-limiting step in the regulation of steroidogenesis is the delivery of cholesterol substrate molecules from the cytoplasm to the mitochondrial inner membrane. While this has been shown to be dependant upon the action of steroidogenic acute regulatory protein (StAR), the precise nature of its involvement in this process has yet to be clearly demontrated. To study the regulation of equine StAR during follicular luteinization, an article annexed to this thesis (Annex I) reports the isolation from a follicular cDNA library of two equine StAR transcripts that differ only in the length of their 3'-UTR. The regulation of StAR mRNA *in vivo* was then studied in preovulatory follicles isolated on a time-course after the administration of an ovulatory dose of hCG. No significant changes in StAR mRNA levels after hCG treatment were found when analyses were performed on intact follicle wall. However, Northern blots performed on isolated granulosa cells showed an induction of StAR at 30h post-hCG, and this was

associated with a rise in follicular fluid concentrations of progesterone. In contrast, StAR mRNA levels were high in theca interna until 33h post-hCG and dropped dramatically thereafter. The net result of these processes is a gradual redistribution of StAR expression from the theca interna to the granulosa cells. This may be linked to a putative degeneration of the theca interna during the ovulatory process in mares, which would require that StAR expression be transferred to the granulosa cells that will form the corpus luteum (CL).

Cholesterol can be converted to progesterone by the successive actions of cytochrome P450 cholesterol side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD). To verify the effects of hCG on the mRNA levels of the progesterone-synthetic enzymes in preovulatory follicles, the second article detailed herein reports the cloning of equine P450scc and 3β-HSD transcripts. Northern blot analyses were performed with preovulatory follicles isolated on a time-course following the administration of hCG. Isolated cellular preparations revealed that theca interna was the predominant site of P450scc expression in follicles prior to hCG. However, transcript levels decreased in theca interna between 30-39h and increased in granulosa cells at 39 h, making the granulosa cell layer the predominant site of P450scc expression at the end of the ovulatory process. Highest levels of expression were detected in corpora lutea. The observed redistribution of P450scc expression within the follicle wall was similar to what was observed for StAR, and may also be related to the putative degeneration of the theca interna. In contrast to other species, expression of 3β-HSD mRNA in equine preovulatory follicles was localized only in granulosa cells, and

transcript levels remained constant throughout the luteinization process. The LH-induced up-regulation of progesterone biosynthesis is therefore related to the redistribution of both StAR and P450scc from the degenerating theca interna to the luteinizing granulosa cell layer.

Progesterone can be converted to estrogens by the successive actions of cytochrome P450 17α-hydroxylase (P45017α) and cytochome P450 aromatase (P450arom). To study the regulation of the equine estrogen-synthetic enzymes during the ovulatory process, the third article presented in this thesis reports the isolation from a follicular cDNA library of equine transcripts encoding P45017α and P450arom. The molecular cloning of P450arom produced two distinct cDNAs, one was derived from promoter 1f and encoded a well-conserved 503-amino acid protein, the other was derived from promoter II and encoded a truncated protein. Northern blot analyses were performed using preovulatory follicles obtained on a time-course after the administration of hCG. P450arom mRNA regulation was biphasic: levels were highest in granulosa cells at 0h post-hCG, decreased significantly during the ovulatory process at 12 and 24h, and increased again between 30–39h post-hCG and in corpora lutea. Probes used to detect specific P450arom mRNA variants then revealed that promoter II usage accounts for the message present in follicles at 0h post-hCG and in corpora lutea, whereas promoter 1f is used exclusively during the ovulatory process. This represents a novel aromatase promoter switching phenomenon, a process that also occurs most notably in breast carcinogenesis to achieve malignant up-regulation of localized estrogen synthesis. The equine preovulatory follicle thus provides an inducible model system for the study of

aromatase promoter switching. Levels of P45017α mRNA were high in theca interna at 0h, but significantly decreased in follicles at 36 and 39h post-hCG and in corpora lutea, which may also be related to the putative degeneration of the theca interna. Overall, these data provide insights into the mechanisms governing the hCG-induced down-regulation of estrogen biosynthesis in luteinizing preovulatory follicles. To provide tools for the further study of P450arom regulation and promoter switching, promoters II and 1f were cloned from a genomic library. Putative transcription start sites were then identified by primer extension assays, and potential regulatory elements were identified by sequence analysis.

The orphan nuclear receptor steroidogenic factor-1 (SF-1) was originally isolated as a transcription factor capable of binding discrete regulatory elements present in the promoters of various steroid hydroxylases. Since then, all of the steroidogenic genes studied in articles 2 and 3 and in Annex I have been shown in other species to be transcriptionally regulated by SF-1 *in vitro* , in apparent contrast with the varied, gene- and cell-specific nature of the observed mRNA regulatory processes. The objectives of the fourth article presented herein were to clone equine SF-1 and to observe the regulation of its transcript in theca interna and granulosa cells following hCG administration, to identify possible links between the regulation of SF-1 mRNA and those of the various steroidogenic genes. The molecular cloning of SF-1 resulted in the serendipitous isolation of a cDNA encoding the highly-related orphan nuclear receptor NR5A2, whose expression in gonadal tissues had not been previously reported. The SF-1 and NR5A2 transcripts were isolated in fragments by a variety of

cloning procedures, and were found to encode proteins that are 60% identical to each other, with nearly identical DNA-binding domains that reflect the indistinguishable DNA-binding specificities of their human homologs. Northern and RT-PCR analysis using various tissues then established a strong link between the expression of SF-1 and that of the steroidogenic genes in most tissues. One exception was the CL, in which SF-1 mRNA levels declined considerably compared to preovulatory follicles despite increased levels of StAR and P450scc expression. High levels of 3β-HSD and P450arom expression in granulosa cells were also in apparent disagreement with low levels of SF-1 mRNA. Interestingly, a role for NR5A2 in regulating steroidogenic genes in granulosa cells and CL was suggested by its level of expression in these tissues, which far surpasses that of SF-1. The regulation of SF-1 and NR5A2 mRNA throughout the ovulatory process was examined using an RT-PCR/Southern blotting technique on ovarian follicular RNA isolated on a time-course after the administration of hCG. In granulosa cells, approximately 3-fold decreases in SF-1 and NR5A2 mRNA levels were attained by 30h post-hCG, similarly to what had been previously observed for SF-1 in rats. These mRNA regulatory processes bore little resemblance to those observed for any of the steroidogenic genes, suggesting that gene-specific mechanisms act downstream of SF-1/NR5A2 mRNA regulation to govern their transcriptional control in this cell type. SF-1 mRNA levels were high in theca interna and dropped significantly 36 hours post-hCG, also in apparent relation with the degeneration of this tissue. Unlike what was observed in granulosa cells, the regulatory pattern of SF-1 in theca interna apparently mirrored those of StAR, P450scc and P45017α,

suggesting a direct link to their transcriptional regulation. Also contrary to what was observed in granulosa cells, NR5A2 appears not to be expressed in theca interna.

Collectively, these studies permit the formulation of a working model of the control of ovarian steroidogenesis in mares. The tissue distribution of steroidogenic gene expression in equine preovulatory follicles demonstrates the predominant use of the Δ5 pathway to direct the biosynthesis of estradiol-17β. Following the onset of the ovulatory process, this pathway is down-regulated by the rapid decline of P450arom gene expression via promoter II. Progesterone biosynthesis is then specifically up-regulated by the consolidation of the expression of the progesterone-synthetic genes in luteinizing granulosa cells. The hCG-induced decline of thecal steroidogenic gene expression occurs in close parallel with the loss of SF-1 transcripts, suggesting a SF-1 dependant mechanism for their transcriptional regulation. The regulation of steroidogenic genes in granulosa cells and CL appears considerably more complex, and may depend in part on NR5A2. Confirmation of this will require the demonstration of the transactivation of these genes by NR5A2.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

ARTICLE ONE

ARTICLE TWO:

ARTICLE THREE

LIST OF ABBREVIATIONS

3β-HSD	3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase
aa	amino acid
Ad4BP	adrenal-4 binding protein
AF-2	activation function-2
AMP	adenosine monophosphate
AP-1/2	activator protein-1/2
AR	androgen receptor
ATP	adenosine triphosphate
C	carbon
C/EBP-β	CCAAT/enhancer-binding protein β
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	complementary deoxyribonucleic acid
CL	corpus luteum
COUP-TF	chicken ovalbumin upstream promoter transcription factor
COX-1/2	cyclooxygenase 1/2
$cPLA_2$	cytosolic phospholipase A_2
CRE	cAMP response element
CREB	cAMP response element binding protein
CRS-1/2	cAMP responsive sequence 1/2
C-terminus	carboxy terminus
DAX-1	dosage-sensitive sex reversal, adrenal hypoplasia congenita, X chromosome, gene 1
eCG	equine chorionic gonadotropin
EGF	epidermal growth factor

ELP 1/2/3	embryonal long terminal repeat-binding protein 1/2/3
FSH	follicle stimulating hormone
GH	growth hormone
GnRH	gonadotropin releasing hormone
GRIP-1	glucocorticoid receptor interacting protein-1
h	hour
hCG	human chorionic gonadotropin
IGF-1	insulin-like growth factor-1
Il-1/1β	interleukin 1/1β
kb	kilobase
kDa	kiloDalton
LCAH	lipoid congenital adrenal hyperplasia
LDL	low density lipoprotein
LH	luteinizing hormone
MIS	mullerian inhibitory substance
ml	milliliter
mm	millimeter
MMP-1/2/9	matrix metalloproteinase 1/2/9
mRNA	messenger ribonucleic acid
NAD^+	nicotinamide adenine dinucletide (oxidized)
NADH	nicotinamide adenine dinucletide (reduced)
NADPH	nicotinamide adenine dinucletide phosphate (reduced)
NF-κB	nuclear factor κB
ng	nanogram
NGF-IB	nerve growth factor-induced gene B
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
N-terminus	amino terminus
OR	cytochrome P450 oxidoreductase

P45017α	cytochrome P450 17α-hydroxylase/17,20 lyase
P450arom	cytochrome P450 aromatase
P450scc	cytochrome P450 cholesterol side-chain cleavage
PAF	platelet-activating factor
pBR	peripheral benzodiazapene receptor
PG(X)	prostaglandin $F_{2\alpha}/E_2/G_2/H_2/D_2$
PGHS-1/2	prostaglandin G/H synthase 1/2
PLA_2	phopholipase A_2
POCS	polycystic ovary syndrome
PR	progesterone receptor
RIIβ	cAMP-dependant protein kinase regulatory subunit type IIβ
RT-PCR	reverse transcription-polymerase chain reaction
SAP	steroidogenesis activator protein
SCP2	sterol carrier protein 2
SF-1/2	steroidogenic factor-1/2
SMRT	silencing mediator of of retinoic acid and thyroid hormone receptor
$sPLA_2$	soluble phospholipase A_2
SR-BI	scavenger receptor, class B/type I
SREBP-1a	sterol regulatory element binding protein-1a

StAR	steroidogenic acute regulatory protein
Stat 5a/5b	signal transducer and activator of transcription 5a/5b
TGF-β	transforming growth factor–β
TIMP-1	tissue inhibitor of metalloproteinases-1
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
USF-1/2	upstream stimulatory factor 1/2
UTR	untranslated region
VEGF	vascular endothelial growth factor
α2M	α2-macroglobulin
μm	micrometer

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INTRODUCTION

1. Overview of the equine estrous cycle

Ovulation, defined as the expulsion of the oocyte from the ovarian follicle, is a key process in the establishment of gestation. It is the culmination of an intricately regulated series of physiological events that constitute the estrous cycle.

The mare is referred to as a seasonal polyestrous animal, as it typically undergoes several cycles of sexual activity during the spring and summer. This is followed by an anestrous season of variable length that is primarily dependant on photoperiod (1), as daylight modulates hypothalamic gonadotropin releasing hormone (GnRH) secretion (2). The transition from the breeding season to the anestrous season is neither sharp nor invariant, and mares may occasionally continue to cycle throughout winter months, albeit at longer and more irregular intervals (1). However, the proportion of mature follicles that actually ovulate declines sharply during the anestrous season.

Unlike other species, the equine estrous cycle is often defined as the duration between two ovulatory events, rather than the duration between the onsets of two periods of estrus (i.e. receptivity to the male) (1). The average duration of this cycle is 21 days (1), including a diestrus phase of 14 to 15 days followed by an estrus of 5 to 7 days (Figure 1). Ovulation typically occurs less than 48 h before the end of estrus (1), and this marks day 0 of the cycle. Following the release of the oocyte, the follicle wall is reorganized into a

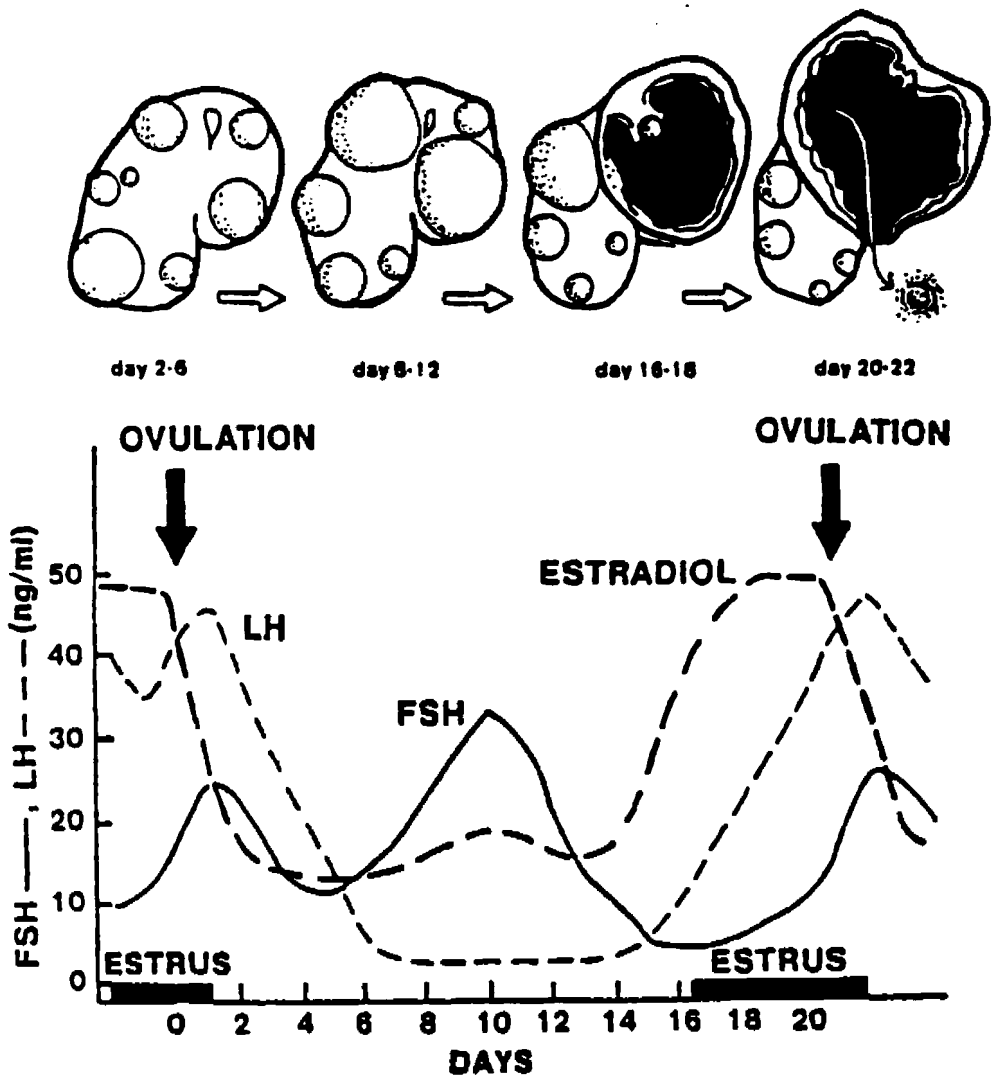

Figure 1. Follicular development, estradiol secretion and circulating gonadotropin levels during the equine estrous cycle. (Adapted from reference 1)

glandular tissue termed the corpus luteum (CL), and this represents the beginning of the luteal phase of the cycle. In most species, the CL is formed from both steroidogenic cell layers of the ovulatory follicle: the granulosa cells and the theca interna. In mares however, the theca interna appears to undergo a degenerative process that commences prior to ovulation and results in a CL that

is derived primarily or exclusively from granulosa cells (3, 4). Structurally formed by day 3 of the cycle, the CL secretes maximal levels of progesterone, its main hormonal product, by day 6 (Figure 2) (1, 5). The extent and duration of progesterone secretion is apparently controlled by luteinizing hormone (LH), and circulating progestone levels correlate with LH receptor expression in the CL (5). In the event that pregnancy does not occur or is not properly recognized, oxytocin-mediated uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release results in the regression of the CL (5) beginning on day 14, with an accompanying decrease in progesterone production (Figure 2). By means that have yet to be clearly elucidated in mares, the presence of the conceptus prevents luteal regression by interfering with $PGF_{2\alpha}$ production (5), thereby insuring the maintenance of progesterone levels required for gestation.

Follicular development is divided into several stages according to specific histological criteria. Ovarian follicles develop to the secondary follicle stage on a continuous basis without apparent need for the gonadotropins LH or follicle stimulating hormone (FSH) (6). The actions of gonadotropins are however required at stages of development beyond the formation of the follicular fluid-filled antrum, which occurs when equine follicles attain approximately 300 μm in diameter (6). Around the time of ovulation, a small group of antral follicles is stimulated mainly by FSH secretion to grow to a diameter of 25-30 mm by the time of luteolysis (1). This degree of follicular growth during the luteal phase is not characteristic of most species, and large (>30 mm) follicles can develop even during the early luteal phase in the mare, though these typically undergo atresia (1). When this occurs, a second wave of follicular growth that will produce the

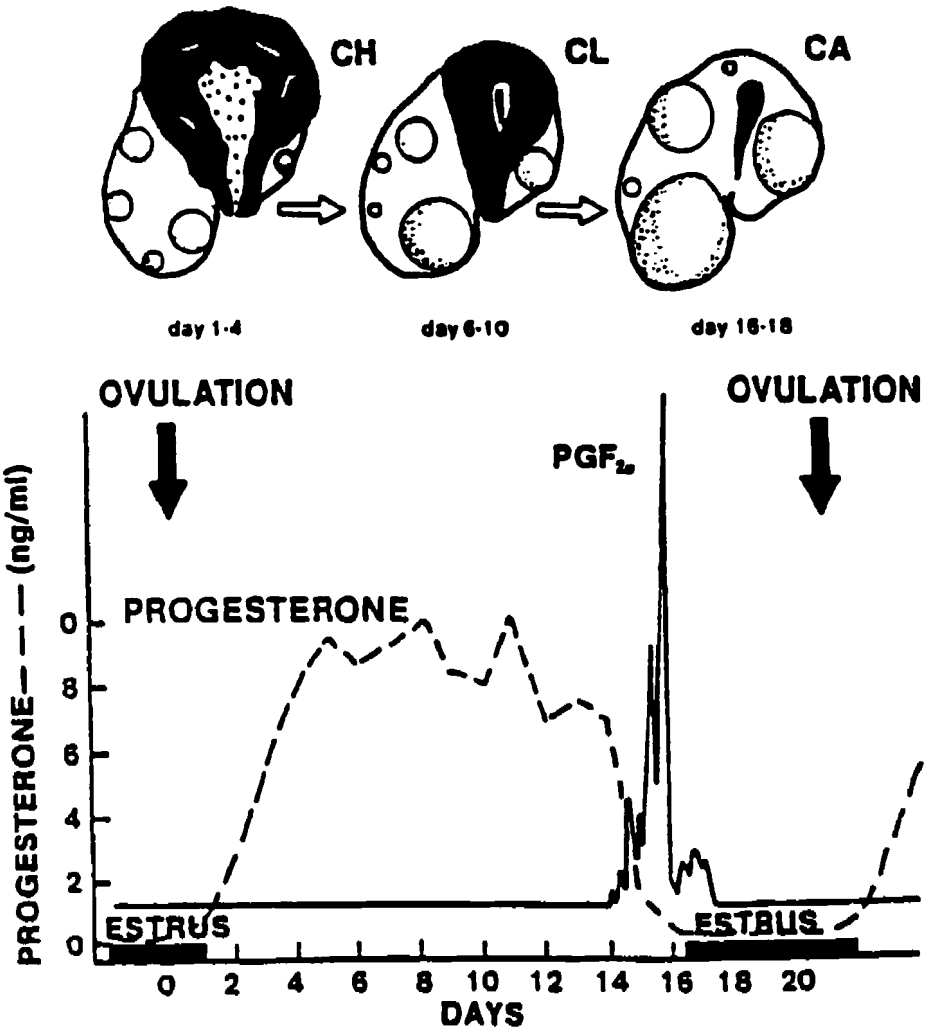

Figure 2. Development of the corpus luteum, progesterone secretion and circulating $PGF_{2\alpha}$ levels during the equine estrous cycle. (Adapted from reference 1)

ovulatory follicle is occasionally initiated in the mid-luteal phase (1). Steroidogenesis in developing follicles occurs via a two-cell mechanism. Thecal cells, under the influence of LH, produce androgens that diffuse across the basement membrane to the granulosa cell layer, which converts them to estrogens as directed by FSH (6, 7). These estrogens then stimulate the mitotic

division of the granulosa cells as well as the expression of further FSH receptors, creating a positive feedback loop (8).

Following the one- or two-wave recruitment process, a single follicle (or occasionally two (1)) is selected and becomes functionally dominant: its growth supplants that of the other follicles, which then undergo atresia. In mares, selection apparently occurs between days 14 and 17 of the cycle (6), and marks the beginning of the follicular phase. The fundamental mechanism underlying this process remains elusive, but circulating FSH and LH levels are clearly involved (6, 8). Rising levels of estrogen and inhibin production combine to inhibit the secretion of FSH, and this is linked to the onset of the atresia of the subordinate follicles. The dominant follicle apparently escapes this fate by expressing LH receptors in its granulosa cell layer, and the control of cellular proliferation and steroidogenesis thus falls under the influence of LH (6, 8). Concurrently, luteolysis relieves the negative feedback of progesterone on LH secretion and its circulating levels begin to rise, thereby stimulating the hypertrophy of the thecal layer (6). This is associated with a massive increase in estrogen biosynthesis, which triggers the onset of behavioral estrus (Figure 1). Estrogen also sets up a positive feedback loop, inducing a further, rapid rise in LH secretion (1, 8). Unlike other species in which LH secretion is induced in a surge beginning approximately 24 h before ovulation (8), the increase of circulating LH in mares is a more gradual process. Levels begin to increase 6 or 7 days before ovulation, and do not peak until 1 to 3 days after ovulation occurs (6) (Figure 1), suggesting that an undetermined ovulatory threshold level of LH must be attained (1). Having attained a diameter of approximately 45 mm by

the end of the follicular phase (6), the follicle ruptures 36-48 h after the attainment of the ovulatory LH threshold, as deduced by the induction of ovulation by administration of human chorionic gonadotropin (hCG) (9, 10). This interval, refered to as the ovulatory process, is considerably longer in mares than in other species, notably the rat and the cow which ovulate 12-14 h and 28 h post-hCG, respectively (11, 12).

1.1 Physiology of the equine ovulatory process

The rupture of the ovulatory follicle is a complex process that is associated with a number of alterations of the follicle wall at the cellular, vascular and connective tissue levels. To permit the expulsion of the follicle's contents at its apex, the integrity of several layers must be compromised, including the granulosa cells, basement membrane, theca interna, theca externa, ovarian stroma, tunica albuginea and germinal epithelium (6). Whereas many processes occur simultaneously within these layers, they are presented herein in spacial order for purposes of clarity.

In the granulosa cell layer, an accumulation of acid mucosubstances occurs in response to the preovulatory rise in LH, resulting in a considerable thickening of the layer and the loosening of the cell's attachment to each other (3). This also occurs in other species (13-15), though not to an extent comparable to the mare, who's granulosa cells become entraped in an expansive mucoid web for reasons that have yet to be determined. At the follicular apex, the granulosa cells that are fixed to the basement membrane lose their characteristic

columnar appearance as collagenolysis causes the membrane to dissociate and fragment. The granulosa cells below this are lost, and the dying cells release hydrolases that contribute to the degradation of the remaining layers (6).

Considerable changes also occur in the thecal layers. In a manner similar to an acute inflammatory reaction (16), increased vascularization, blood flow, leukocytic infiltration and vascular fenestration and dilation are observed. These lead to hyperæmia, œdema and the formation of zones of hæmorrhage as the moment of ovulation approaches (3). As is observed in pigs, eosinophils are the predominant leukocytes that infiltrate the equine follicle wall (3, 17). Neither the chemotactic agent involved nor the precise purpose for this eosinophilia has been determined, though it has been suggested that infiltrating leukocytes could secrete paracrine hormones such as cytokines and/or enzymes involved in follicle wall rupture (18). This is contradicted however by leukocyte depletion studies in sheep that indicate that circulatory eosinophils are not likely to be required for normal ovulation to occur (19). In the equine theca interna, a unique degenerative process is induced by LH that is characterized by pyknosis and reductions in cell size and number, leading to an overall thinning of the layer (3). It has been proposed that this process is related to the decreased need for thecal androgen production at the end of the follicular phase (3), although why this does not also occur in other species remains unresolved.

Other degradative processes concurrently act upon the outer layers. In the theca externa and tunica albuginea, extensive collagenolysis leads to an overall decrease in the tensile strength of the follicle wall (6). Softening and deformability of the follicle can thus be detected by palpation as ovulation

becomes imminent (3). Ultrasonography also shows changes in the appearance of the follicle, which tends to elongate with the apex pointing towards the ovulatory fossa; a distinctive site on the equine ovary's surface at which all ovulatory events occur (6). The ovarian stroma between the theca externa and tunica albuginea is stretched and pressed aside during follicle growth to an extent that it likely provides no resistance to ovulation (6). At the apex, the germinal cells become pyknotic and loosen from the tunica albuginea, providing an additional source of hydrolases for the degradation of underlying layers (6). Vasoconstriction also occurs at the apex, hastening the local breakdown of tissues.

All of the aforementioned degradative processes result in the thinning of the apical follicle wall, forming a stigma which is eventually overcome by the hydrostatic pressure of the follicular fluid. While smooth muscle contractions have been observed at the base of the follicle during ovulation in mares (6), increases in intrafollicular pressure have not been observed, and are not believed to be involved in inducing ovulation (6).

1.1.1 Molecular aspects of follicle wall rupture

The observable histological changes in the follicle wall that accompany the ovulatory process can be roughly categorized into vascular and proteolytic effects (18). Studies performed mostly with rodent models have shown that these are induced by distinct but overlapping sets of molecular mediators.

A wide variety of vasoactive agents is believed to be involved in the ovulatory process. Among these, vascular endothelial growth factor (VEGF) apparently acts in response to the relative hypoxia of the inner follicular compartments by stimulating angiogenesis and increasing vascular permeability (18). Ovarian production of the cytokine interleukin-1 (Il-1) has been shown to be required for ovulation to occur, and may act by inducing the synthesis of hyaluronic acid, nitric oxide and prostaglandins and by activating gelatinase (18). Inhibition of nitric oxide (NO) synthesis can suppress ovulation, indicating potentially critical roles for NO in ovarian vasodilation as well as steroidogenesis and suppression of apoptosis (18). Reductions in ovulation rates have also been observed upon antagonism of additional vasoactive agents, such as platelet activating factor (PAF), bradykinin and oxygen free radicals (18). The involvement of eicosanoids in ovulation has been extensively studied, and major roles have been proposed notably for prostaglandins E (PGE) and $F_{2\alpha}$ ($PGF_{2\alpha}$) in mediating vasodilation in the follicle wall and vasocontriction at the apex, respectively (6). Evidence for this now exists at the physiological, pharmacological and genetic levels. Prostaglandins have long been known to accumulate in the follicular fluid with the approach of ovulation (18), and this has been associated with the induction of prostaglandin G/H synthase-2 (PGHS-2) in several species (11, 12, 20). Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit PGHS-2 activity (21), block ovulation in part by interfering with vascular responses to LH/hCG (18). Most convincingly however, ovulation has been found to be severely impaired in PGHS-2-null mice (22).

Many proteolytic activities have been identified within the follicle wall and follicular fluid. Among the first identified were the tissue-type and urokinase-type plasminogen activators (tPA and uPA), which are induced by LH and are able to activate collagenase (18). However, the relative importance of these enzymes has recently been downplayed by the generation of tPA/uPA double knockout mice, whose ovulation rate is only slightly reduced (23). More recently, zymographic analysis of LH-induced collagenolytic activity has implicated interstitial collagenase (now known as matrix metalloproteinase-1 or MMP-1) and collagenase type IV (MMP-2) (18) in the breakdown of the connective tissue of the follicle wall. Fittingly, increases in MMP-1 and MMP-2 mRNAs have been observed in rats following the initiation of the ovulatory process (18), along with MMP-9 which degrades collagen type V (24). The significance of these data has however been blurred by additional findings, such as the presence and activity of MMP-2 in follicles even before the LH surge, the decrease of MMP-2 activation by LH, and that MMP-9 null mice are fertile (24). Furthermore, tissue inhibitor of metalloproteinases-1 (TIMP-1), a potent collagenase antagonist, is induced in parallel with MMPs (18), although it may act primarily to protect surrounding tissues. In addition to their effects on the follicular vasculature, prostaglandins have also been proposed to mediate the proteolytic breakdown of the follicle wall in response to LH. NSAIDs have been shown to inhibit follicular collagenolysis and can suppress the induction of MMP-1 (18), suggesting that prostaglandins might be required to induce collagenase activity at the gene level.

Two additional molecular mediators have been proposed to play key roles in ovulation. One of these is progesterone, as pharmacological inhibition of its synthesis and action (18) has been shown to block ovulation. Furthermore, progesterone synthesis increases dramatically during the ovulatory process (25), along with progesterone receptor expression (PR) (24). Most importantly, PR null mice show an anovulatory phenotype (26). It has been proposed that progesterone could act both by activating proteolysis (18) and by affecting vascular responses, possibly by activating a kallikrein activity that would generate a vasoactive kinin (24). The second potentially important mediator of the ovulatory response is cyclin D2, as mice lacking this gene also exhibit an anovulatory phenotype (27). Rather than from affecting the expression of other key genes, lack of ovulation in these mice apparently arises from insufficient numbers of granulosa cells, which fail to proliferate adequately. This gives rise to the notion that a threshold number of granulosa cells must be present within the follicle to permit the onset of the ovulatory process (24).

1.2 Cellular and biochemical aspects of luteinization

Luteinization can be broadly defined as a differentiation process by which cellular components of the follicle wall acquire the morphological and functional characteristics of the luteal cells that constitute the CL. This process is initiated by the preovulatory rise in LH, and begins prior to ovulation (28). In the mare, the granulosa cells are believed to be the major or sole cellular contributors to the CL; these differentiate into a cell type refered to as granulosa-

lutein cells or large luteal cells (3, 4). At the cellular level, hypertrophy and increased cytoplasm:nuleus ratio is observed, and large luteal cells thereby become the largest steroidogenic cell type in the body (28). Further changes include increases in smooth endoplasmic reticulum and golgi apparatus, as well as in mitochondrial size and complexity (28). Gap junctions decrease in number when luteinization is initiated, contributing to the dispersion of the granulosa cells (28). As with all differentiation processes, decreased proliferative potential is also observed. While some degree of cellular division has been noted during luteal development in sheep (28), no mitotic figures were observed in equine luteinizing granulosa cells following hCG treatment (3), and rat granulosa cells are considered to undergo terminal differentiation and complete cessation of division after the LH surge (24). The most important functional change that accompanies luteinization however is the shift of the major ovarian steroid hormone product from estradiol-17β to progesterone (Figures 1 and 2).

1.2.1 Luteinization-associated modulation of gene expression

Luteinization involves the reprogramming of the follicular cells to express a distinct, luteal-specific complement of genes (25). Most studies addressing this have examined the genes implicated in ovarian steroidogenesis. These have shown that several genes that are required for progesterone synthesis are induced following the LH surge. Namely, steroidogenic acute regulatory protein (StAR) mRNA is markedly induced in rat granulosa cells during luteinization (29-31), reflecting an increased need for cholesterol

precursors. Similarly, cytochrome P450 cholesterol side-chain cleavage (P450scc) mRNA levels are considerably higher in the CL than in preovulatory follicles in several species (32-38) (Figure 3). However, this induction apparently follows a different timecourse from one species to another, and a transient downregulation of P450scc mRNA by the LH surge has been observed in bovine preovulatory follicles (4, 30, 32, 33, 39, 40). The expression of 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) cannot be detected in pig and sheep preovulatory granulosa cells, but is abundant in CL (33, 39, 41). Consistent with this, a four order of magnitude 3β-HSD activity increase was observed when bovine preovulatory follicles were compared to corpora lutea (42). As the luteinization process results in the upregulation of progesterone production at the gene level, the downregulation of estradiol-17β synthetic genes also occurs. Notably, the follicular expression of cytochrome P450 17α-hydroxylase (P45017α) drops sharply following the LH surge in most species (32, 39, 43) (Figure 3), though levels apparently remain stable in humans, likely reflecting a species-specific variation in CL steroidogenesis (37). However, a decrease in cytochrome P450 aromatase (P450arom) mRNA occurs in all species reported so far (32, 34, 39, 44) (Figure 3).

Other noteworthy luteinization-associated gene regulation processes have been identified in rodent granulosa cell models. Among these is the loss of cyclin D2 expression, with concurrent increases in $p21^{cip1}$ and $p27^{kip1}$ (24). This shift in the balance between cell cycle activators and inhibitors has been proposed to be the molecular basis for the cessation of granulosa cell proliferation following the LH surge (24). The type IIβ regulatory subunit of

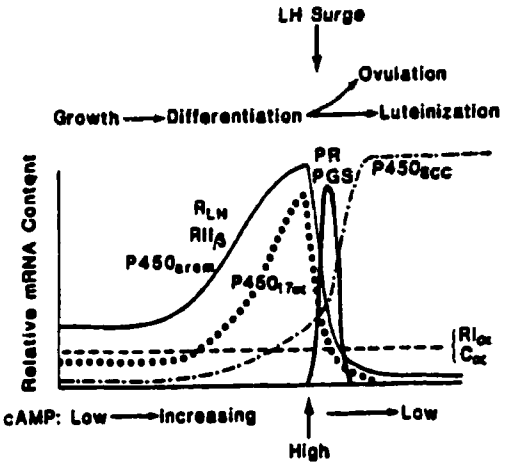

Figure 3. Changes in ovarian gene expression associated with luteinization. (Adapted from reference 25)

cAMP-dependant protein kinase (RIIβ) is believed to limit the gonadotropin responsiveness of granulosa cells, and is sharply downregulated by the LH surge (25) (Figure 3). It had therefore been postulated that RIIβ could function to prevent premature luteinization (and/or ovulation) (25), although the fertile phenotype of subsequently generated RIIβ null mice (45) apparently contradicts this notion. Another potentially important gene is α2-macroglobulin (α2M), which is induced during the luteinization process (25). While its precise function in ovarian physiology remains undertermined, α2M is known to be a secretory protein that can trap and inactivate proteases, cytokines and growth factors (28). It could thus play a role in limiting the activities of collagenases in a manner similar to TIMP-1, thereby contributing to the tissue remodeling involved in ovulation and luteinization. In addition, it could sequester cytokines and growth factors, either to inactivate them or to release them

elsewhere to increase their local action (28). PR has been shown to be induced on a timecourse similar to PGHS-2 in rats (25) (Figure 3), and pharmacological evidence has shown that progesterone action is required for luteinization to occur (25). This was further demonstrated at the genetic level with the generation of PR null mice which, in addition to being anovulatory, also show defects in the luteinization process (26).

2 Prostaglandins

Prostaglandins (PGs) are members of the eicosanoid biomolecule family, which also includes prostacyclin, thromboxane, leukotrienes and lipoxins (46). All of these are derived from open chain, 20-carbon polyunsaturated fatty acids, typically arachidonic acid. Whereas leukotrienes and lipoxins are formed directly from their parent fatty acid, prostaglandins, prostacyclin and thromboxanes are derived from a cyclic endoperoxide intermediate (Figure 4). Various classes of prostaglandins can be synthesized from this transitional compound, and these are categorized into subfamilies according to specific structural criteria. While all prostaglandins consist of a cyclopentane ring to which two side chains are attached at positions 8 and 12, it is the nature of the additional substituents on the ring that categorizes them into a particular series, which are named with the letters A through J. For instance, PGF compounds have hydroxyls at the C-9 and C-11 positions, and PGE compounds are characterized by a hydroxyl and a ketone group. Additional information in subscripts indicates the number of double bonds in the molecule, and the letter

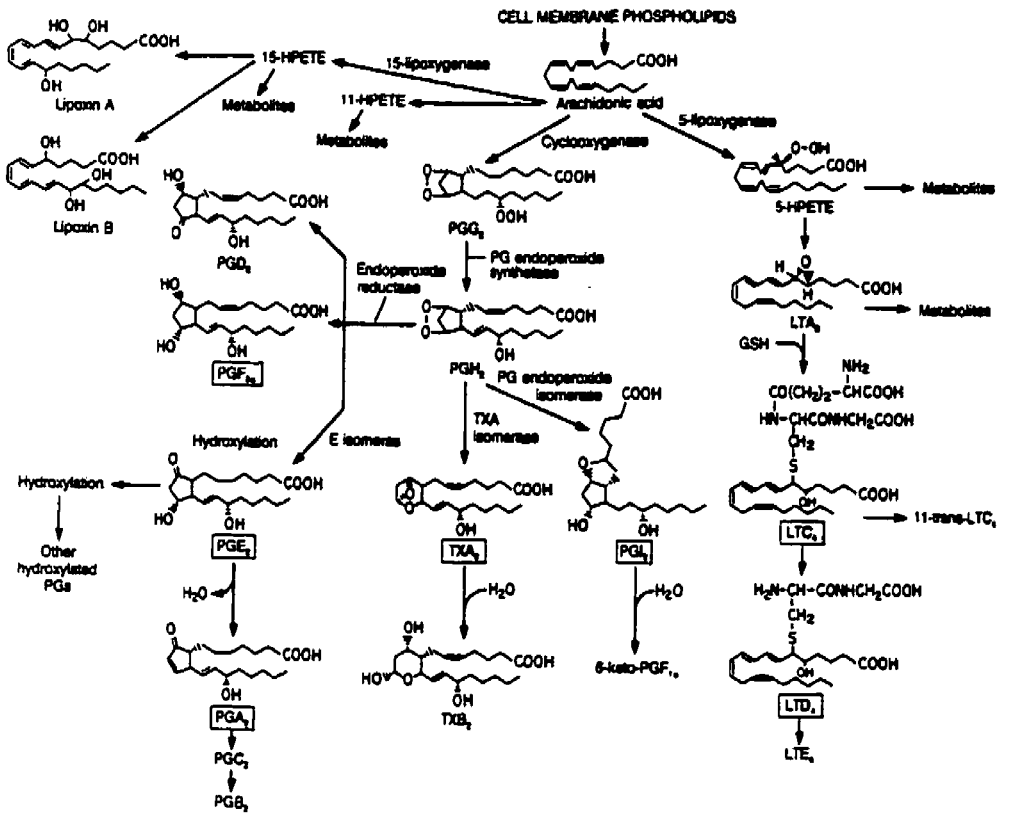

Figure 4. Structure and biosynthetic pathways of selected eicosanoids. (Adapted from reference 46)

α can be employed to denote that the C-9 substituent is located behind the plane of the ring, as in $PGF_{2\alpha}$ (Figure 4) (46).

2.1 Roles of prostaglandins in physiological and pathological processes

Prostaglandins have long been known to function as potent autocrine/paracrine hormones. They are believed to be synthesized by virtually

every cell type in the body, and have wide-ranging effects in processes ranging from normal metabolism to tumor growth (21).

Among the well established roles for prostaglandins is their modulation of glomerular hemodynamics in the renal medulla (21), resulting in an antihypertensive effect (46). Additional roles in renal physiology include the modulation of sodium and water reabsorption, regulation of renin secretion (21), as well as potential involvement in the embryological development of the kidney itself (47, 48). Similarly, prostaglandins are required for the maintenance of normal blood flow in the gastric mucosa (49). They are also involved in the maintenance of intestinal glandular architecture and local resistance to infection (21). Bone metabolism is mediated by prostaglandins, which can stimulate both its resorption and formation (21). This occurs at many levels, including the induction of osteoclast and osteoblast differentiation and activity, and both stimulation and inhibition of collagen synthesis (21). Involvement of prostaglandins in the nervous system is wide-ranging, and includes important roles in brain development, selective synapse loss, fever response, hyperalgesia, and both inhibition and potentiation of adrenergic transmission (21, 46). In addition to their normal physiological roles, prostaglandins have been implicated in a number of pathological processes. These include inflammation and arthritis (21), Alzheimer's disease (21), and colorectal, prostate and other cancers (21, 50-54). Proposed mechanisms by which prostaglandins may contribute to tumorigenesis include the increase of cellular adhesion to the extracellular matrix, augmented resistance to apoptosis, increased cellular proliferation, immunosuppression and stimulation of angiogenesis (55-58).

Another system in which prostaglandins are known to play key roles is the female reproductive tract. Intrafollicular accumulation of prostaglandins is required for ovulation to proceed normally (22), and these may mediate both vascular and proteolytic aspects of follicle wall rupture (see section 1.1.1). Following fertilization, roles have been proposed for prostaglandins in the preparation of the uterus to receive the embryo, as well as in mediating the embryo-uterine interactions required for implantation (21). Confirmation of this has come from the analysis of PGHS-2 null mice, who exhibit defects in ovulation, implantation and decidualization (22). $PGF_{2\alpha}$ production is required for the initiation of luteolysis, and this originates from the uterus at the end of the luteal phase (see section 1) or from the fetus with the approach of parturition (59). Luteolysis results in the loss of progesterone synthesis, which induces myometrial oxytocin receptor expression, which in turn permits the contractile response required to initiate parturition. Prostaglandins also stimulate uterine contractions more directly (21).

2.2 Molecular biology of ovarian prostaglandin biosynthesis

Arachidonic acid, the principal substrate for prostaglandin biosynthesis, is principally stored in an esterified state at the *sn*2 position of cell membrane phospholipids (60). The obligatory first step involved in prostaglandin formation in all tissues therefore involves the hydrolytic release of arachidonate (Figure 5). This is believed to occur by the stimulus-induced activation of one or more isoforms of phospholipase A_2 (PLA_2), making arachidonate release the first

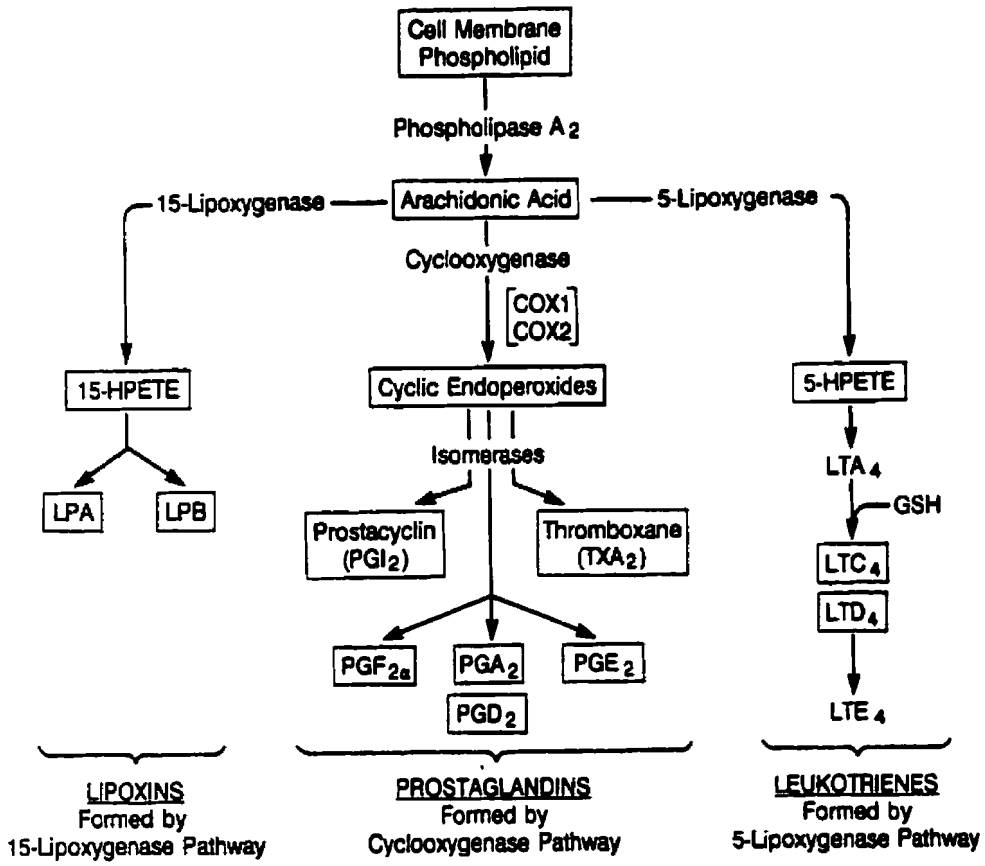

Figure 5. Schematic overview of the biosynthesis of prostaglandins and other eicosanoids. (Adapted from reference 46)

regulated step in the acute phase of prostaglandin biosynthesis (60). Existing data tend to implicate cytosolic PLA_2 ($cPLA_2$) in the acute/early phase of prostaglandin biosynthesis, as evidenced by overexpression studies and its rapid, Ca^{2+}-inducible association with membrane fractions (60, 61). Conversely, late-phase prostaglandin formation may result from a second isoform, termed soluble PLA_2 ($sPLA_2$). Unlike cPLA2, sPLA2 is a secretory protein that acts upon the surface of target cells, and its inhibition results in greatly reduced prostaglandin synthesis in cases requiring a priming or activation period (60).

Evidence also exists for the involvement of a third isoform, cardiac PLA_2, in certain tissues (60).

Following its release, arachidonate is converted to a cyclic endoperoxide by the action of prostaglandin G/H synthase (PGHS) (Figure 5), which is situated on the luminal surface of the endoplasmic reticulum and the outer envelope of the nuclear membrane (60). Recent studies have demonstrated the existence of two distinct genes encoding isoforms of PGHS, named PGHS-1 (or cyclooxygenase-1, COX-1) and PGHS-2 (or COX-2). While both forms catalyze the same reactions and share an overall 60% homology, they differ in other respects, including gene structure, substrate usage, gene regulation, tissue distribution, and mRNA splicing, stability and translational efficiency (21, 60). In almost all cases, PGHS-1 is considered to play a housekeeping role, as it is constitutively expressed at low levels in most tissues (21). It is therefore associated with the homeostatic levels of prostaglandin production required for the maintenance of blood perfusion in the kidney and gastric mucosa, as well as for thromboxane synthesis in platelets (21). PGHS-2 however is normally absent from most tissues except specific regions of the kidney (62), prostate and brain (60), but is readily inducible by hormones, cAMP, inflammatory factors, growth factors, tumor promoters and cytokines in a variety of cell systems (21, 60). Notable examples of PGHS-2 function in physiological and pathological processes include kidney development, tumorigenesis, embryonic implantation and mediation of inflammation, fever and pain (21). In the ovary, PGHS-1 is constitutively expressed in theca interna (63). The preovulatory rise in prostaglandin accumulation in the follicular fluid has however been

unambiguously associated with the induction of PGHS-2 mRNA and protein in the granulosa cells of several species (11, 12, 20). This is evidenced by the simultaneous nature of the rises in follicular prostaglandins and PGHS-2 expression, as well as the lack of ovulatory prostaglandin induction in PGHS-2 null mice (22, 47).

Following PGH_2 formation by PGHS, specific prostaglandins are synthesised in given tissues according to the particular downstream enzymatic machinery that they express (21) (Figure 5). High levels of both PGE_2 and $PGF_{2\alpha}$ accumulate in the follicular fluid prior to ovulation (12, 64-68). However, mice lacking the $PGF_{2\alpha}$ receptor (59) or different PGE_2 receptor subtypes (69) ovulate normally, rendering uncertain the identity and mechanism of action of the prostaglandin(s) involved in follicle wall rupture. PGE synthase activity has not been widely studied, and two isoforms are believed to exist in rats that differ by their tissue distributions and dependance on glutathione (70). Similarly, two distinct proteins were isolated from sheep seminal vesicles that have different sizes and catalytic properties (71). A recent study has reported the cloning of a human PGE synthase (72), although whether it corresponds to the enzyme responsible for PGE_2 synthesis in preovulatory follicles remains to be determined. Conversely, PGF synthase activity has been extensively characterized, and two distinct transcripts have been cloned, known as the lung (73) and liver (74) forms. It remains to be determined however if these originate from distinct genes (75) or if either is involved in follicular prostaglandin synthesis. Furthermore, two alternative pathways for $PGF_{2\alpha}$ synthesis from

PGE_2 and PGD_2 have been proposed (75), and enzymes that might catalyze these reactions have yet to be identified.

2.2.1 Prostaglandin G/H synthase-2

Shortly after the cloning of PGHS-1, discrepancies were noted when hormone and cytokine-induced prostaglandin synthesis could not be associated with increases in PGHS-1 expression in fibroblast and granulosa cell systems (21, 25). This led investigators to suspect the existence of a second PGHS isoform, and two antigenically and structurally distinct PGHS proteins were identified in rat tissues (63, 76). Shortly thereafter, PGHS-2 cDNAs were cloned by several groups (11, 77, 78).

2.2.1.1 Biochemistry and enzymology of PGHS-2

The human, rat and bovine genes encoding PGHS-2 have been cloned and characterized (79-81), and all consist of a highly conserved 10 exon structure spread over approximately 8 kilobases (kb) of genomic DNA. The transcripts vary in size in a species-specific manner from approximately 3.4 (human) (82) to 4.0 kb (rat, mouse) (78, 83), with differences arising mainly in the length of their 3'-untranslated regions (UTR). All contain several repeats of the Shaw-Kamen's sequence in their 3'-UTRs, which have been associated with mRNA instability (84, 85). The PGHS-2 protein has an apparent molecular weight of approximately 72000 despite a calculated size of 67000, a discrepancy which has

been attributed to N-linked glycosylations (86). Crystallography studies have revealed the three-dimensional structure of the PGHS-2 protein (87), which is highly similar to that of PGHS-1 (88). Both consist of an amino-terminal epidermal growth factor module, a central region with four α-helices that serves as a membrane-spanning domain, and a carboxy-terminal catalytic domain. Two such proteins associate as head-to-tail homodimers, with each contributing two histidine residues that coordinate a heme group (88).

Both PGHS enzymes are known to have dual enzymatic activities that sequentially convert arachidonic acid to PGG_2 and then to PGH_2, thereby forming the cyclic endoperoxyde molecule which serves as a substrate for the subsequent synthesis of all prostaglandins (Figure 6). A model describing the catalytic mechanisms involved has been proposed (60), and is supported by structural information provided by crystallography studies. This model describes a distinct active site for both the cyclooxygenase and the peroxydase activities. These sites are believed to act coordinately; while the former is occupied by arachidonic acid, the latter binds an alkyl hydroperoxide. An arachidonyl radical is then formed by an electron transfer chain involving a key tyrosyl residue and the iron atom in the heme group, and this intermediate then reacts with oxygen to form PGG_2 (60). Interestingly, the cyclooxygenase active site is the pharmacological target of aspirin (89), which functionally inactivates both PGHS-1 and –2 by acetylating them at a single serine residue. Newer generations of NSAIDs however exploit the subtle differences in the substrate access channels of the different PGH synthases in order to more selectively

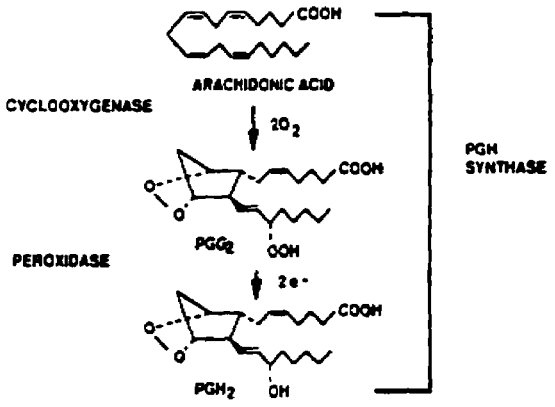

Figure 6. Dual enzymatic activities of prostaglandin G/H synthase. (Adapted from reference 60).

target PGHS-2, thereby decreasing side-effects attributable to unwanted PGHS-1 inhibition (87, 90).

2.2.1.2 Hormonal regulation of ovarian PGHS-2 expression

A wide variey of bioactive substances (see section 2.2), including several hormones, have been shown to induce PGHS-2 expression both *in vitro* and *in vivo* in a number of cell types. Examples of these include osteoblasts, bone marrow cells, monocytes, synovial tissue, brain endothelial cells, microglial cells, intestinal epithelial cells and endometrial cells (21, 91, 92). Another well-documented cell type in which PGHS-2 can be readily induced is ovarian granulosa cells. In culture, LH, FSH, GnRH, forskolin and IL-1β can all induce PGHS-2 expression in this cell type (11, 67, 93-96). *In vivo*, a transient induction of PGHS-2 has been observed in rat, bovine and equine granulosa cells in

response to the endogenous LH surge or the administration of hCG (11, 12, 20, 67, 95). The timecourse of PGHS-2 induction varies however from one species to another, occuring 2-4 h post-hCG in rats (11) versus 18 h in cows (12, 95) and 30-33 h in mares (20). Interestingly, these times relate directly to the length of the ovulatory process in these species (see section 1), as in each case PGHS-2 is induced roughly 10 h before follicle wall rupture occurs. It has therefore been suggested that PGHS-2 induction is a fundamental determinant of the length of the ovulatory process (20). Variations in length of the ovulatory process amongst species thus likely result from underlying differences in PGHS-2 transcriptional mechanisms, although these have yet to be completely elucidated.

2.2.1.3 Transcriptional regulation of PGHS-2 in the ovary

Many functional transcriptional elements have been identified in the PGHS-2 promoters of several species. The use of particular elements varies however as a function of cell type and the nature of the agonist involved. For instance, a nuclear factor-κb (NFκB) element is implicated in osteoblast response to tumor necrosis factor α (97), a cAMP response element (CRE) is required for PGHS-2 transcription in fibroblasts and vascular endothelium in response to certain stimuli (98, 99), and an activator protein-1 (AP-1) site is involved in response to agonists such as bile acids and okadaic acid in certain cell types (100, 101). These and other studies therefore suggest that the PGHS-2 promoter responds to various signalling pathways by the use of distinct transcriptional

elements and mechanisms. In ovarian granulosa cells, two distinct elements have been implicated in the induction of PGHS-2 transcriptional activity in response to LH/hCG. The first is a CAAT box present in the rat promoter that binds CCAAT/enhancer-binding protein β (C/EBPβ), which is itself induced by the LH surge (102). While this provided a simple mechanism for the transcriptional induction of PGHS-2, the involvement of the CAAT box was subsequently called into doubt by a similar study employing a larger fragment of the promoter (103). Interestingly, PGHS-2 is induced normally in response to hCG in C/EBPβ null mice, but high mRNA levels are maintained following induction rather than dropping promptly again, as seen in wild-type animals (104). This suggests that the true role of C/EBPβ may be to repress PGHS-2 expression before and after its induction. In support of this theory, a recent study has shown a transient decrease in C/EBPβ protein levels in bovine granulosa cells that coincides temporally with the induction of PGHS-2 (79).

The second important element is an E-box that has been characterized in both rats and cows (46, 79). This region is absolutely required for PGHS-2 induction in granulosa cells, and binds upstream stimulatory factor (USF). In addition to both the USF-1 and USF-2 proteins, significant amounts of amino-terminal truncated USF-2 (also known as mini-USF-2) have been shown to bind the bovine E-box prior to PGHS-2 induction (79). As mini-USF-2 lacks a transactivation domain, it has been proposed that it serves to repress transcription in a dominant negative fashion by competing with transcriptionally active USF for the binding site (79). Fittingly, PGHS-2 induction coincides with the disappearance of mini-USF-2 from bovine

granulosa cells (79). On the contrary, similar studies in rats showed no evidence of ovarian mini-USF proteins (103). It has therefore been postulated that species with long ovulatory processes delay the induction of PGHS-2 by expressing mini-USF proteins to repress its transcriptional activation until the appropriate moment (79). On the other hand, species with short ovulatory processes such as the rat may have different or no such mechanisms. Confirmation of this will require the analysis of the PGHS-2 transcriptional mechanisms in other species with short and long ovulatory processes.

3. Steroid hormones

Steroids are a subclass of an expansive family of chemical compounds known as terpenoids (46). Structurally, all steroids are based on cyclopentanoperhydrophenanthrene, a saturated 17 carbon molecule arranged in a four ring configuration, to which are added various substituents (Figure 7). For purposes of classification and nomenclature, the six families of steroid compounds that exhibit hormonal activity in mammals are considered to be derived from parent molecules. Namely, progestins, glucocorticoids and mineralocorticoids are related to pregnane, androgens to androstane, estrogens to estrane and vitamin D to cholesterol (Figure 8). Specific compounds are then named by addition of the subtituent suffixes and their positions to the parent molecule, with the addition of the greek letter α if the substituent is above the plane of the ring, β if it is below. For instance, addition to an androstane parent molecule of a hydroxyl group below the plane of ring D at position 17, a keto

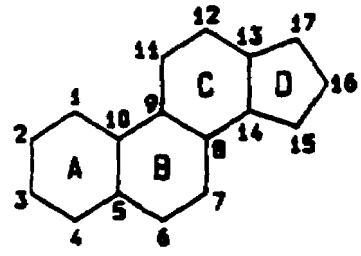

Figure 7. Cyclopentanoperhydrophenanthrene. (Adapted from reference 46)

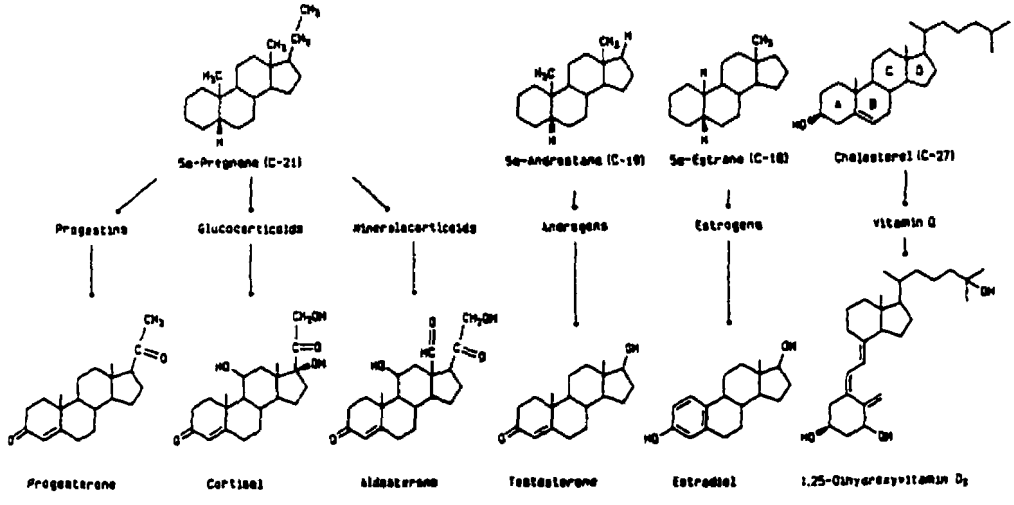

Figure 8. The six classes of mammalian hormonal steroids, their parent molecules and representative compounds. (Adapted from reference 46)

group at position 3 and unsaturation at position 4 results in a compound systematically named 17β-hydroxyandrost-4-en-3-one, trivially known as testosterone (Figure 8) (46).

Categorization of a hormonal steroid into a specific family is done according to both structural and biological criteria. Glucocorticoids and

mineralocorticoids are 21-carbon molecules with oxo groups on carbons 3 and 20, a hydroxyl group on carbon 21, a two-carbon side-chain at position 17 and a double bond at position 4. Glucocorticoids may also have additional hydroxyl groups at positions 11 and/or 17, whereas mineralocorticoids are characterized by a hydroxyl at position 11 and an aldehyde group at position 18. Vitamin D is in fact a secosteroid, characterized by breakage of the bond between carbons 9 and 10. Androgens are 19-carbon compounds characterized by oxygen functions on carbons 3 and 17. Estrogens have 18 carbons, oxygen functions at positions 3 and 17 and an aromatic A ring. Progestins, as represented by the naturally occuring progesterones, all consist of 21 carbons and oxo functionalities at positions 3 and 20 (46).

3.1 Roles of steroid hormones in physiological processes

Unlike prostaglandins, steroid hormones are synthesized by a few highly specialized tissues and exert their effects on a limited number of targets, which are typically distant from the site of secretion. Glucocorticoids are synthesized in the zona reticularis and zona fasciculata of the adrenal cortex and are important mediators of intermediary metabolism. Their specific effects include the stimulation of hepatic gluconeogenesis and glycogenolysis, decreased peripheral glucose utilization, increased lipolysis and protein catabolism, and augmented water diuresis (105). Mineralocorticoids are produced in an adjacent region of the adrenal cortex, the zona glomerulosa. These target mainly the distal tubules of the kidney and promote sodium retention and potassium and

hydrogen secretion, thereby exerting powerful effects on electrolyte balance and blood pressure (105). The synthesis of bioactive vitamin D requires the coordinate action of the skin, liver and kidney. Its main target tissues are bone and the mucosal cells of the gastrointestinal tract, where it increases circulating calcium levels by promoting its movement from bone and uptake from dietary sources (105). Testicular androgens are crucial for a series of developmental processes in the male, including the differentiation and growth of the reproductive tract, external genitalia and regions of the brain. At maturity, they also direct the development and maintenance of male secondary sex characteristics and sexual behavior, and are required for spermatogenesis. Furthermore, they exert anabolic effects in both sexes, as low levels of adrenal androgen production also occurs in females (46).

Estrogens and progestins are the two major steroid hormone products of the ovary. While the former are synthesized mainly by developing follicles, the latter are produced predominantly by the corpus luteum. The physiological roles of estrogens are varied and are required throughout the reproductive cycle and certain phases of gestation in all species. They serve to modulate gonadotropin secretion, thereby coordinating follicular development and the rise in LH levels that is associated with ovulation (see section 1). Concurrently, they are required for the expression of behavioral estrus in most species, including the mare (105, 108). Estrogen's effects on the tubular genitalia vary amongst species, and typically include proliferation of the uterine endometrium and fluid accumulation (46, 107, 108). This has not been reported in the mare however, although changes in the cervix and fluid accumulation in the oviduct

and vagina have been noted during estrus (108). During gestation, massive amounts of estrogen synthesis by the fetoplacental unit have been noted in several species such as horses and pigs. In pigs, these estrogens act as a luteostatic agent, thereby assuring maternal recognition and maintenance of pregnancy (106, 109). While estrogens are also believed to be required for successful pregnancy in the mare, they apparently do not act in a luteotropic or luteostatic fashion (109). At parturition, estrogens act to initiate prostaglandin synthesis, resulting in the start of the acute phase of delivery (105, 110). Many of the proposed physiological roles of estrogens were confirmed by the generation of mice with null mutations of cytochrome P450 aromatase (P450arom), the gene responsible for the final step in estrogen biosynthesis (111). Notably, these mice were anovulatory, their ovaries contained numerous atretic follicles and no corpora lutea, and had high levels of circulating gonadotropins. Unexpectedly however, male P450arom-null mice also exhibit reduced fertility, which has been associated with a progressive reduction in spermatogenesis (112). Coupled to data obtained in an estrogen receptor gene knockout analysis (112, 113), these results now indicate a previously unsuspected role for estrogens in male reproductive functions.

Progesterone is the main hormone responsible for progestin bioactivity in mammals (46). Traditionally, progesterone action has been associated primarily with the establishment and maintenance of pregnancy (26, 46, 115). This has been demonstrated in many species including the mare, in which progesterone coordinately affects the development of the embryo and the uterus so as to permit implantation (26, 114). Subsequently, continued progesterone secretion

by the CL is an absolute requirement for the maintenance of pregnancy, likely due to its stimulation of uterine secretions (109). Further roles for progesterone have been proposed in the modulation of gonadotropin secretion, the control of sexual behavior, ovulation and luteinization, and mammary gland development during gestation (25, 46, 115) (see sections 1.1.1 and 1.2.1). These have been confirmed in a convincing fashion by the analysis of the phenotype of progesterone receptor-null mice, which are characterized by ovulatory failure, impaired luteinization, impaired response of mammary tissues to hormonal stimulation and failure to demonstrate sexual receptivity response (26). Collectively, these data have expanded the roles of progesterone well beyond its established functions in gestation, redefining it as a pleiotropic coordinator of diverse reproductive processes.

3.2 Molecular biology of ovarian steroid hormone biosynthesis

All naturally occuring hormonal steroids are synthesized from cholesterol (46). While it may be synthesized de novo from acetate by steroidogenic cells, it is believed that plasma lipoproteins represent the major supply of cholesterol for steroidogenesis (116). Consistent with this, hormonal induction of steroidogenesis stimulates the low density lipoprotein (LDL) receptor and accelerates LDL cholesterol uptake (36, 116). Selective cholesterol uptake in steroidogenic tissues also occurs from high density lipoproteins, in part by the action of scavenger receptor, class B/type I (SR-BI) (117). The rate-limiting step of steroid hormone synthesis is catalyzed by cytochrome P450

cholesterol side-chain cleavage enzyme (P450scc), which is situated in the inner mitochondrial membrane (118). Cholesterol delivery from intracellular pools to the mitochondria is therefore an important procedure which is likely to be regulated by hormonal stimuli (118). It has been proposed that this occurs by the transit of lipid vesicles along intermediate filaments that then dock with mitochondria (118). Phosphorylation of cytoskeletal subunits has been suggested as a mechanism by which the rate of delivery of the vesicles may be modulated (118). By a means yet to be clearly elucidated, sterol carrier protein 2 (SCP2) then likely functions to promote the transfer of cholesterol from vesicles to mitochondria (118). Steroidogenesis activator protein (SAP), much like SCP2, can serve to accelerate steroid hormone production, and has been proposed to act in a manner similar to a heat shock protein, serving as a chaperon to help cholesterol transport proteins to interact with the mitochondria (118).

While P450scc limits the rate of the chemical reactions involved in steroid hormone production, it has long been known that the acute response to steroidogenic stimuli is limited by the rate of cholesterol transfer from the outer to the inner mitochondrial membrane. One critical mediator of this process is steroidogenic acute regulatory protein (StAR), a labile and tightly regulated protein which is believed to act at points of contact between the inner and outer membrane to promote cholesterol desorption from one membrane to another (119). Another protein known to be required for this transfer to occur is the peripheral benzodiazepine receptor (pBR) (120). It has been proposed that the membrane-spanning regions of pBR serve to form a pore through which cholesterol could be transferred (121). However, the precise mechanisms of

action of StAR and pBR, as well as any cooperative mechanism that may exist between them, remain to be demonstrated.

Following its insertion into the inner mitochondrial membrane, cholesterol is cleaved by the P450scc enzyme complex to form pregnenolone (Figure 9) (116). In the corpus luteum, pregnenolone is then converted by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) to progesterone, its main hormonal product (46). In mature follicles, granulosa cell-derived pregnenolone can be metabolized by two alternative steroidogenic pathways (123). Via the Δ4 pathway, progesterone is formed by 3β–HSD, and successively converted to 17α-hydroxyprogesterone, then to androstenedione by cytochrome P450 17α-hydroxylase/17-20 lyase (P45017α). In the Δ5 pathway, 3β–HSD and P45017α act in the reverse order, converting pregnenolone to 17α-hydroxypregnenolone, then to dehydroepiandrosterone and finally (by 3β–HSD) to androstenedione (Figure 9). Although ovarian expression patterns of these enzymes suggest a functional redundancy between the pathways, species-specific variations appear to exist that dictate the preferential usage of one or the other. Specifically, human, cow and sheep P45017α appears inefficient at, or incapable of, using 17α-hydroxyprogesterone as a substrate, creating a functional blockage of the Δ4 pathway (123). Conversely, 17α-hydroxypregnenolone is a poor substrate for guinea pig P45017α, therefore requiring predominant use of the Δ4 pathway (123). Similar studies in pigs and rats indicate no detectable substrate preference, suggesting that both pathways are used in these species (123). Equine ovarian steroidogenesis has not been extensively characterized, and evidence exists pointing to the preferential use of

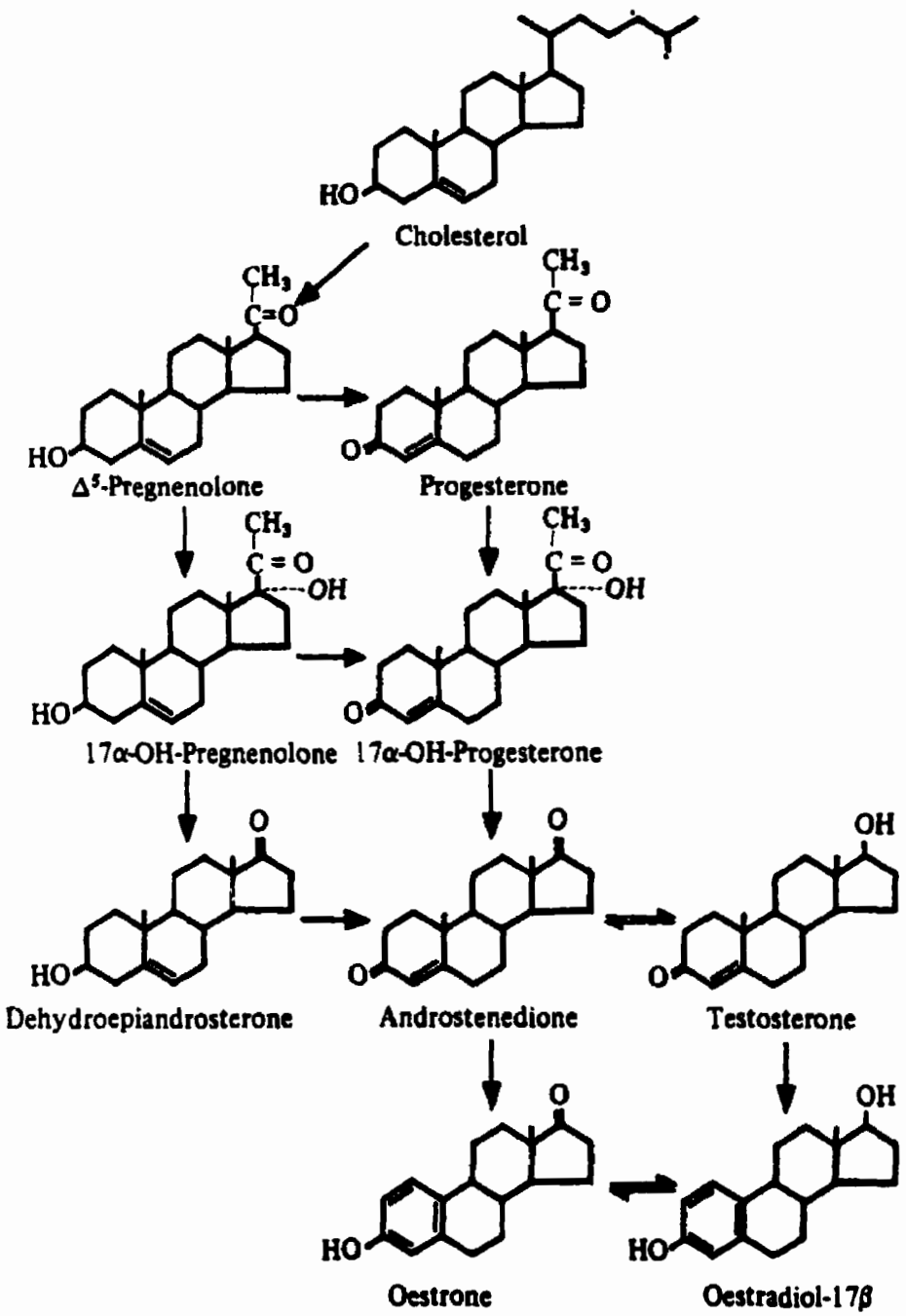

Figure 9. Simplified representation of ovarian steroidogenesis.

(Adapated from reference 122)

both pathways. While analysis of follicular fluid steroids suggests Δ4 usage

(108), the relative absence of 3β–HSD activity in equine theca interna compared to granulosa cells proposes that the opposite may be true (34, 40).

The expression of P45017α is restricted to the theca interna (41, 123), making it the obligatory site for ovarian androgen synthesis. These steroids then diffuse to the granulosa cell layer, which is the site of cytochrome P450 aromatase (P450arom) expression in all species, including the mare (124). P450arom and 17-ketosteroid reductase then convert androgens to estradiol-17β (46), the major and most potent of the estrogens synthesized in the ovary (108, 110) (Figure 9).

3.2.1 Steroidogenic acute regulatory protein

The steroidogenic response to hormonal stimuli has long been known to occur in two phases; an acute phase which requires new protein synthesis and occurs within minutes (118) and a secondary phase which begins approximately one hour afterwards if the stimulus is maintained (118). Further studies identified the rate-limiting step in the acute steroidogenic response as being the transfer of cholesterol from the mitochondrial outer membrane to the inner membrane, where it becomes accessible to P450scc (125). While the search for the labile protein responsible for this activity yielded several candidates (126), the most compelling evidence now implicates steroidogenic acute regulatory protein (StAR). StAR mRNA is rapidly induced in steroidogenic cells in response to hormonal stimuli and cAMP, and this closely parallels increases in steroid production (125). Furthermore, the introduction of a StAR cDNA into

steroidogenic as well as non-steroidogenic cells resulted in increased steroid production in absence of hormonal stimulus (125). The distribution of StAR expression is limited almost exclusively to steroidogenic cells (127-129), in which the protein has been localized to contact sites between the inner and outer mitochondrial membranes (130). Further genetic evidence of the role of StAR in steroidogenesis came with the discovery that lipoid congenital adrenal hyperplasia (LCAH), a rare human autosomal recessive disorder, results from mutations in the StAR gene (131). LCAH patients suffer notably from profound adrenocortical insufficiency and male pseudohermaphroditism caused by insufficient fetal testosterone synthesis during development (132). This phenotype was closely mimicked when targeted StAR gene disruption experiments were performed in mice, who are all born with female external genitalia and die rapidly from respiratory failure, presumed to result from adrenocorticoid deficiency (133).

3.2.1.1 Biochemistry and enzymology of StAR

The human StAR gene consists of seven exons spread over approximately 8 kb (134), and is transcribed into a 1.6 kb major transcript along with minor 4.4 and 7.5 kb messages (127, 129). Considerable variability has been observed in the number and sizes of StAR transcripts in other species, likely resulting from differences in 3' untranslated regions that have been proposed to influence translation (31, 132, 135-139). StAR mRNAs are translated into highly conserved proteins of 284 or 285 amino acids which feature an N-terminal mitochondrial

targeting sequence that is cleaved following mitochondrial import, resulting in a mature protein of 30 kDa (140). Mutational analyses (141, 142) as well as genetic data collected from LCAH patients (143) have indicated that the enzymatic activity of StAR resides within its C-terminus. In addition, phosphorylation of StAR occurs *in vivo* at least at two protein kinase A sites, one of which appears to be required for full activity (144).

An initial model describing the mechanism of StAR action suggested that it forms a conduit for the transfer of cholesterol down the chemical gradient that exists between the mitochondrial membranes (126). This was consistent with its rapid mitochondrial import and processing and its localization at contact sites of the inner and outer mitochondrial membranes. Recent data disproves this model however, as it has been clearly shown that targeting to the mitochondria is not required for StAR action, suggesting that it acts on their outer membrane (141, 142). In addition, it has been demonstrated that StAR can function as a sterol transfer protein, likely enhancing the desorption of cholesterol molecules from one membrane to another (119). Current models therefore suggest that while StAR is directed to the mitochondria via its N-terminus, its C-terminus interacts with the outer mitochondrial membrane to direct cholesterol transfer (145) (Figure 10). Final import into the mitochondria may therefore represent the mechanism by which StAR is ultimately inactivated (125, 132).

3.2.1.2 Hormonal regulation of ovarian StAR expression

It is now well established that StAR expression in steroidogenic cells is

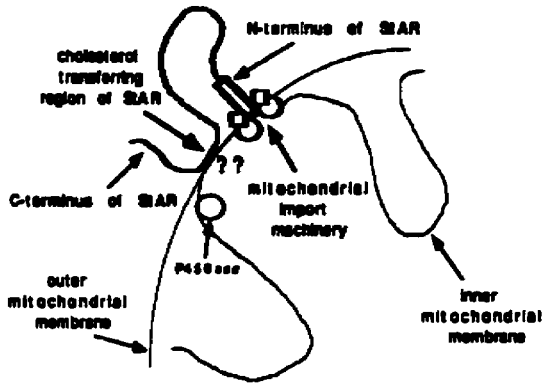

Figure 10. The known mechanism of StAR action. (Adapted from reference 125)

regulated by tropic hormones which act via the cAMP/protein kinase A (PKA) signalling pathway (125, 132). This was demonstrated notably in corpora lutea, as StAR expression in hypophysectomized sheep increased in response to exogenous LH (146). Further studies in other species confirmed that gonadotropins stimulate luteal StAR expression (142, 147). Conversely, luteolytic stimuli such as PGF2α (31, 38, 146, 148) or estradiol-17β deprivation in rabbits (149) resulted in losses of StAR expression. The granulosa cells of maturing follicles express low levels of StAR (29, 30), but this is markedly induced *in vitro* and *in vivo* by cAMP (29, 150), eCG/hCG (30, 31, 151), FSH, forskolin and IGF-1 (152-154). Studies conducted in an immature rat model have indicated that high levels of StAR expression in granulosa cells are induced in parallel with the onset of the luteinization process, and this is presumably triggered *in vivo* by the LH surge (30, 132).

3.2.1.3 Transcriptional regulation of StAR in the ovary

Initial evidence for the implication of the orphan nuclear receptor steroidogenic factor-1 (SF-1) in the transcriptional regulation of StAR came when it was noticed that SF-1 null mice do not express StAR mRNA (155). Sequence analysis of rat, human, bovine and mouse StAR promoters revealed several potential SF-1 binding sites, though transient transfection and electrophoretic mobility shift assays subsequently demonstrated vastly different degrees of functional importance for each element (150, 155-158). While these studies have established a role for SF-1 in the basal transcription of StAR in several species, it remains unclear if or how cAMP acts upon SF-1 to up-regulate StAR expression in response to hormonal stimuli. This is underscored by one study indicating that an SF-1 element in the murine StAR promoter is not required for hormonal induction (155), though data from other reports imply that species- and cell type-specific variations may exist (134, 156-158).

Recent data have identified additional transcription factors that may be responsible for the hormonal inducibility of the StAR promoter. Two C/EBP response elements have been identified in both the mouse and human promoters, and C/EBPβ binds one of these with high affinity (159, 160). C/EBPβ binding was further shown to be required for both basal and inducible StAR transactivation, and it has been proposed to functionally interact with SF-1 (125, 159, 160). Sterol regulatory element binding protein-1a (SREBP-1a) also stimulates StAR promoter activity in transient transfections, although its binding site remains to be identified (161). Conversely, the transcription factor dosage-sensitive sex reversal, adrenal hypoplasia congenita, X chromosome,

gene 1 (DAX-1) binds a stem-loop structure in the StAR promoter, thereby inhibiting transcription of the StAR gene (162). Furthermore, DAX-1 may also downregulate StAR expression by a direct, protein-protein interaction with SF-1 (163). While both SF-1 and C/EBPβ have been implicated in the regulation of the StAR promoter in the adult ovary as detailed above (150, 158, 160), it remains to be shown if SREBP-1a, DAX-1 or other factors play important roles in this tissue.

3.2.2 Cytochrome P450 cholesterol side-chain cleavage

Early studies suggested that the sequential conversions of cholesterol to 22-hydroxycholesterol, 20,22-hydroxycholesterol and pregnenolone were mediated by distinct enzymes (116). However, *in vitro* reconstitution assays employing purified proteins ultimately demonstrated that all steps could be attributed to a single protein complex, termed cytochrome P450 cholesterol side-chain cleavage (P450scc) (116).

3.2.2.1 Biochemistry and enzymology of P450scc

The human gene encoding P450scc spans over 20 kb and consists of nine exons (164). This is transcribed into a single mRNA species of approximately 2.0 kb that encodes a 520 aa protein (165) which is highly conserved throughout evolution, as demonstrated by the subsequent cloning of P450scc from species ranging from rodents (166) to fish (167). The protein consists of an N-terminal

mitochondrial leader sequence, followed by putative functional domains proposed to bind cholesterol substrate molecules, coordinate a heme group and associate with components of the electron transfer chain (168). Following translation, P450scc is translocated to the mitochondrial inner membrane by a poorly understood, ATP-dependant mechanism, and the leader sequence is cleaved by a specific peptidase (169). P450scc then associates with a larger complex of over 850 kDa that consists of 16 subunits (116). These mitochondrial import and maturation steps are apparently obligatory, as improperly-targeted P450scc proteins fail to produce pregnenolone (170).

P450scc catalyzes pregnenolone formation by the successive actions of three distinct catalytic activities; 22-hydroxylase, 20-hydroxylase and 20,22-lyase (Figure 11) (116). Electrons required for these oxidations to occur are donated by NADPH, and three such molecules are required per molecule of pregnenolone generated (Figure 11). Rather than being used directly by P450scc, the electrons flow along a chain, and are initially accepted by the flavoprotein adrenodoxin reductase, which is loosely adherent to the inner mitochondrial membrane. These are transferred to adrenodoxin, an iron/sulfur protein which diffuses freely in the mitochondrial matrix and ultimately binds P450scc, which then functions as the terminal oxidase (116, 171).

3.2.2.2 Hormonal regulation of ovarian P450scc expression

A variety of hormonal agents have been shown to modulate P450scc mRNA levels in cultured granulosa and thecal cells. These include up-

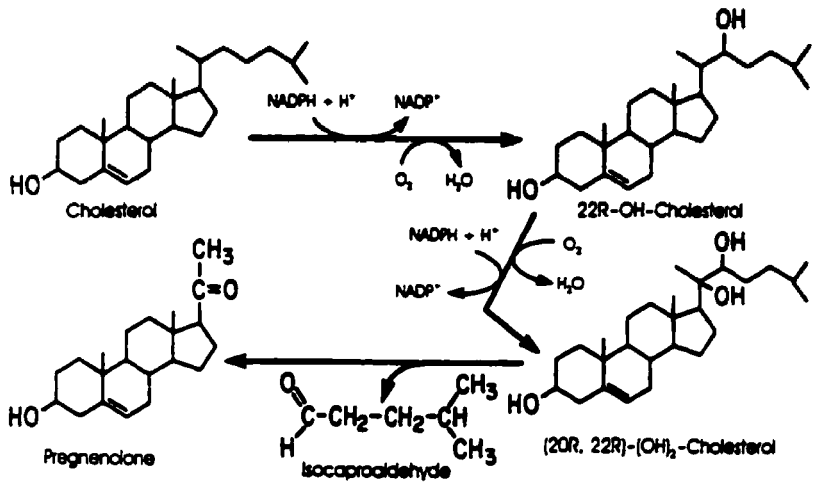

Figure 11. Enzymatic reactions catalyzed by the cytochrome P450 cholesterol side-chain cleavage complex. (Adapted from reference 46)

regulations by FSH/IGF-1 (172-176), activin (177), GH (178), cAMP (174, 179-181), estradiol (35, 182), prolactin (166), EGF (181) and LH (179), whereas long-term phorbol ester treatment (183) and angiotensin II (181) cause a down-regulation. Studies performed *in vivo* have demonstrated that P450scc expression in rat granulosa cells increases in response to gonadotropins and cAMP (181) but, following the LH surge, it becomes cAMP-independant and responds to different hormonal agents (166). The LH-induced luteinization process also leads to a marked up-regulation of P450scc mRNA in several species, when levels present in luteal cells are compared to preovulatory granulosa cells (32-38). The LH surge itself however has species-specific effects on P450scc expression in the short term, as a transient down-regulation has been observed in cows and monkeys (32, 40, 184), but no significant change has been

observed in pigs or sheep (33, 39), while P450scc mRNA levels rise rapidly in rats (166).

3.2.2.3 Transcriptional regulation of P450scc in the ovary

The transcriptional mechanisms underlying the hormonal regulation of P450scc in granulosa cells have been extensively studied in several species. Several key cis-acting elements have been identified, including an SF-1 element required for tissue-specific, cAMP-induced P450scc expression (185-188). A link to the basal transcriptional machinery is then provided by the coactivator CREB binding protein (CBP)/p300, which has been shown to functionally bind SF-1 on the P450scc promoter and enhance transcription (189, 190). Another nearby element binds the transcription factor Sp1 (187, 191-194), and a direct, synergistic interaction between SF-1 and Sp1 has been proposed to occur in response to cAMP (190). Activator protein-2 (AP-2) binds Sp1 to activate P450scc transcription in cells that do not express endogenous SF-1 (194), but whether or not it plays a role in modulating P450scc expression in granulosa cells remains to be determined.

3.2.3 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase

The conversion of Δ5-3β-hydroxysteroids to Δ4-3-ketosteroids is catalyzed by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD), and is a required step in the biosynthesis of all essential adrenal and gonadal steroid

hormones (195) (Figure 12). Numerous cases of 3β-HSD deficiency have been identified in humans. Confoundingly, while these patients suffer from adrenal hyperplasia and male pseudohermaphroditism resulting from insufficient fetal testosterone production, both sexes show signs of virilization at puberty (196). Also unexpectedly, 3β-HSD expression was found in non-steroidogenic tissues, such as sebaceous glands, kidneys and brain (196). Taken together, these data suggested the existence of a primary adrenal/gonadal 3β-HSD isoform, along with one or more peripheral isoform(s) that could partially compensate for primary 3β-HSD deficiency (195, 196). This notion was confirmed by molecular cloning, as two distinct 3β-HSD genes have been identified in humans, along with six genes in rats and mice and likely incomplete sets of orthologs in other species (195-197). These isoforms differ notably by their tissue distributions and mechanisms of gene regulation (197). The 3β-HSD gene expressed in adrenal and gonadal tissues is refered to as type II in humans and type I in all other species, reflecting the chronology of their cloning rather than their being distinct genes (197).

3.2.3.1 Biochemistry and enzymology of 3β-HSD

The structure of human type II 3β-HSD has been characterized, and is split into four exons and three introns spread over approximately 7.8 kb (195). This is transcribed into a 1.7 kb mRNA (37, 198) that encodes a highly conserved 372 aa protein (195). Other 3β-HSD genes encode proteins of apparent molecular weights varying from 40 to 46 kDa (196, 197) that share the structural

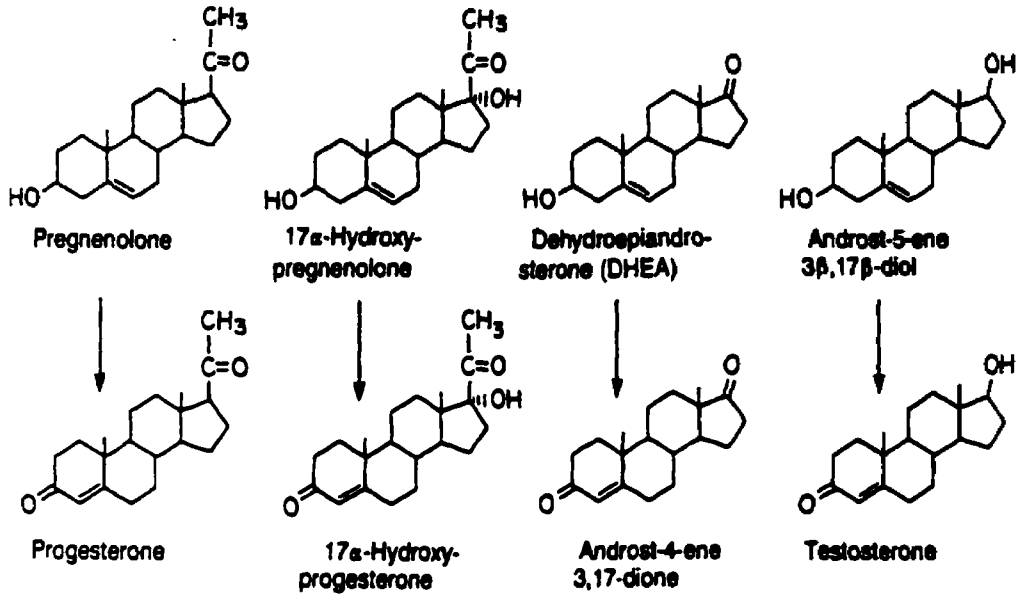

Figure 12. Enzymatic reactions catalyzed by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase in gonadal tissues. (Adapted from reference 46)

hallmarks of the primary gonadal/adrenal isoform (195). Putative functional domains include two transmembrane segments (195) which anchor the protein to both the endoplasmic reticulum and mitochondrial inner membrane (130, 197). As is characteristic of members of the short chain alcohol dehydrogenase superfamily, 3β-HSD also possesses an N-terminal GXXGXXG motif that is believed to bind the AMP moiety of the cofactor NAD^+ (195). Another characteristic of this superfamily is the YXXXK motif that localizes to the catalytic active site, and two such elements are present in most 3β-HSD homologs (195). Furthermore, one of these motifs is located within a region identified by labelling studies to be the site of steroid binding and isomerase activities (195).

The conversion of Δ5-3β-hydroxysteroids to Δ4-3-ketosteroids involves the successive actions of 3β-hydroxysteroid dehydrogenase and Δ5-Δ4 isomerase activities. Protein purification experiments as well as later *in vitro* expression studies were required to demonstrate that both activites reside within a single 3β-HSD protein (195). A model describing the catalytic mechanism has been developed from affinity radiolabeling and inhibition data (195). This model suggests the existence of a single, bifunctional catalytic site that functions initially as a 3β-hydroxysteroid dehydrogenase that uses NAD^+ as a cofactor. The NADH molecule that is generated by this reaction would then serve to induce a conformational change that activates the latent isomerase activity (195). The non-adrenal/gonadal 3β-HSD isoforms have been shown to possess markedly different catalytic properties with respect to rate of catalysis, cofactor preference and affinity for various substrates (196). Interestingly, mouse type IV and V and rat type III 3β-HSD apparently function as NADPH-dependant 3-ketosteroid reductases (196). As these are expressed in the kidney and male liver, this suggests that their principal role may be to inactivate steroid hormones such as dihydrotestosterone (196).

3.2.3.2 Hormonal regulation of ovarian 3β-HSD expression

Several hormonal agents have been reported to modulate 3β-HSD expression in cultured ovarian cells, including transforming growth factor-β (123), insulin (123), insulin-like growth factor-β (173), activin (123, 177), FSH (173, 177, 199), hCG/LH (200, 201) angiotensin II (123) and cAMP (123, 200, 201).

Studies *in vivo* examining the regulation of 3β-HSD in ovarian interstitial and luteal cells have reported that hormonal stimuli such as LH and prolactin can up-regulate its expression (202-206). Conversely, luteolytic (i.e. high dose) treatments of prolactin (203, 204) or $PGF_{2\alpha}$ (33, 207, 208) have the opposite effect on corpora lutea. Much like P450scc, higher levels of 3β-HSD mRNA are present in corpora lutea when compared to preovulatory follicles in several species (33, 37, 39, 41, 42, 209), indicating that the LH-induced luteinization process up-regulates 3β-HSD gene expression. However, the LH surge has species-specific effects in the short term, as it induces a transient down-regulation of follicular 3β-HSD mRNA in cows (32), but a rapid increase in monkeys (184).

3.2.3.3 Transcriptional regulation of 3β-HSD in the ovary

Reports of the transcriptional regulation of 3β-HSD have to this date been limited to the adrenal/gonadal isoform, and have examined only adrenal (210) and non-steroidogenic (206, 210) cell lines. Results have identified an SF-1 element within the 3β-HSD promoter that is required for a synergistic response to cAMP and phorbol ester (210). As gonadotropins act via both the protein kinase A an C pathways (211), it has therefore been inferred that gonadotropin-mediated control of 3β-HSD expression occurs through SF-1 (206). Similarly, a response element that binds signal transducer and activator of transcription-5 (Stat5) has been implicated in the up-regulation of 3β-HSD in response to prolactin (206). The notion that this could also apply to ovarian cells is

supported by the fact that Stat5a/Stat5b double knockout mice are infertile and do not form corpora lutea (212). However, a direct demonstration of the use of either the SF-1 or Stat5 elements in ovarian cell 3β-HSD transcriptional response to hormonal stimuli has not been reported as yet.

3.2.4. Cytochrome P450 17α-hydroxylase

Cytochrome P450 17α-hydroxylase (P45017α) is another bifunctional steroidogenic enzyme, as it catalyzes androgen formation by the successive actions of its 17α-hydroxylase and 17,20 lyase activities (116) (Figure 13). In spite of irrefutable demonstrations that a sole protein is indeed responsible for both enzymatic activities, P45017α also acts as a single-function 17α-hydroxylase in the adrenal cortex to direct the synthesis of glucocorticoids and mineralocorticoids (116). It has thus been proposed that tissue- and developmental stage-specific mechanisms differentially regulate each activity, and P45017α is the only cytochrome P450 enzyme in which this is believed to occur (213). P45017α is therefore refered to as the qualitative regulator of steroidogenesis, as it determines the identity of the final hormonal product by selectively applying either one or both of its enzymatic activities (213).

3.2.4.1 Biochemistry and enzymology of P45017α

Human P45017α is encoded by a single, 6.0 kb gene that is split into eight exons (116), and is transcribed into an mRNA of approximately 1.8 kb (37). This

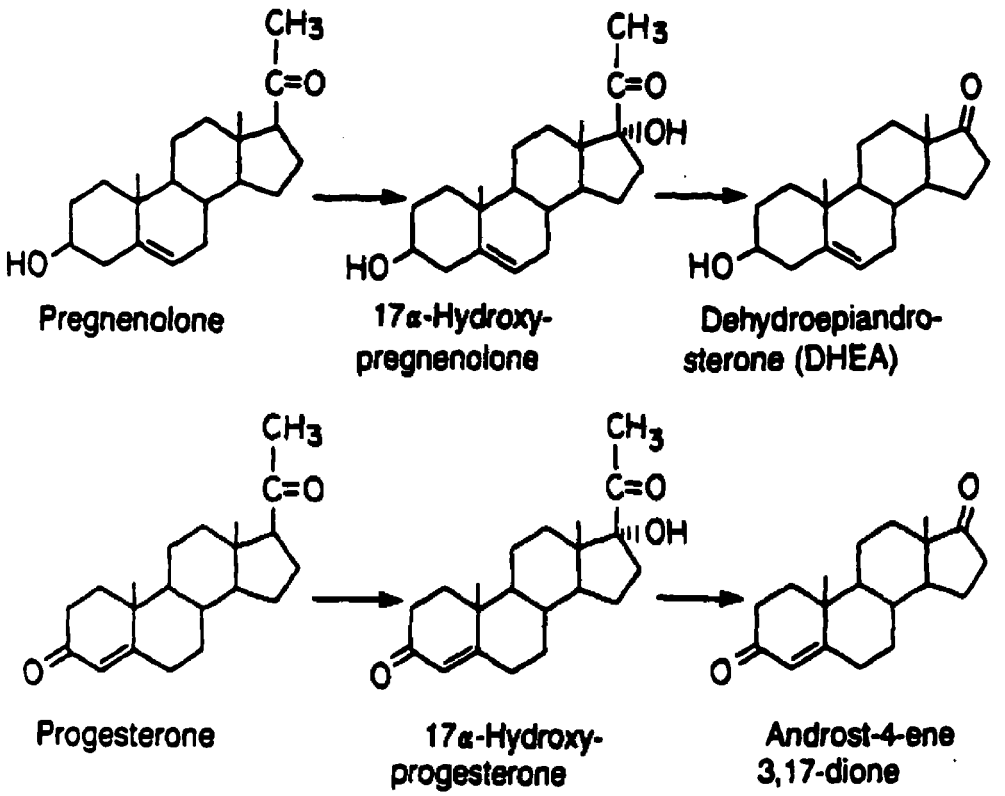

Figure 13. Enzymatic reactions catalyzed by cytochrome P450 17α-hydroxylase in the ovary. (Adapated from reference 46)

is translated into a protein with a single, bifunctional catalytic site and that features all of the structural hallmarks of the cytochrome P450 family, including a heme binding site, a sterol binding site, and a domain that interacts with its electron transfer partner (116, 213). Unlike P450scc, P45017α protein localizes to the smooth endoplasmic reticulum, and therefore accepts electrons from a distinct chain (116). While the electron transfer protein cytochrome b_5 can selectively augment P45017α activity, the flavoprotein P450 oxidoreductase (OR) alone suffices to deliver electrons from NADPH to P45017α, and is believed to be its sole redox partner under physiological conditions (213).

The mechanism(s) by which the activities of P45017α are differentially regulated have been extensively studied, and are believed to involve only post-transcriptional mechanisms (213). As OR is considerably more abundant in testis than in the adrenal gland, it was suggested that a higher OR: P45017α molar ratio could favor the 17,20 lyase reaction, thereby explaining the higher rate of androgen production in gonadal tissues (213). This notion is supported by *in vitro* kinetic analyses in yeast that demonstrated that the 17,20 lyase reaction is more severely impeded than the 17α-hydroxylase reaction when OR concentrations are low (214). Furthermore, human patients suffering from 17,20 lyase deficiency were shown to have mutations in the region of the P45017α protein responsible for interacting with OR, resulting in severely impaired 17,20 lyase activity but only mildly diminished 17α-hydroxylase function (215). Another mechanism involved in the differential regulation of P45017α activities is cAMP-inducible serine/threonine phosphorylation, which is associated with a selective increase in 17,20 lyase activity (216). While the mechanism by which phosphorylation accomplishes this remains to be elucidated, an increase in P45017α affinity for OR has been proposed (213). Interestingly, polycystic ovary syndrome (POCS), a disorder characterized notably by ovarian and adrenal hyperandrogenism in women (217, 218), is associated with kinase hyperactivity that results in insulin receptor hyperphosphorylation (219). It has therefore been postulated that a similar mechanism may result in P45017α hyperphosphorylation in POCS patients, thereby causing inapproprite 17,20 lyase activity levels and ensuing excess androgen production (220).

3.2.4.2 Hormonal regulation of ovarian P45017α expression

The expression of P45017α, which is essentially limited to the theca interna of ovarian follicles (32, 34, 39, 221), is believed to be regulated *in vivo* primarily by LH during follicular development (220). The subsequent LH surge has species-specific effects on P45017α expression, varying from a slight down-regulation in humans (37) to near-silencing in cows, pigs and rats (32, 39, 43). This apparently reflects the distinct nature of the human corpus luteum, which retains the ability to synthesize significant amounts of aromatizable androgens (37). Several additional factors have been identified that modulate P45017α mRNA levels in various *in vitro* systems, including insulin (222), IGF-1 (220) and inhibin (223), which all stimulate its expression. The opposite effect has been observed upon addition of TGF-β (224, 225), corticotropin-releasing factor (226) or activin (223).

3.2.4.3 Transcriptional regulation of P45017α in the ovary

Two distinct regions of the human and bovine P45017α promoters have been associated with cAMP responsiveness (227, 228). The first, termed CRS1, binds at least four nuclear proteins, two of which have been identified as being the TALE homeodomain transcription factors Pbx and Meis1 (220, 227, 230, 231). While CRS1 is clearly required for inducible P45017α expression in adrenal cells (232), studies in bovine thecal cells indicate that the second cAMP responsive region, CRS2, plays a more important role in the ovary (233). CRS2 consists of

an overlapping set of binding sites for the transcription factors SF-1 and chicken ovalbumin upstream promoter transcription factor (COUP-TF), which acts as a negative regulator by preventing SF-1 binding (227, 234). A functionally similar sequence has been identified in the rat promoter that binds two additional positive transcriptional regulators; nerve growth factor-induced gene B (NGF-IB) and the proto-oncogene SET (235, 236). While a role for SF-1 has been proposed in both the basal and cAMP-induced transactivation of the P45017α promoter, evidence has also been obtained that its purpose is restricted to basal transcription in ovarian cells (227-229, 233, 234). Indeed, an additional element that binds Sp1 is apparently required for cAMP-induced P45017α transcription in thecal cells (237). Androgen receptor (AR) can bind to the CRS1 in testicular Leydig cells, thereby providing a mechanism for androgens to limit their own synthesis by downregulating P45017α transcriptional activity (238). Similarly, Müllerian inhibitory substance (MIS), a hormone implicated in sexual differentiation, can downregulate testicular androgen synthesis at the level of P45017α gene transcription (239). However, roles for AR and/or MIS in modulating ovarian steroidogenesis at the transcriptional level have not been reported thus far.

3.2.5 Cytochrome P450 aromatase

The conversion of androgens to estrogens is performed by cytochrome P450 aromatase (P450arom). As evidenced by the phenotype of P450arom null mice, this activity is absolutely required for normal reproductive functions,

including the regulation of gonadotropin secretion, follicular development, ovulation and spermatogenesis (see section 3.1). In addition, P450arom expression in the non-gonadal tissues of vertebrate species suggests further roles, such as the local synthesis of brain estrogens that plays a role in determining sexually dimorphic reproductive behavior (240). Other sites of estrogen synthesis include the placenta, liver, bone, skin and adipose tissue of certain species (241). While the physiological roles of these locally-produced estrogens remain to be elucidated, dysregulation of P450arom expression has been observed in several pathological conditions, including endometriosis (242) and uterine and hepatic tumors (243-245). Most notably however, a dramatic up-regulation of P450arom expression can occur in breast tissues during carcinogenesis, thereby generating estrogens that stimulate tumor growth in an autocrine/paracrine manner (246). This discovery has stimulated the search for specific pharmacological inhibitors of P450arom activity, which has been aided significantly by the development of computer models of the active site (246).

3.2.5.1 Biochemistry and enzymology of P450arom

The coding sequences of human P450arom are split amongst nine exons (numbered II through X) that span over 75 kb (247-249). Remarkably, at least eight distinct non-coding first exons are known to exist, which are spread over at least 40 kb and named I.1, I.2, I.3, I.4, I.5, I.6, 2a and If (250, 251). These are alternatively spliced to exon II at a common site, giving rise to transcripts that differ only in their 5'-untranslated regions. Use of each exon I is presumably

linked to a distinct promoter, and transcription can also be initiated from the region immediately upstream of exon II, refered to as promoter II (252). Each P450arom promoter that has been characterized to date is structurally and functionally distinct, and includes elements involved in the response to particular stimuli (250). One consequence of this convoluted regulatory mechanism is that promoter usage occurs in a tissue-specific manner. For instance, FSH-responsive ovarian P450arom expression in the ovary is directed mainly by promoter II (252, 253), whereas promoter I.4 controls glucocorticoid-stimulated transcription in adipose tissue (250), and promoter If has been described as brain-specific and androgen-inducible (254, 255). Regulation of the P450arom gene is rendered even more complex by several physiological and pathological processes that induce promoter switching. For instance, the P450arom transcripts present in porcine placental tissues in early pregnancy result from the use of a different promoter than those found in midpregnancy (256). Similarly, while promoter I.4 is used in normal adipose tissue, transcripts derived from promoters I.4, I.3 and II are found in cancerous tissue; a switching mechanism that is likely critical for the carcinogenic process itself (246, 257-261). While best described in humans, the structure of the P450arom gene appears equally complex in other mammalian species, who all show evidence of alternative exon I usage (263-266). Furthermore, while P450arom is believed to be a single-copy gene in humans (250), evidence exists for several distinct genes in pigs (267).

The human P450arom gene is transcribed into two mRNAs of 2.9 and 3.4 kb that differ in the lengths of the 3'-untranslated regions (37), although the

number and size of observable transcripts varies amongst species (181). The primary structure of the P450arom protein is highly conserved over considerable evolutionary distance, as evidence by its cloning from species ranging from human to chicken to trout (262, 268, 269). It consists of 503 amino acids in mammalian species, and includes domains proposed to bind heme, serve as a membrane-spanning region and bind substrate (250, 270), along with a site at which N-linked glycosylation occurs (271, 272). P450 arom localizes to the endoplasmic reticulum, where it associates with the flavoprotein P450 oxidoreductase (273). It is this protein that is reponsible for electron transfers from the three molecules of NADPH that required for P450arom action, which results in the loss of the carbon group at position 19 and the aromatization of the A ring (Figure 14). While the catalytic mechanism remains to be conclusively elucidated, it is believed that two oxygen molecules oxidize the C19 carbon to form a 19-oxo intermediate. A third molecule would most likely then form an unusual iron-peroxide intermediate with the heme group iron atom, which would in turn attack the 19-oxo carbon, resulting in a one-step deformylation/aromatization (274). This mechanism is supported by molecular modeling techniques, which place key threonine (T310) and glutamate (E309) residues in sufficient proximity to the heme-iron to participate in the catalytic process (250).

3.2.5.2 Hormonal regulation of ovarian P450arom expression

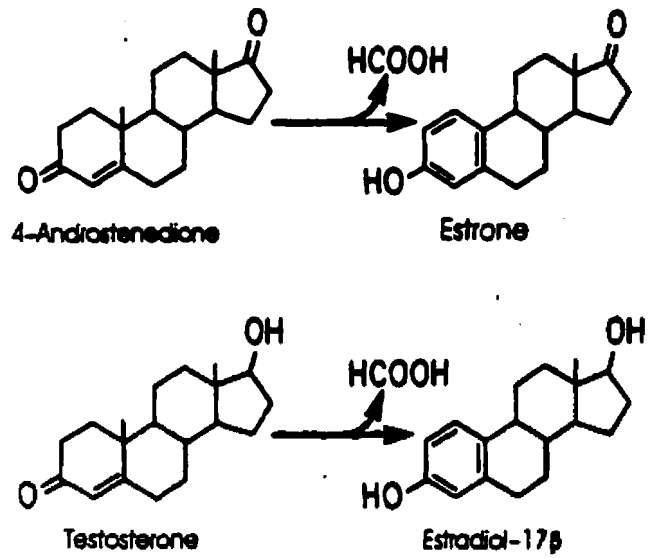

Figure 14. Enzymatic reactions catalyzed by cytochrome P450 aromatase in the ovary. (Adapted from reference 46)

Experiments conducted *in vivo* using an immature rat model have demonstrated that P450arom expression in the granulosa cells of developing follicles is controlled by FSH, and can be synergistically augmented by estrogen (181). The LH surge then rapidly and dramatically decreases P450arom mRNA levels in all species examined thus far (32, 34, 39, 44, 275), and this effect can be reproduced *in vitro* with cultured granulosa cells by treatment with LH, hCG or forskolin (181). Interestingly, luteinized granulosa cells maintain a reduced level of P450arom expression via a cAMP-independant mechanism (181), leading to the speculation that a switching mechanism occurs from cAMP-responsive to cAMP-independant promoter usage (257). However, this was later disproven by a study demonstrating that promoter II is the major regulator of P450arom expression in both granulosa cells and the corpus luteum (252).

In vitro studies have identified additional hormonal agents capable of modulating P450arom expression. These include prolactin, which can increase P450arom mRNA in granulosa cells and decrease it in luteal cells (181). Similarly, while EGF treatment results in a down-regulation of P450 expression in granulosa cells, IGF-1 has the opposite effect, augmenting P450arom mRNA in synergy with FSH (181).

3.2.5.3 Transcriptional regulation of P450arom in the ovary

Whereas numerous regulatory elements have been identified that modulate P450arom expression in response to various stimuli (250), only two have been identified in promoter II, the predominant promoter influenced by gonadotropin stimulation in the ovary (252, 253). As FSH action is mediated by cAMP, initial searches centered around the identification of a cAMP-response element. This lead to the description of a site in the human, rat and bovine promoters that binds SF-1 and is responsible for both basal and inducible P450arom promoter activity (275-277). Subsequently, a sequence that differs only slightly from a classic cAMP response element (CRE) was identified in human and rat that binds cAMP response element binding protein (CREB) and acts in synergy with SF-1 to promote inducible P450arom expression (276-278). Interestingly, the CRE element present in the bovine and porcine promoters features a 1 bp deletion that renders it non-functional (279-281), indicating significant species-specific differences in how P450arom is regulated. Studies in eutopic endometrial cells have shown that COUP-TF can compete with SF-1 for

its binding site, thereby silencing P450arom expression (282). Conversely, endometreotic tissues show inappropriate high levels of P450arom mRNA, and this apparently results from SF-1 activating transcription by displacing COUP-TF (282). No evidence however exists as yet for COUP-TF-mediated regulation of promoter II in granulosa cells.

3.2.6 Steroidogenic factor-1

Steroidogenic factor-1 (SF-1), also known as adrenal-4 binding protein (Ad4BP), is a member of the NR5A subclass of nuclear receptors (283). SF-1 was originally isolated as a transcription factor capable of binding discrete regulatory elements present in the promoters of various steroid hydroxylases (284-287). Since then, its known scope of action has been largely expanded. Notably, embryological expression studies and a subsequent gene knockout analysis have demonstrated a critical role for SF-1 in the development of the steroidogenic organs, as SF-1 null mice exhibit adrenal and gonadal agenesis and male-to-female sex reversal (288, 289). Similar studies further demonstrated that SF-1 is required for the proper development of all levels of the hypothalamic-pituitary-gonadal axis (290). Specifically, SF-1 null mice are characterized by virtual ablation of their pituitary gonadotrope cells and regions of their dorsomedial and ventromedial hypothalamic nuclei, resulting in inadequate LH, FSH and GnRH secretion (290). In adult tissues, SF-1 has been implicated in the transcriptional regulation of a number of functionally unrelated genes. In addition to the cytochrome P450 steroid hydroxylases (185,

187-190, 234-236, 268, 277, 282, 291-297), the list of SF-1 target genes now includes 3β-HSD (210), StAR (150, 155-159), adrenocorticotropic hormone receptor (298-300), Müllerian inhibiting substance (301, 302), glycoprotein subunit α (303), LH β-subunit (304-310), GnRH receptor (311-313), N-methyl-d-aspartate receptor subunit NR2C (314), prolactin receptor (315), Leydig insulin-like gene (316), SR-BI (317), α2-macroglobulin (318), high density lipoprotein receptor (319), anti-Müllerian hormone type II receptor (320), small heterodimer partner (321), oxytocin (322, 323) and DAX-1 (285, 324, 325). The scope of SF-1 action therefore defines it as a pleitropic regulator of diverse metabolic and developmental processes.

3.2.6.1 Molecular biology of SF-1

SF-1 is encoded by a structurally complex gene named after its *Drosophila* homolog fushi tarazu factor 1 (*Ftz-F1*). *Ftz-F1* has been cloned in several species, and all genes identified thus far encode at least two transcripts (291). For instance, murine *Ftz-F1* is transcribed into four distinct mRNA species, designated SF-1, embryonal long terminal repeat-binding protein 1 (ELP1), ELP2 and ELP3, which arise from the use of different promoters and splicing mechanisms (291). While the ELP transcripts are characterized by differences in expression patterns, their functions remain largely unknown (291). The SF-1 transcript encodes a 461 or 462 aa protein (depending on species) that features an N-terminal DNA-binding domain consisting of two zinc finger modules, known as the P box and D box (291). While the P box of most nuclear receptors

determines the site to which they bind, SF-1 employs an additional domain, the A box, to contact DNA regions 5' of the hexamer half-site and dictate its overall DNA-binding specificity (326). Also unconventionally, SF-1 and a few other structurally related transcription factor are able to interact with DNA as monomers (326). Near the C-terminus of SF-1 is a region homologous to the ligand-binding domains of ligand-activated nuclear receptors (291). While SF-1 is refered to as an orphan nuclear receptor due to its lack of a recognized ligand, one study has suggested that it may be activated by oxysterol binding (327). Recent studies however propose that this domain is involved in mediating protein-protein interactions that modulate its transactivational activity (301, 328-330). Another functional domain at the C-terminus of SF-1 is a short alpha helix known as activation function-2 (AF-2). AF-2 has been shown to modulate the transactivation activity of SF-1, and is absolutely required for the cAMP-induced transcriptional up-regulation of the P450$17\alpha$ target gene (331).

Additional mechanisms of SF-1 protein activity regulation have been elucidated. A serine residue that is apparently phosphorylated by mitogen-activated protein kinase has been implicated in recruiting the transcriptional cofactors GRIP1 and SMRT, and this is required for maximal SF-1 activity (332). Phosphorylation of SF-1 at a putative C-terminal protein kinase A site has also been proposed as a means by which its transactivational properties may be activated (276, 291, 297). Additional studies have also shown that SF-1 activity can be modulated by specific protein-protein interactions. Notably, synergistic interactions between SF-1 and proteins such as Ptx1 (333), Wilms' tumor 1 (334), steroid receptor coactivator-1 (328, 330), SOX9 (301) and p300/CBP (189, 330) are

required for the transcriptional activation of specific target genes. Conversely, SF-1 mediated transactivation has been shown to be antagonized by its association with DAX-1 (163, 329, 334) and the nuclear receptor corepressor N-CoR (329).

3.2.6.2 Hormonal regulation of SF-1 expression

Few physiological processes have been shown to impact significantly upon the transcriptional activity of the SF-1 gene. Endogenous GnRH was shown to have an up-regulatory effect on SF-1 expression in the pituitary of gonadectomized rats, and a similar effect was observed when exogenous GnRH was administered to GnRH-deficient animals (335). In the rat ovary, the LH surge was shown to cause a transient down-regulatory effect on SF-1 mRNA, protein and binding activity levels in granulosa cells (44, 286, 336). As this occurs in parallel with the LH-induced loss of P450arom expression and SF-1 is known to transactivate P450arom promoter II (see section 3.2.5.3), it has been proposed that the down-regulation of SF-1 mRNA is the direct cause of the silencing of the P450arom gene (286, 336).

3.2.6.3 Transcriptional regulation of SF-1

The transcriptional mechanisms underlying SF-1 expression have been studied in different cell culture systems. An E box in the SF-1 promoter that binds USF has been shown to be required for maximal expression in

steroidogenic cells (337-339). Additional elements within 90 bp of the transcriptional start site bind Sp1 and CAAT binding factor (338), although these do not appear to be required for promoter activity.

HYPOTHESES AND OBJECTIVES

It is well established that the LH surge induces both ovulation and luteinization, and that these processes are associated with increases in follicular prostaglandin and progesterone biosynthesis, as well as a decrease in estradiol-17β production. The overall hypothesis of the work presented herein is that these events result from the modulation of the expression of specific genes.

The general objective of this thesis was to elucidate some of the gene regulation events induced by the LH surge that result in the preovulatory accumulation of prostaglandins in the follicular fluid and the changeover of the predominant steroid hormone product from estradiol-17β to progesterone. The studies also aimed to establish the mare as a model for the study of ovarian gene expression in monoovulatory species, as features of the equine ovarian follicle render it particularly well suited for such analyses. Specific objectives were:

1. To clone and characterize cDNAs encoding equine PGHS-2, StAR, P450scc, 3β-HSD, P45017α, P450arom and SF-1.
2. To study the mRNA regulation of these genes during the ovulatory process, as well as their cellular distribution within the follicle.
3. To integrate these data into models describing the regulation of ovarian prostaglandin and steroid hormone biosynthesis, thus identifying the key genes involved in determining or rate-limiting steps.
4. To generate the tools needed to investigate the transcriptional regulation of these key genes. This involved the cloning and characterization of segments of the PGHS-2 and P450arom genes and promoters.

Summary of article #1

Title: Molecular characterization of equine prostaglandin G/H synthase-2 and regulation of its messenger ribonucleic acid in preovulatory follicles. **Derek Boerboom** and Jean Sirois. Endocrinology 139(4):1662-1670 (1998).

Thesis author's contribution to the work: As the primary author, I was responsible for all aspects of the production of this article.

Summary:

- Equine PGHS-2 was cloned from a follicular cDNA library designed for that purpose.
- Sequence analysis revealed an open reading frame that encodes a 604-amino acid protein that is more than 80% identical to PGHS-2 species homologs, as well as numerous repeats of the Shaw-Kamen's sequence, a motif typically indicative of a short mRNA half-life.
- The complete equine PGHS-2 gene was isolated from a genomic library and sequenced. The equine PGHS-2 gene structure is similar to its human homolog except for lacking sequence elements in introns 4, 8, and 9 and in the 3'-UTR region of exon 10. Primer extension analysis indicated the transcription start site.
- To characterize the regulation of PGHS-2 mRNA in equine follicles before ovulation, preovulatory follicles were isolated during estrus on a time-course

after an ovulatory dose of hCG. Results from Northern blots showed a transient induction of PGHS-2 mRNA starting 30 hours post-hCG which occurs selectively in granulosa cells.

Work's contribution to the advancement of science:

- This study provides for the first time the primary structure of the equine PGHS-2 gene, transcript, and protein.
- It demonstrates a delayed induction of PGHS-2 gene expression in equine granulosa cells, thus providing a model to study the molecular basis for the late transcriptional activation of PGHS-2 in species with long ovulatory processes.
- Overall, it supports the putative role of PGHS-2 as a determinant of the mammalian ovulatory clock, contributing to our knowledge of the fundamental mechanisms governing the ovulatory process.

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Molecular Characterization of Equine Prostaglandin G/H Synthase-2 and Regulation of Its Messenger Ribonucleic Acid in Preovulatory Follicles*

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ABSTRACT

To increase our understanding of the molecular control of PG synthesis in equine preovulatory follicles, the specific objectives of this study were to clone and determine the primary structure of equine prostaglandin G/H synthase-2 (PGHS-2) and to characterize the regulation of PGHS-2 messenger RNA (mRNA) in follicles before ovulation. A complementary DNA (cDNA) library prepared from follicular mRNA and a genomic library were screened with a mouse PGHS-2 cDNA probe to isolate the equine PGHS-2 cDNA and gene, respectively. The expression library yielded three nearly full-length clones that differed only in their 5'-ends; clones 3, 5, and 6 were 2946, 3138, and 3398 bp in length, respectively. The longest clone was shown to start 9 bp downstream of the transcription initiation site, as determined by primer extension analysis, and to contain 120 bp of 5'-untranslated region (UTR), 1812 bp of open reading frame, and 1466 bp of 3'-UTR. The open reading frame encodes a 604-amino acid protein that is more than 80% identical to PGHS-2 homologs in other species. Numerous repeats (n = 11) of the Shaw-Kamen's sequence (ATTTA) are present in the 3'-UTR, a motif typically indicative of mRNAs with a short half-life. The complete equine PGHS-2 gene was isolated and sequenced from a ~17-kilobase clone obtained from the genomic library. The equine PGHS-2 gene structure (10 exons and 9 introns; total length of 6991 bp) is similar to its human homolog except for lacking sequence elements in introns 4, 8, and 9 and in the 3'-UTR region of exon 10. To characterize the regulation of PGHS-2 mRNA in equine follicles before ovulation, preovulatory follicles were isolated during estrus, 0, 12, 24, 30, 33, 36, and 39 h (n = 4–5 follicles/time point) after an ovulatory dose of hCG. Results from Northern blots showed significant changes in steady state levels of PGHS-2 mRNA in preovulatory follicles after hCG treatment ($P < 0.05$). The transcript remained undetectable between 0–24 h post-hCG, first appeared (~4 kilobases) only at 30 h, and reached maximal levels 33 h post-hCG. PGHS-2 mRNA was selectively induced in granulosa cells and not in theca interna. Thus, this study provides for the first time the primary structure of the equine PGHS-2 gene, transcript, and protein. It also demonstrates that the induction of PGHS-2 gene expression in equine granulosa cells is a long molecular process (30 h post-hCG), thereby providing a model to study the molecular basis for the late transcriptional activation of PGHS-2 in species with a long ovulatory process. (*Endocrinology* **139**: 1662–1670, 1998)

PGs, PROSTACYCLINS, and thromboxanes are members of the prostanoid family, a group of potent biological mediators involved in various physiological and pathological processes (1–3). Their synthesis from arachidonic acid is dependent on the expression of prostaglandin G/H synthase (PGHS; also known as cyclooxygenase), the first rate-limiting enzyme of the PG biosynthetic pathway (1). Two isoforms of PGHS, referred to as PGHS-1 and PGHS-2, have been identified (4, 5). Although encoded by different genes, the two isozymes share a relatively conserved primary structure, as evidenced from an overall 60% identity observed at the amino acid level in sheep (6, 7), chickens (8), rats (9), mice (10, 11), humans (12, 13), and guinea pigs (14). PGHS-1 and PGHS-2 have the same homodimer/coordinated heme group structure and dual enzymatic activities, and both isoforms are sensitive to nonsteroidal antiinflammatory drugs. However, mounting evidence points to distinct biological roles for each isoform as their patterns of expression and regulation differ greatly (3–5). Also, targeted gene disruption studies have revealed different phenotypes in PGHS-1 *vs.* PGHS-2 null mice (15, 16).

In recent years, several studies have implicated PGHS enzymes in various reproductive functions, including luteolysis (17), embryonic development and implantation (18–23), and parturition (24–31). Ovulation is another physiological process during which PG synthesis is required. In rat preovulatory follicles, there is a selective induction of PGHS-2 messenger RNA (mRNA) and protein in granulosa cells before ovulation (32–36). The induction is rapid (~2–4 h post-hCG) and transient, and precedes follicular rupture by approximately 10 h (36). This molecular process is also present in species with a long ovulatory process, such as cows (37, 38). However, PGHS-2 induction in bovine granulosa cells is relatively delayed compared with that in rats, being expressed only 18 h after hCG treatment (37). Interestingly, as the interval from PGHS-2 induction to follicular rupture is remarkably conserved in both species (~10 h), we proposed that PGHS-2 could be one of the determinants involved in dictating the species-specific length of the ovulatory process (37).

Marked differences in the time course of PGHS-2 induction among species have underscored the need to characterize the distinct molecular mechanisms involved in PGHS-2 gene expression in large monoovulatory species with a long

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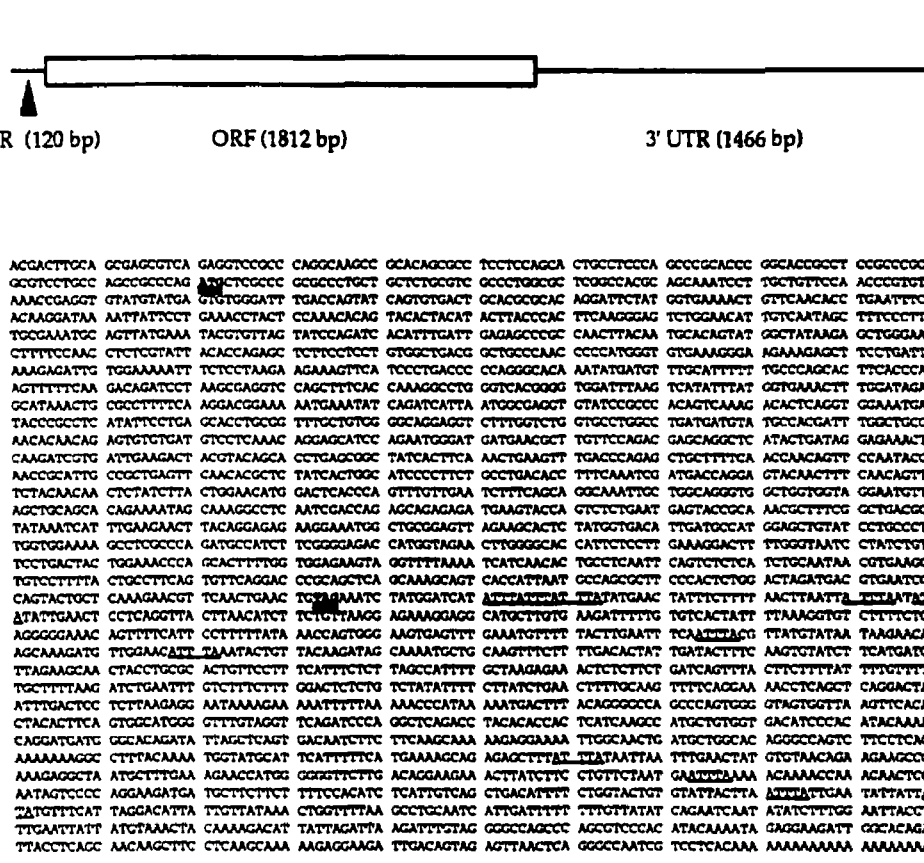

FIG. 1. Primary structure of the equine PGHS-2 cDNA. A, The equine PGHS-2 cDNA is composed of a 5'-UTR of 120 bp, an open reading frame of 1812 bp, and a 3'-UTR of 1466 bp. B, The complete nucleotide sequence was derived from clone 6 as described in *Materials and Methods*. The translation initiation (ATG) and stop (TAG) codons are highlighted in *bold*, repeats of the Shaw-Kamen's sequence (ATTTA) in the 3'-UTR are *underlined*, and numbers on the *left* refer to the first nucleotide on that line. The nucleotide sequence was submitted to GenBank (accession no. AF027334).

ovulatory process. The mare is a valuable model to study the hormonal control of follicular PG synthesis before ovulation. The preovulatory follicle reaches a relatively large size (40–45 mm) and can be identified *in vivo* by ultrasonography up to 7 days before ovulation (39–41). Ovulation can be induced by administration of hCG, and the interval from gonadotropin injection to follicular rupture is approximately 36–48 h (42, 43). It was recently shown that the induction of PG synthetic activities in equine follicles before ovulation is associated with the selective induction of PGHS-2 protein in granulosa cells (44). Its time course of induction (30 h post-hCG) is further delayed compared with that in cows, but the interval between PGHS-2 induction and ovulation remains similar (~10 h), supporting the hypothesis that PGHS-2 induction could serve as an important signal to control the mammalian ovulatory clock (44, 45).

To further increase our understanding of the molecular control of PG synthesis in equine follicles, the general objective of this study was to characterize the regulation of follicular PGHS-2 mRNA before ovulation. The specific objectives were to clone and determine the primary structure of equine PGHS-2, and to characterize the expression of PGHS-2 mRNA in a developmental series of equine preovulatory follicles isolated between 0–39 h after the administration of an ovulatory dose of hCG.

Materials and Methods

Materials

Diethyldithiocarbamic acid was purchased from Sigma Chemical Co. (St. Louis, MO); Lutalyse was obtained from Upjohn (Kalamazoo, MI); hCG was purchased from The Buttler Co. (Columbus, OH); Rompun was obtained from Haver (Bayvet Division, Shawnee, KS); Torbugesic was purchased from Fort Dodge Laboratories (Fort Dodge, IA); Dormosedan was obtained from SmithKline Beecham, Animal Health (West Chester, PA); Biotrans nylon membranes (0.2 μm) were purchased from ICN Pharmaceuticals (Montreal, Canada); [α-^{32}P]deoxy (d)-CTP, [α-^{32}P]dATP, [γ-^{32}P]ATP, and [^{35}S]dATP were obtained from Mandel Scientific-New England Nuclear Life Science Products (Mississauga, Canada); QuikHyb hybridization solution, Poly(A) Quick mRNA purification kit, and ZAP-cDNA/Gigapack cloning kit were purchased from Stratagene Cloning Systems (La Jolla, CA); TRIzol total RNA isolation reagent, RNA ladder [0.24–9.5 kilobases (kb)], 1-kb ladder, synthetic oligonucleotides, and culture media were obtained from Life Technologies (Gaithersburg, MD); RNAsin, Prime-a-Gene labeling system, DNA 5'-End Labeling System, and AMV reverse transcriptase were purchased from Promega (Madison, WI); Kodak X-Omat AR film was obtained from Eastman Kodak (Rochester, NY); electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA); T4 polynu-

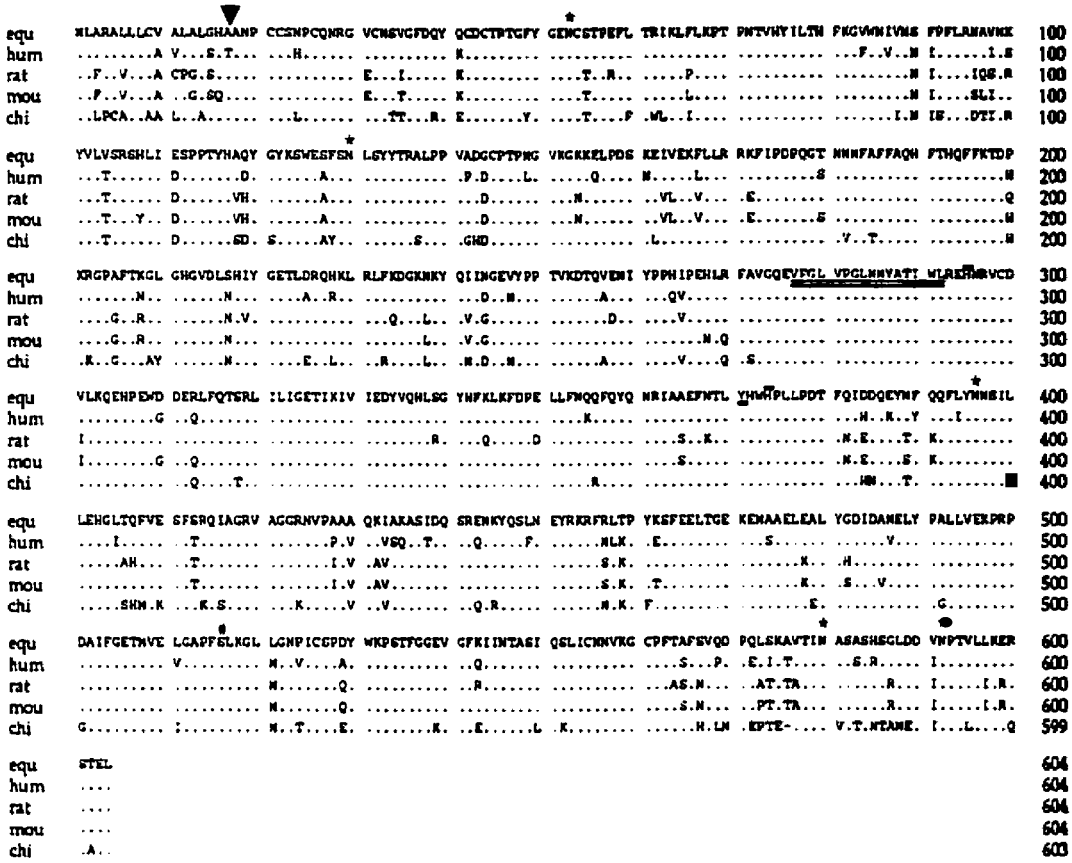

FIG. 2. Predicted amino acid sequence of equine PGHS-2 and comparison with the human, rat, mouse, and chicken homologs. Identical residues are indicated by a printed period. The signal peptide cleavage site is indicated with an *inverse triangle* and the putative transmembrane region is *double underlined*. The tyrosine (Tyr^{371}) associated with the cyclooxygenase active site is *underlined*, heme coordination residues (His^{296} and His^{374}) are *overlined*, and the aspirin-acetylated serine residue (Ser^{516}) is indicated by a *number sign*. Potential *N*-glycosylation sites are marked with an *asterisk*; note that residue 90 in the equine protein is a serine and therefore cannot be subject to *N*-linked glycosylation as reported for PGHS-2 in other species. Sequences of equine (Equ), human (hum), rat (rat), mouse (mou), and chicken (chi) PGHS-2 were obtained from GenBank.

cleotide kinase and all sequencing reagents were obtained from Pharmacia Biotech (Baie D'Urfé, Canada).

Cloning and sequencing of the equine PGHS-2 complementary DNA (cDNA) and gene

To clone the equine PGHS-2 cDNA, an equine expression library was made with RNA extracted (TRIzol, Life Technologies) from a preovulatory follicle isolated during estrus, 36 h after hCG treatment (44). Polyadenylated [poly(A)$^+$] RNA was purified with the Poly(A) Quick mRNA purification kit (Stratagene), and the library was constructed using the ZAP-cDNA/Gigapack cloning kit (Stratagene) following the manufacturer's protocol. One round of 300,000 plaques was screened with a 1.2-kb 5'-fragment of the mouse PGHS-2 cDNA generated by *Eco*RI digestion (46). The probe was labeled with [α-^{32}P]dCTP using the Prime-a-Gene labeling system (Promega) to a final specific activity greater than 1×10^8 cpm/μg DNA, and hybridization was performed at 55 C with QuikHyb hybridization solution (Stratagene). Positive clones were plaque purified through secondary and tertiary screening, and pBluescript phagemids containing the cloned DNA insert were excised *in vivo* with the Ex-Assist/SOLR system (Stratagene).

To clone the equine PGHS-2 gene, a genomic library (Stratagene) was screened according to the manufacturer's protocol with the mouse PGHS-2 cDNA fragment described above. Seven positive clones were identified from an initial round of 400,000 phage plaques screened. They were purified and initially analyzed by restriction endonuclease mapping with *Sac*I and *Xba*I and by Southern blot analyses with the mouse PGHS-2 cDNA probe. Fragments yielding a positive signal were subcloned into the vector pGEM 3ZF(−), partially sequenced and compared with the human homolog (47). One of the initial clones, clone 3-1b, was shown to contain the complete equine PGHS-2 gene as well as upstream and downstream DNA sequences. Additional restriction fragments from clone 3-1b were subcloned in pGEM 3ZF(−), and the entire gene (exons and introns) was sequenced. The exon/intron borders were determined by comparison between the genomic and cDNA sequences.

DNA sequencing was performed by the Sanger dideoxy nucleotide chain termination method (48) using the T7 Sequencing Kit (Pharmacia); vector-based primers (T3, T7, or SP6) and specific oligonucleotide primers synthesized as internal PGHS-2 sequences were obtained. Nucleotide and amino acid analyses were performed using the FASTA program of WI Package version 9.0 (Genetics Computer Group, Madison, WI) and the MacDNASIS software version 2.0 (Hitachi, Hialeah, FL).

Primer extension analysis

Primer extension analysis was performed in aqueous buffer as described previously (49, 50). Briefly, total RNA was extracted with TRIzol (Life Technologies) from preovulatory follicles isolated 0 h (negative control) or 36 h after administration of hCG. A 24-mer antisense oligonucleotide (5'-GGCTGGGAGGCAGTGCTGGAGGAG-3') designed

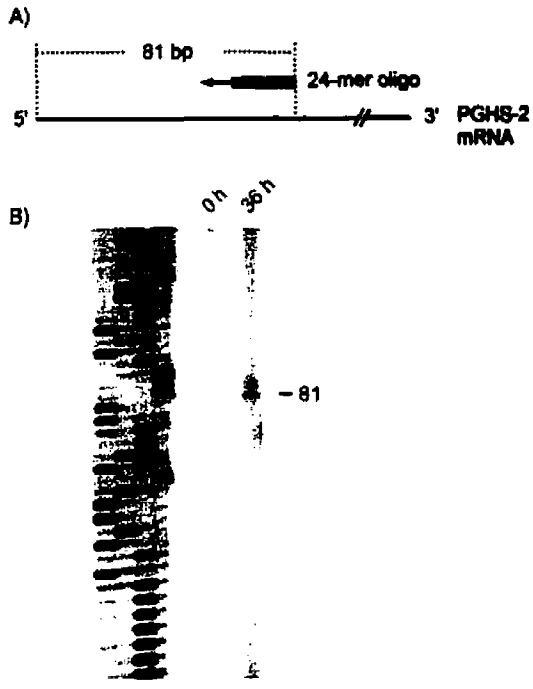

FIG. 3. Primer extension analysis of equine PGHS-2 mRNA. *A*, Schematic representation of the strategy employed in primer extension analysis. The labeled antisense 24-mer primer was hybridized to RNA samples containing (follicle isolated 36 h post-hCG) and not containing (follicle 0 h post-hCG) PGHS-2 mRNA. The *arrow* indicates the direction of reverse transcription, and the reaction was performed as described in *Materials and Methods*. *B*, The extended product was analyzed on a 6% polyacrylamide gel, and its size was determined by comparison with the products of an unrelated sequencing reaction shown on the *left*. Results with follicular RNA isolated 36 h post-hCG show a doublet, with the most intense of the bands representing a product of 81 bp. The size of the extension product was confirmed by comparison with the those of products of a sequencing reaction containing the same oligonucleotide used for primer extension and an equine PGHS-2 genomic clone spanning this region (data not shown). No extension product was detected with RNA isolated 0 h post-hCG (negative control).

from the equine PGHS-2 cDNA and located between +50 and +73 bp from the beginning of the longest cDNA clone was end labeled and hybridized (50,000 cpm/reaction) to 50 μg total RNA at 30 C overnight in 30 μl buffer (1 M NaCl; 167 mM HEPES, pH 7.5; and 0.33 mM EDTA, pH 8.0). After precipitation, primer extension was performed by adding 3.5 μl 4 mM dNTPs, 2.5 μl 10 × RT buffer (0.5 M Tris-Cl, pH 8.2; 50 mM $MgCl_2$; 50 μM dithiothreitol; 0.5 M KCl; and 0.5 mg/ml BSA), 1.25 μl RNAsin, 18 μl H_2O, and 40 U AMV reverse transcriptase and incubating at 42 C for 90 min. After extraction and precipitation, the extension product was analyzed by electrophoresis on a 6% polyacrylamide-7 M urea gel, and its size was determined by comparison with two sequencing reactions run in adjacent lanes. One reaction involved the same oligonucleotide used for primer extension and an equine PGHS-2 genomic clone spanning this region, whereas the other sequencing reaction used an unrelated primer and a template of known sequence.

Isolation and dissection of equine preovulatory follicles

Standardbred and Thoroughbred mares were teased daily with a pony stallion for detection of estrus, and ovarian follicular development was monitored daily by transrectal real-time ultrasonography, as previously described (44). During estrus and when the preovulatory follicle reached 35 mm in diameter, hCG (2500 IU, iv) was administered, and ovariectomy was performed via colpotomy 0, 12, 24, 30, 33, 36, and 39 h post-hCG treatment with a chain ecraseur (n = 4–5 follicles/time point) (44). During the procedure, neuroleptanalgesia was induced with a combination of xylazine (Rompun; 0.65 mg/kg, iv), butorphanol (Torbugesic; 0.005 mg/kg, iv), and detomidine (Dormosedan; 0.02 mg/kg, iv), as previously described (44). The recovered ovary was immersed in ice-cold Eagles's MEM supplemented with penicillin (50 U/ml)-streptomycin (50 μg/ml; Life Technologies), L-glutamine (2.0 mM; Life Technologies), and nonessential amino acids (0.1 mM; Life Technologies). All animal procedures were approved by the comité de déontologie animale of the University of Montreal.

The preovulatory follicle was dissected into three cellular preparations using a methodology previously described (44). They included pieces of follicle wall (theca interna with attached granulosa cells) and isolated preparations of theca interna and granulosa cells. All samples were stored at −70 C until RNA extraction.

RNA extraction and Northern blot analysis

Total cellular RNA was extracted from equine tissues using TRIzol (Life Technologies) and a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Fairlawn, NJ). For Northern analysis, RNA samples (10 μg) were denatured at 55 C for 15 min in 50% deionized formamide-6% formaldehyde, electrophoresed in a 1% formaldehyde-agarose gel, and transferred onto a nylon membrane as previously described (36, 37). A ladder of RNA standards was run with each gel, and ethidium bromide (10 μg) was added to each sample before electrophoresis to compare RNA loading and determine the migration of standards. The membrane was first hybridized to the ^{32}P-labeled equine PGHS-2 cDNA probe using QuikHyb solution (Stratagene) as described above. After stripping the radioactivity with 0.1% SSC (standard saline citrate)-0.1% SDS for 30 min at 100 C, the same blot was subsequently hybridized with a rat elongation factor Tu (EFTu) cDNA as a control gene for RNA loading and transfer (51).

Statistical analysis

Changes in relative levels of mRNA during the ovulatory process were quantified by determining on autoradiograms the optical density of the PGHS-2 band with a computer-assisted image analysis system (Collage Macintosh program, Fotodyne, New Berlin, WI). The EFTu signal was also scanned and used to normalize results. For each cellular preparation, data were expressed as ratios of PGHS-2 mRNA to EFTu and are presented as the mean ± SEM (n = 4 follicles/time point). One-way ANOVA was used to test the effect of time after hCG treatment on relative PGHS-2 mRNA levels. When ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple comparisons with the control (0 h post-hCG). Data were transformed to logarithms before analysis when heterogeneity of variance was observed with the Hartley test. Statistical analyses were performed using the JMP Sortware (SAS Institute, Cary, NC).

Results

Characterization of the equine PGHS-2 cDNA

To clone the equine PGHS-2 cDNA, a follicular cDNA library was screened with a 5'-fragment of the mouse PGHS-2 cDNA probe (46). Twelve positive clones were isolated from an initial screen of approximately 3.0×10^5 plaques. Three of these primary candidates (clones 3, 5, and 6) were purified through secondary and tertiary screens, sequenced, and shown to be near full-length clones that differed only in their 5'-ends; clones 3, 5, and 6 were 2946, 3138, and 3398 bp in length, respectively. The longest clone (clone 6) contained 120 bp of 5'-untranslated region (UTR), an open reading frame of 1812 bp, and a 3'-UTR of 1466 bp (Fig. 1). The large 3'-UTR was found to contain numerous (n = 11) repeats of the Shaw-Kamen's sequence (ATTTA) (52), a motif typically associated with short half-life of mRNAs (Fig. 1B).

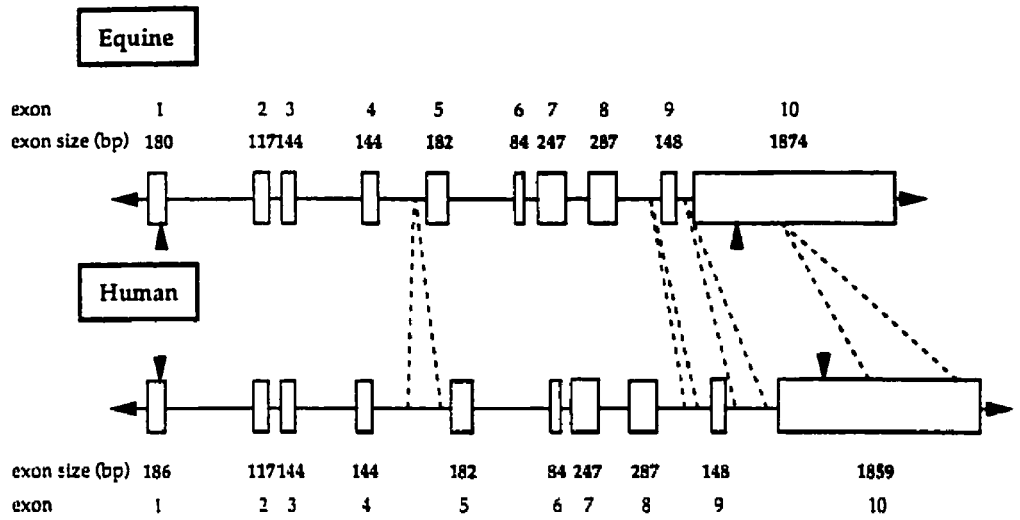

FIG. 4. Comparative analysis of the equine and human PGHS-2 gene structures. The complete equine PGHS-2 gene sequence was derived from genomic clone 3-1b and was submitted to GenBank (accession no. AF027335). Exon sequences are represented as *boxes*, and size is stated in base pairs. Introns are shown as *lines* connecting the exons. All structural elements are drawn to scale. *Dashed lines* designate the approximate locations of regions present in the human, but not the equine, gene. *Arrowheads* show the positions of the translation start (exon 1) and stop (exon 10) codons.

FIG. 5. Exon/intron boundaries of the equine PGHS-2 gene. Exonic sequences are presented in *uppercase letters*; intronic sequences are shown in *lowercase letters*. Numbers in superscript indicate the first and last nucleotides of each exon according the their positions in the full-length cDNA [the first eight nucleotides of exon 1 (5'-GTTGTCAA-3') were derived from a genomic fragment, whereas the rest of the cDNA is shown in Fig. 1). Sizes of introns are indicated in *parentheses* and were precisely determined by sequencing.

Exon		Intron		Exon
			G^{001}TTGTC...	1
1	...ACGCAG^{160}gtaggt...	1 (802 bp)	...ctgtagC^{181}AAATC...	2
2	...CAACAC^{297}gtaagt...	2 (119 bp)	...ttgcagC^{298}TGAAT...	3
3	...TAGTAT^{441}gtaagt...	3 (647 bp)	...tttcagC^{442}CAGAT...	4
4	...TGAAAG^{585}gtgagt...	4 (395 bp)	...tttcagG^{586}GAAGA...	5
5	...CACGGG^{767}gtaaga...	5 (683 bp)	...ttctagG^{768}TGGAT...	6
6	...TATCAG^{851}gtttgt...	6 (129 bp)	...ttatagA^{852}TCATT...	7
7	...TGATAG^{1098}gtaagc...	7 (282 bp)	...aaacagG^{1099}AGAAA...	8
8	...GGCAGG^{1385}gtacgc...	8 (377 bp)	...tcacagG^{1386}TGGCT...	9
9	...TTACAG^{1533}gtgaga...	9 (151 bp)	...tcccagG^{1534}AGAGA...	10
10	...AAAAAA^{3406}			

Similarities between the equine PGHS-2 coding sequence and other species homologs

The amino acid sequence of equine PGHS-2 was deduced from the coding region of the cDNA, and comparisons were made with the human (13), rat (9), mouse (11), and chicken (8) homologs (Fig. 2). Results showed that the open reading frame encodes an equine protein that is identical in length (604 amino acids) to PGHS-2 of other mammalian species and is 1 amino acid longer than chicken PGHS-2. The equine enzyme appears to share all the important structural and functional domains implicated in PGHS function (Fig. 2). Comparisons between equine PGHS-2 and other mammalian homologs revealed more than 80% identity at the amino acid and nucleic acid levels.

Primer extension analysis

To characterize the complete 5'-UTR of the equine PGHS-2 mRNA and identify the transcription initiation site, primer extension analysis was performed (Fig. 3A) using a 24-mer antisense oligonucleotide (5'-GGCTCCGAGGCAGTGCTGGAGGAC-3') and RNA extracted from a preovulatory follicle isolated 36 h post-hCG treatment. The extension products were resolved on a 6% denaturing polyacrylamide gel, and their sizes were characterized by comparison with an adjacent sequencing reaction. Results showed the presence of two extension products that differed by only 1 base, with their sizes corresponding to 81 and 82 nucleotides (Fig. 3). The 81-nucleotide extension product was considered as the principal transcription initiation site because the band had a greater intensity (Fig. 3B). Therefore, we conclude that the longest cDNA clone (clone 6) begins 9 bp downstream of the start site, and that the full-length 5'-UTR of the equine PGHS-2 mRNA has 128 bp. No extension product was detected when follicular RNA isolated 0 h post-hCG was used (Fig. 4B).

Primary structure of the equine PGHS-2 gene

An equine genomic library (Stratagene) was screened with a mouse PGHS-2 cDNA probe to clone and characterize the primary structure of the equine PGHS-2 gene. Seven positive

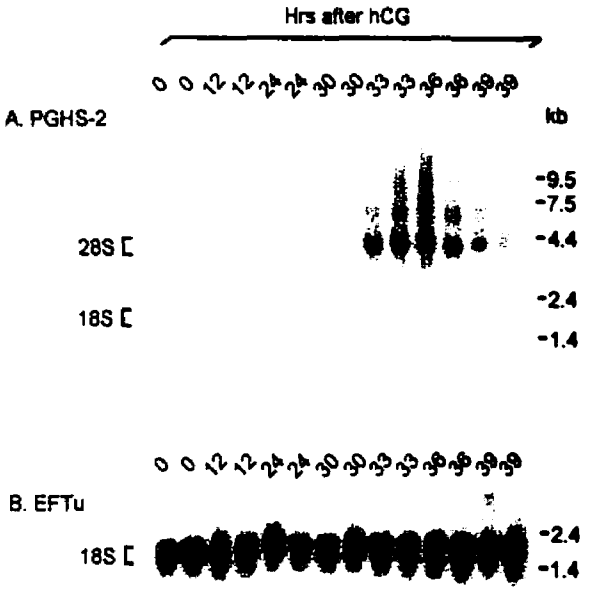

FIG. 6. Time-dependent regulation of equine PGHS-2 mRNA by hCG in equine follicles during the ovulatory process. Preparations of follicle wall (theca interna with attached granulosa cells) were obtained from preovulatory follicles isolated 0, 12, 24, 30, 33, 36, and 39 h after hCG, as described in *Materials and Methods*. Samples of total RNA (10 μg/lane; two follicles per time point) were analyzed by Northern blotting using a ^{32}P-labeled equine PGHS-2 cDNA probe (A). The same blot was stripped of radioactivity and hybridized with a cDNA encoding rat EFTu as a control gene for RNA loading (B). *Brackets* on the *left* show the migrations of 28S and 18S ribosomal bands, and markers on the *right* indicate the migrations of RNA standards. Filters in A and B were exposed to film at −70 C for 8 and 2 h, respectively.

clones isolated from an initial round of 4×10^5 phage plaques were purified and characterized by restriction endonuclease mapping and Southern blot analysis. One clone (3-1b) containing an insert of approximately 17 kb was used to sequence the complete PGHS-2 gene. The results showed that the equine gene is composed of 10 exons and 9 introns and is 6991 bp in length. The first 8 bp in the 5′-UTR of exon 1, which were absent in the longest cDNA clone, were shown to correspond to 5′-GTTGTCAA-3′. The structure of the equine gene is very similar to that of the human gene, except for sequence elements lacking in introns 4, 8, and 9 and in the region of exon 10 corresponding to the 3′-UTR (Fig. 4). The sizes of all internal exons (exons 2–9) and the coding region of exons 1–10 are identical between the 2 species. However, the length of the 5′-UTR of exon 1 and that of the 3′-UTR of exon 10 differed (Fig. 4). The coding nucleotide sequence of the genomic clone was identical to that of the cDNA clones. Figure 5 shows that intron size varies from 119 bp (intron 2) to 802 bp (intron 1), and that each splice site agrees with the consensus donor/acceptor (GT/AG) sequence.

Regulation of PGHS-2 mRNA in preovulatory follicles

To characterize the gonadotropin-dependent regulation of PGHS-2 mRNA during the ovulation process in mares, a series of preovulatory follicles was isolated during estrus, 0, 12, 24, 30, 33, 36, and 39 h after an ovulatory dose of hCG. Samples of total RNA extracted from the follicle wall (theca interna with attached granulosa cells) were analyzed by Northern blotting using an equine PGHS-2 cDNA probe generated from clone 6. The results showed a marked regulation of steady state levels of PGHS-2 transcript in equine follicles after hCG treatment (Fig. 6A). No PGHS-2 mRNA was detected between 0–24 h post-hCG. Transcripts (~4 kb) first appeared 30 h post-hCG treatment, reached maximal levels at 33 h, and progressively decreased thereafter. Hybridization of the same membrane with a cDNA encoding rat EFTu confirmed uniform RNA loading (Fig. 6B).

To determine which cell type within the follicle wall expresses PGHS-2 mRNA, isolated preparations of granulosa cells and theca interna were obtained from preovulatory follicles collected between 0–39 h after hCG treatment. Total RNA was extracted and analyzed by Northern blots as described above. The results clearly showed a selective expression of PGHS-2 in granulosa cells and followed a pattern similar to that seen in the follicle wall (Fig. 7). No transcripts were detected in theca interna, except for a relatively weak signal in one sample (36 h post-hCG; Fig. 7) that probably resulted from contamination by granulosa cells.

To quantify changes in relative levels of PGHS-2 mRNA during the ovulatory process, Northern blots of total RNA extracted from follicle wall, granulosa cells, and theca interna were scanned and subjected to a densitometric analysis (n = 4 follicles/time point between 0–39 h post-hCG; Fig. 8). In addition to PGHS-2, the EFtu band was also scanned to normalize for RNA loading and transfer. The results showed a significant increase ($P < 0.05$) in PGHS-2 mRNA levels between 30–36 h post-hCG in follicle wall and granulosa cells compared with levels present at 0 h (Fig. 8, A and B). In contrast, PGHS-2 signals remained very low or undetectable in all theca interna samples, and no significant differences were observed at any time point (Fig. 8C).

Discussion

This study characterizes for the first time the primary structure of the equine PGHS-2 gene, transcript, and protein, and sequencing results further underscore the highly conserved nature of PGHS-2 across species. Comparative analyses showed that the deduced amino acid sequence of the equine protein was more than 85% identical to those of the human (13), rat (9), mouse (11), and guinea pig (14) homologs. The equine PGHS-2 protein consists of 604 amino acids as in other mammalian species. The enzyme has all putative structural and functional domains involved in PGHS function, including the axial and distal histidines involved in heme binding (53), a tyrosine residue essential for cyclooxygenase function (54), and a serine known as the acetylation site for aspirin (54). Four putative *N*-linked glycolysation sites are conserved, whereas one site present in PGHS-2 of other species is not present in the horse. The biological significance of this difference, if any, remains unknown.

One interesting structural feature of the equine PGHS-2 transcript is the presence of numerous Shaw-Kamen's se-

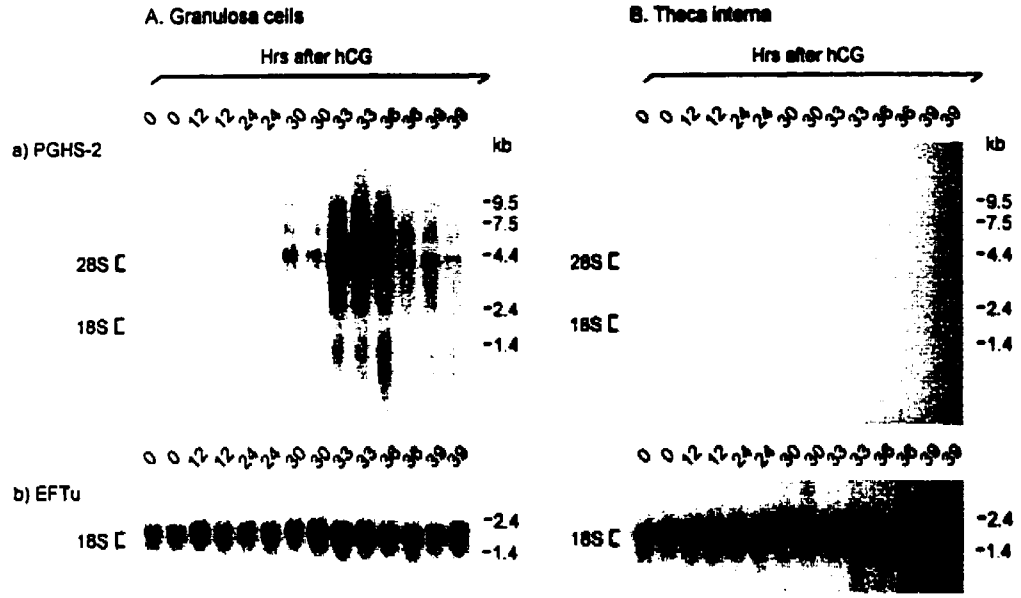

FIG. 7. Cell-specific induction of PGHS-2 mRNA in equine preovulatory follicles. Isolated preparations of granulosa cells (A) and theca interna (B) were obtained from equine preovulatory follicles isolated between 0–39 h after hCG treatment, as described in *Materials and Methods*. Samples of total RNA (10 μg/lane; n = 2 follicles/time) were analyzed by Northern blotting using a ^{32}P-labeled equine PGHS-2 cDNA as probe (*upper panels*). The same blots were stripped of radioactivity and hybridized with a cDNA encoding rat EFTu as a control gene for RNA loading (*lower panels*). *Brackets* on the *left* show the migrations of 28S and 18S ribosomal bands, and markers on the *right* indicate the migrations of RNA standards. Filters in *upper panels* were exposed to film at −70 C for 8 h; filters in *lower panels* were exposed for 2 h.

quences (5′-ATTTA-3′) in the 3′-untranslated region. This motif has previously been shown to be present in several immediate early genes and to confer instability to mRNAs (52, 55). The number of repeats in equine PGHS-2 (n = 11) is comparable to the numbers observed in PGHS-2 of other species (n = 8–16) (8, 9, 11, 13, 14). Interestingly, although the position of several motifs varies among species, a group of five Shaw-Kamen's repeats is consistently found within the first 80 nucleotides downstream of the translation termination codon of all PGHS-2 transcripts (9, 11, 13, 14), suggesting their greater relative importance in mediating mRNA degradation. Rapid turnover of PGHS-2 mRNA has previously been shown in different cell types and probably relates to the need for a tight regulation of gene expression considering the potent biological effects of prostanoids (3–5).

To date, the primary structure of the PGHS-2 gene had been characterized only in human (56) and mouse (57). This study documents the exon/intron organization of the equine gene. Compared with those of mouse and human PGHS-2, the genomic structure of equine PGHS-2 is highly conserved, with 10 exons and nine introns (56, 57). Internal exons 2–9 and the coding regions of exons 1 and 10 of the equine gene are identical in size to their human and mouse counterparts. However, differences are observed in the size of the untranslated region of exon 1: 129, 134, and 122 nucleotides in length for the equine, human, and mouse genes, respectively (11, 56). Transcription of the equine PGHS-2 gene starts at an adenosine residue, which is identical to the that in the rat (50) and mouse (11) but distinct from the human cap site identified as a cytidine (56). Also, important variations are observed among species in the length of the 3′-UTR in exon 10, which correlates with overall differences observed in the sizes of cloned cDNAs. Although rat and mouse PGHS-2 cDNAs are approximately 4.0 kb (9, 11), equine and human cDNAs are relatively shorter, only 3.4 kb (13). Results from genomic sequencing in the horse show that the stretch of adenosines found at the end of our cDNA clones may, in fact, correspond to a 21-base adenosine repeat present in the corresponding region of the PGHS-2 gene. This finding suggests that our cDNA clones may have been reverse transcribed from an internal poly(A)$^+$ sequence in the 3′-UTR instead of the poly(A)$^+$ tail, and therefore, the full-length cDNA could be longer than reported herein. Similar conclusions can be drawn for the apparent small size of the human PGHS-2 cDNA (13, 56).

A unique time course of induction of PGHS-2 mRNA was observed in a series of equine preovulatory follicles isolated between 0–39 h after hCG treatment. Induction of PGHS-2 transcript in granulosa cells was first detected only 30 h post-hCG. This impressive delay in agonist induction of PGHS-2 gene expression is unprecedented. In other cell types, the regulation of PGHS-2 is more rapid, being induced within 1 h by 12-*O*-tetradecanoylphorbol-13-acetate or lipopolysaccharide in fibroblasts, macrophages, endothelial cells, and mesangial cells (11, 13, 58, 59). Although PGHS-2 is considered an early response gene in fibroblasts, its very delayed induction in equine granulosa cells suggests that it does not serve this role in ovarian cells. Wong *et al.* (60) have shown that induction of PGHS-2 transcript by gonadotropins in rat granulosa cells is dependent on protein synthesis, which shows that it is not an early response gene in follicular cells. The cellular localization of PGHS-2 mRNA in equine follicles complements a similar result recently reported for the PGHS-2 protein (44). Also, the delayed induction of the

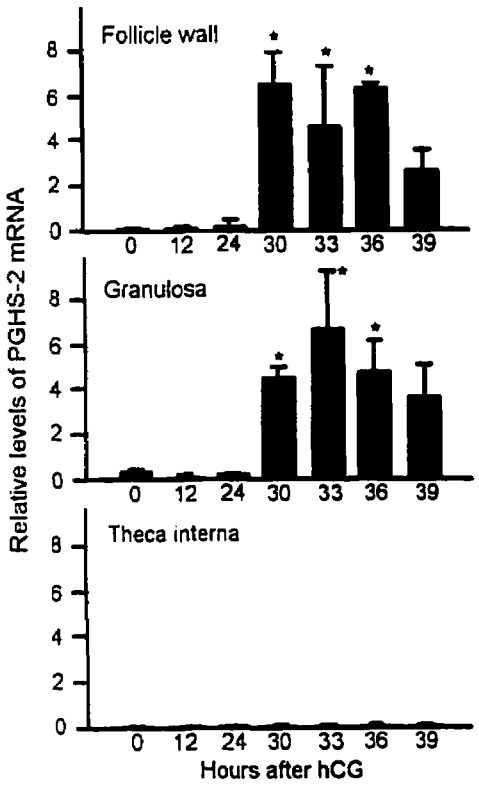

FIG. 8. Relative levels of PGHS-2 mRNA in equine preovulatory follicles isolated between 0 and 39 h after hCG treatment. Samples (n = 10 μg) of total RNA extracted from follicle wall (A), granulosa cells (B), and theca interna (C) were analyzed by Northern blotting with the equine PGHS-2 cDNA and subsequently with rat EFTu cDNA as a control gene for RNA loading. After autoradiography (films not shown), the PGHS-2 signal intensity was quantified by densitometric analysis and normalized with the control gene EFTu. Results are presented as PGHS-2 mRNA levels relative to EFTu (mean ± SEM; n = 4 follicles/time point). Columns marked with an *asterisk* are significantly different ($P < 0.05$) from the 0 h post-hCG value.

transcript coincides with the late detection of the protein and of follicular PG synthetic activities (44, 61). Collectively, these results clearly suggest that the transcriptional regulation of the PGHS-2 gene in equine granulosa cells is a relatively long molecular process (30 h post-hCG) compared with its regulation in rat (2–4 h post-hCG) (36) and bovine (18 h post-hCG) (37) preovulatory follicles. The apparent relationship between the progressively delayed induction of PGHS-2 transcripts in species with long ovulatory processes further supports a putative role of PGHS-2 as a determinant of the mammalian ovulatory clock (45).

In summary, this study documents for the first time the primary structure of the equine PGHS-2 gene, transcript, and protein, and comparative analyses further underscore the highly conserved structure of the enzyme across species. Studies of the regulation of PGHS-2 mRNA in equine follicles during the ovulatory process reveal a time-dependent (30 h post-hCG) and granulosa cell-specific induction of the transcript. The regulation of PGHS-2 gene expression in equine granulosa cells is a uniquely delayed molecular event compared with its regulation in follicles of other species with shorter ovulatory processes and its rapid agonist-dependent induction in other cell types. The characterization of the equine PGHS-2 promoter and the development of homologous ovarian cell culture system are currently underway to provide a model to study the molecular basis for the delayed transcriptional activation of PGHS-2 in species with a long ovulatory process.

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Summary of article #2

Equine P450 cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase: molecular cloning and unique regulation of their messenger ribonucleic acids in equine follicles during the ovulatory process. **Derek Boerboom** and Jean Sirois. Biology of Reproduction (in press).

Thesis author's contribution to the work: As the primary author, I was responsible for all aspects of the production of this article.

Summary:

- Cloning, sequencing and primer extension analyses were used to reveal the structure of the equine P450SCC and 3β-HSD transcripts.
- P450SCC and 3β-HSD open reading frames were found to encode 520 and 373 amino acid proteins, respectively, that are highly conserved (68-79% identity) when compared to homologs of other mammalian species.
- Northern blot analyses were performed with preovulatory follicles isolated on a time-course following the administration of hCG. Isolated cellular preparations revealed that theca interna was the predominant site of P450SCC expression in follicles prior to hCG. However, transcript levels decreased in theca interna between 30-39h and increased in granulosa cells at 39 h, making the granulosa cell layer the predominant site of P450SCC expression

at the end of the ovulatory process. Highest levels of expression were detected in corpora lutea.

- In contrast to other species, expression of 3β-HSD mRNA in equine preovulatory follicles was localized only in granulosa cells. 3β-HSD transcript levels remained constant throughout the luteinization process.

Work's contribution to the advancement of science:

- This study characterizes for the first time the complete structure of equine P450SCC and 3β-HSD mRNA.
- It identifies novel patterns of expression and regulation of these transcripts in equine follicles prior to ovulation. The redistribution of P450scc expression within the follicle wall was similar to what was observed for StAR, and may be related to the putative degeneration of the theca interna.
- The study offers significant insight as to how the overall regulation of progesterone biosynthesis occurs throughout the ovulatory process.
- This paper also integrates mRNA regulation data from article #3 and annex I into a working model of the overall regulation of follicular steroid hormone biosynthesis in mares.

Equine P450 Cholesterol Side-Chain Cleavage and 3ß-Hydroxysteroid Dehydrogenase/Δ5-Δ4 Isomerase: Molecular Cloning and Regulation of their Messenger Ribonucleic Acids in Equine Follicles during the Ovulatory Process[1]

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Short title: Regulation of P450SCC and 3ß-HSD in equine follicles

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SUMMARY

The preovulatory LH rise is the physiological trigger of follicular luteinization, a process during which the synthesis of progesterone is markedly increased. To study the control of follicular progesterone biosynthesis in mares, the objectives of this study were to clone and characterize the equine cholesterol side-chain cleavage cytochrome P450 (P450SCC) and 3ß-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3ß-HSD), and describe the regulation and cellular localization of their transcripts in equine follicles during hCG-induced ovulation. Complementary DNA (cDNA) cloning and primer extension analyses revealed that the equine P450SCC transcript is composed of a 5'-untranslated region (UTR) of 52 nucleotides, an open reading frame (ORF) of 1560 nucleotides, and a 3'-UTR of 225 nucleotides, whereas the equine 3ß-HSD mRNA consists of a 5'-UTR of 61 nucleotides, an ORF of 1119 nucleotides, and a 3'-UTR of 432 nucleotides. The equine P450SCC and 3ß-HSD ORF encode 520 and 373 amino acid proteins, respectively, that are highly conserved (68-79% identity) when compared to homologs of other mammalian species. Northern blot analyses were performed with preovulatory follicles isolated 0, 12, 24, 30, 33, 36 and 39 h post-hCG, and corpora lutea obtained on day 8 of the cycle. Results showed that levels of P450SCC mRNA in follicular wall (theca interna with attached granulosa cells) decreased after hCG treatment (30-39 h *versus* 0 h post-hCG, $P < 0.05$), and increased again after ovulation to reach their highest levels in corpora lutea ($P < 0.05$). Northern blots on isolated cellular preparations revealed that theca interna was the predominant site of P450SCC expression in follicles prior to hCG ($P < 0.05$). However, transcript levels decreased in theca interna between 30-39 h ($P < 0.05$) and increased in granulosa cells at 39 h ($P < 0.05$), making the granulosa cell layer the predominant site of P450SCC expression at the end of the ovulatory process. A different pattern of regulation was observed for 3ß-HSD, as transcript levels remained constant throughout the luteinization process ($P > 0.05$). Also, in contrast to other species, expression of 3ß-HSD mRNA in equine

preovulatory follicles was localized only in granulosa cells, and not in theca interna. Thus, this study characterizes for the first time the complete structure of equine P450SCC and 3ß-HSD mRNA, and identifies novel patterns of expression and regulation of these transcripts in equine follicles prior to ovulation.

INTRODUCTION

The cholesterol side-chain cleavage cytochrome P450 (P450SCC), and its associated electron-transport chain, is the first rate-limiting and hormonally regulated step in the biosynthesis of steroids from cholesterol (1, 2). The enzyme is located on the matrix side of inner mitochondrial membranes, and catalyzes the conversion of substrate cholesterol to pregnenolone, a common precursor to all steroid hormones. The primary structure of P450SCC has been deduced from its cloning in various species (3-7). In humans, the P450SCC gene spans more than 20 kilobases (kb), is split into nine exons, and encodes a transcript of about 2.0 kb and a protein of 521 amino acids (4, 8).

Once produced from cholesterol, pregnenolone proceeds either via the Δ5 steroidogenic pathway and undergoes 17α-hydroxylation to become 17-hydroxypregnenolone, or enters the Δ4 pathway and is converted to progesterone (1, 2). The enzyme 3ß-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3ß-HSD) catalyzes the synthesis of progesterone from pregnenolone, as well as the conversion of other Δ5-3ß-hydroxysteroids into the corresponding Δ4-3-ketosteroids (9-12). Thus, 3ß-HSD is essential for the biosynthesis of all classes of steroid hormones, including progesterone, androgens, estrogens, glucocorticoids and mineralocorticoids (9-12). The enzyme, located in the endoplasmic reticulum and in mitochondrial membranes (13-15), is expressed to high levels in classic steroidogenic tissues (i.e. gonads, adrenal cortex and placenta), as well as in various peripheral tissues where it could play an important role in intracrine steroid synthesis (9-12, 16). Multiple genes encoding distinct 3ß-HSD isoforms have been characterized in humans, rats, mice and hamsters (reviewed in 9-12). They are expressed in a tissue-specific manner and are under distinct mechanisms of regulation (9-12). The adrenal/gonadal 3ß-HSD isoform in humans is referred to as type II, whereas the same isoform is designated type I in other species, in reference to the chronology of their cloning (10).

The LH preovulatory rise is the physiological trigger of follicular luteinization, a process during which progesterone biosynthesis is markedly increased (17). The molecular basis of this event has been studied in various species, with results often revealing differences in the regulation and cellular distribution of key steroidogenic enzymes across species (18-24). The transcriptional regulation of three steroidogenic genes, including the steroidogenic acute regulatory protein (StAR), P450SCC and 3ß-HSD genes, are thought to play a major role in the control of progesterone biosynthesis (9, 25, 26). A recent study in the horse documented a unique inverse regulation of StAR mRNA in theca interna and granulosa cells of equine follicles prior to ovulation (27). However, in contrast to other species, the gonadotropin-dependent control of P450SCC and 3ß-HSD expression in equine follicular cells has remained largely uncharacterized. Therefore, the general objective of this study was to describe the regulation of P450SCC and 3ß-HSD in equine preovulatory follicles. The specific objectives were to clone and characterize the primary structure of equine P450SCC and 3ß-HSD, and describe the regulation and cellular localization of their transcripts in equine follicles during human chorionic gonadotropin (hCG)-induced ovulation.

MATERIALS AND METHODS

Materials

Rompun was obtained from Haver (Bayvet Division, Shawnee, KS); Dormosedan was purchased from SmithKline Beecham, Animal Health (West Chester, PA); hCG was obtained from The Buttler Company (Columbus, OH); Torbugesic was purchased from Fort Dodge Laboratories Inc (Fort Dodge, IA); Lutalyse was purchased from UpJohn (Kalamazoo, MI); [α-^{32}P]dCTP, [γ-^{32}P]ATP and [^{35}S]dATP were obtained from Mandel Scientific NEN Life Science Products (Mississauga, Ontario); TRIzol total RNA isolation reagent, RNA ladder (0.24-9.5 kilobases), synthetic oligonucleotides and culture media were purchased from Life Technologies Inc (Gaithersburg, MD); AMV reverse transcriptase, RNAsin, DNA 5'-End Labeling System, Prime-a-Gene labeling system and AMV reverse transcriptase were obtained from Promega (Madison, WI); Biotrans nylon membranes (0.2 μm) were purchased from ICN Pharmaceuticals (Montreal, Quebec); QuikHyb hybridization solution was obtained from Stratagene Cloning Systems (La Jolla, CA); T4 polynucleotide kinase and all sequencing reagents were purchased from Pharmacia Biotech Inc (Baie D'Urfé, Québec); Electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA); and Kodak film X-OMAT AR was obtained from Eastman Kodak Company (Rochester, NY).

Cloning of equine cytochrome P450scc and 3β-HSD cDNAs

The equine P450scc and 3ß-HSD cDNAs were cloned using an expression library prepared with mRNA extracted from an equine preovulatory follicle isolated during estrus and with the ZAP-cDNA/Gigapack cloning kit (Stratagene), as previously described (28). Approximately 100,000 phage plaques were screened with a 1.2 kb *Eco RI* restriction fragment of the rat P450scc cDNA (5), and a 1.5 kb *Eco RI* restriction fragment of the bovine 3ß-HSD cDNA (29). Probes were labeled with [α-

^{32}P]deoxy-CTP using the Prime-a-Gene labeling system (Promega) to a final specific activity greater than 1×10^8 cpm/μg DNA, and hybridization was performed at 55 C with QuikHyb hybridization solution (Stratagene). Positive clones were purified through secondary and tertiary screening, and pBluescript phagemids containing the cloned DNA insert were excised *in vivo* with the Ex-Assist/SOLR system (Stratagene). DNA sequencing (30) was performed using the T7 Sequencing Kit (Pharmacia), vector based primers (T3 and T7) and custom oligonucleotide primers (Gibco BRL). Nucleotide and amino acid analyses were performed with the FASTA program of Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI) and the MacDNASIS software version 2.0 (Hitachi, Hialeah, FL).

Primer extension analysis

Primer extension assays were performed in aqueous buffer, as described (28, 31). The primers included a 30-mer antisense oligonucleotide 5'-CTTTGACCAAGACTGAGCGCAGAGGAAGCC-3' corresponding to the region located between 28 and 57 bp from the beginning of the longest P450scc cDNA clone, and a 30-mer antisense oligonucleotide 5'-CACCCAGCCATGGGTAAACCTG TTAGAGTG-3' corresponding to the region located between 21 and 50 bp from the beginning of the longest 3ß-HSD cDNA clone. The primers were end-labelled (DNA 5'-End Labeling System, Promega) and hybridized (50,000 cpm/reaction) to 50 μg of total RNA extracted from a corpus luteum (10 μg; day 8 of cycle), and RNA extracted from spleen (negative control) at 30 C overnight in 30 μl of buffer (1 M NaCl, 167 mM HEPES pH 7.5 and 0.33 mM EDTA pH 8.0). After precipitation, primer extension was performed by adding 3.5 μl of 4 mM dNTPs, 2.5 μl of 10X RT buffer (0.5 M Tris-Cl, pH 8.2, 50 mM $MgCl_2$, 50 μM DTT, 0.5 M KCl, 0.5 mg/ml BSA), 1.25 μl RNAsin, 18 μl H_2O, 40 U AMV reverse transcriptase and incubating at 42 C for 90 min. After extraction and precipitation, extension products were analyzed by electrophoresis on a 6% polyacrylamide/7M urea gel, and their size was determined by

comparison with the products of either an unrelated equine sequencing reaction that served as a nucleotide ladder (3ß-HSD), or a sequencing reaction that used a corresponding equine P450SCC genomic clone (obtained by genomic library screening; Boerboom and Sirois, unpublished data).

Isolation of equine preovulatory follicles and corpora lutea

Equine preovulatory follicles and corpora lutea were isolated from Standardbred and Thoroughbred mares at precise stages of equine estrous cycle, as previously described (28, 32). Ovulation was induced with hCG (2500 IU, iv) during estrus when the preovulatory follicle reached 35 mm in diameter. The ovary bearing the presumptive preovulatory follicle was removed via colpotomy 0, 12, 24, 30, 33, 36 and 39 hours post-hCG with a chain ecraseur (n = 4-5 follicles per time point)(28). In this model, ovulation occurs between 39 and 42 h after hCG treatment (28, 32). Additional hemiovariectomies were performed during the luteal phase (day 8 of cycle) to isolate three corpora. The recovered ovary was kept in ice-cold Eagles's Minimal Essential Medium (MEM) supplemented with penicillin (50 U/ml)-streptomycin (50 µg/ml), L-glutamine (2.0 mM) and nonessential amino acids (0.1 mM). Preovulatory follicles and corpora lutea were dissected from the surrounding ovarian tissues with a scalpel. Follicles were dissected into three cellular preparations using a methodology previously described (28, 33). Briefly, the follicle was cut into several pieces, and under a dissecting microscope, the theca externa and other surrounding tissues were dissected away from the theca interna using fine forceps. The resulting theca interna with attached granulosa cells was subsequently referred to as a follicular wall preparation. Some pieces of follicular wall were further dissected into isolated preparations of granulosa cells and theca interna by gently scraping the theca interna with a bent glass Pasteur pipette. Granulosa cells were recovered by centrifugation. With this approach, the relative purity of each cellular preparation is thought to exceed 95% based on the selective expression of P450 17α-hydroxylase/C17-20 lyase and P450 aromatase

mRNAs by theca interna and granulosa cells, respectively (34). All samples were stored at -70 C until RNA extraction. Animal procedures were approved by the institutional animal use and care committee.

RNA extraction and Northern blot analysis

RNA was extracted from equine tissues using TRIzol (Life Technologies) and a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific). Northern blot analyses were performed as described (28, 35). RNA samples (10 μg) were denatured at 55 C for 15 min in denaturing buffer, electrophoresed on a 1% formaldehyde-agarose gel and transferred by capillarity to a nylon membrane (28, 35). A ladder of RNA standards was run with each gel and ethidium bromide (10 μg) was added to each sample prior to electrophoresis to compare RNA loading and determine migration of standards. Hybridization was performed using QuikHyb solution (Stratagene) and the following cDNA probes: a 0.7 kb *Hind III/Pst I* fragment of the equine P450SCC cDNA, a 0.9 kb *Hind III/Sac I* fragment of the equine 3ß-HSD cDNA, and the rat elongation factor Tu cDNA (EFTu) as a control gene for RNA loading (36). Each cDNA was labeled by random oligonucleotide-primed synthesis to a final specific activity greater than 1×10^8 cpm/μg DNA using [α-^{32}P]dCTP and the Prime-a-Gene labeling system (Promega). and following the manufacturer's protocol. Stripping of hybridization signal between successive rounds of probing was achieved by soaking filters in 0.1% SSC-0.1% SDS for 20 min at 100 C.

Statistical analysis

Relative levels of P450SCC, 3ß-HSD and EFTu mRNAs were quantified by densitometric analysis of autoradiogram bands using a computer-assisted image analysis system (Collage Macintosh program, Fotodyne Inc., New Berlin, WI). Data were expressed as ratios of P450SCC to EFTu, and 3ß-HSD to EFTu prior to analyses (n = 4 follicles (or mares)/time point). Statistical analyses were performed using JMP

Software (SAS Institute Inc, Cary, NC). One-way ANOVA was used to test the effect of time after hCG on relative levels of P450scc and 3ß-HSD mRNAs. When ANOVAs indicated significant differences ($P<0.05$), the Tukey-Kramer test was used to compare individual means.

RESULTS

Characterization of equine cytochrome P450scc

Twelve positive clones isolated from the primary screening were selected for purification and *in vivo* excision, and extensive DNA sequencing was performed on the three longest cDNA clones. Results revealed that the longest equine P450scc cDNA consisted of a 5'-untranslated region (5'-UTR) of 14 bp, an open reading frame (ORF) of 1560 bp, and a 3'-UTR of 225 bp (Fig. 1). The coding region encodes a 520 amino acid protein, which is identical in length to that of goat (7), sheep (7), cow (3) and pig (6) P450scc, but one and six amino acid shorter than that of the human (4) and rat protein (5), respectively (Fig. 2).

Primer extension analysis was used to determine the size of the complete P450scc 5'-UTR. A major 95-nucleotide extension product was produced when the primer was hybridized to a sample known to contain P450scc mRNA (corpus luteum) (Fig. 3A). From this result, it is deduced that the longest isolated P450scc cDNA appeared to lack the first 38 nucleotides of the full-length transcript, giving a complete 5'-UTR contains 52 bp.

Characterization of equine 3ß-HSD

The three longest cDNAs obtained from an initial group of 12 positive clones were used for the characterization of equine 3ß-HSD. Sequencing results showed that the longest equine 3ß-HSD clone consisted of a 5'-UTR of 39 bp, an ORF of 1119 bp,

and a 3'-UTR of 432 bp (Fig. 4). The 3ß-HSD coding region encodes a 373 amino acid protein that is highly homologous to the adrenal/gonadal 3ß-HSD isoform of other mammalian species (29, 37-41) (Fig. 5). Putative functional regions include two YXXXK motifs that are characteristic of short-chain alcohol dehydrogenase active sites, an amino-terminal GXXGXXG motif thought to form a hydrophobic pocket involved in binding NAD+, and two hydrophobic domains involved in anchoring 3ß-HSD to membranes (9, 11, 42, 43) (Fig. 5).

The length of the complete 5'-UTR of the equine 3ß-HSD mRNA was determined by primer extension analysis. Results showed that a single 72-nucleotide extension product was produced when the primer was hybridized to RNA extracted from a corpus luteum (Fig. 3B). Therefore, our longest 3ß-HSD cDNA clone appeared to lack the first 22 nucleotides of the full-length transcript, giving a complete 5'-UTR of 61 bp.

Regulation of equine P450SCC and 3ß-HSD mRNAs in preovulatory follicles

To characterize the regulation of P450SCC and 3ß-HSD mRNAs during the equine ovulatory process, Northern blot analyses were performed with preovulatory follicles isolated between 0 and 39 h after an ovulatory dose of hCG, and corpora lutea obtained on day 8 of the cycle (Fig. 6). The equine P450SCC mRNA appeared primarily as a transcript of 2.0 kb in size, but a less abundant transcript of approximately 4.0 kb was detected in samples containing more P450SCC (Fig. 6). Two transcripts of comparable intensities, 1.8 and 3.9 kb, were observed for 3ß-HSD in follicular extracts (Fig. 6). When data from all follicular wall samples (n = 4/time point) were quantified by densitometric analyses and corrected with the control gene EFTu, results showed significant changes in levels of P450SCC but not in 3ß-HSD during the ovulatory process. Administration of hCG caused a decrease in follicular P450SCC mRNA, with levels at 30, 33, 36 and 39 h being significantly lower than at 0 h ($P < 0.05$). Following ovulation, a significant increase in P450SCC mRNA was

observed in corpora lutea ($P < 0.01$), whereas levels of 3ß-HSD remained constant during this period ($P > 0.05$).

Cellular localization of P450SCC and 3ß-HSD expression in equine follicles

Northern blot analyses were performed on isolated preparations of granulosa cells and theca interna to study the relative contribution of each steroidogenic cell type in follicular P450SCC and 3ß-HSD mRNA expression. Prior to hCG treatment (0 h), theca interna was clearly the predominant site of P450SCC expression in the follicle, as levels of transcripts were higher in theca than in granulosa cells ($P < 0.01$, Fig. 7). Levels of P450SCC mRNA remained unchanged between 0 and 24 h in theca interna, but a significant decrease was observed at 30-39 h post-hCG ($P < 0.05$, Fig. 7). In granulosa cells, levels of P450SCC transcripts were relatively low and remained unchanged between 0 and 36 post-hCG, but a significant increase was observed at 39 h post-hCG ($P < 0.05$, Fig. 7). Interestingly, the predominant site of P450SCC mRNA expression in the preovulatory follicle switched from the theca interna layer at 0 h to the granulosa cell layer at 39 h post-hCG (Fig. 7).

The cellular localization and regulation of 3ß-HSD transcripts in equine ovarian cells differed from that observed for P450SCC. Expression of 3ß-HSD mRNA was observed predominantly, if not exclusively, in granulosa cells (Fig. 7). The administration of hCG had no significant effect on 3ß-HSD transcripts, with levels remaining unchanged in granulosa cells throughout the ovulatory process ($P > 0.05$, Fig. 7). A very weak 3ß-HSD signal was detected in few theca interna samples, which likely resulted from contaminating granulosa cells.

DISCUSSION

In contrast to other species, the molecular control of follicular steroidogenesis

has remained largely uncharacterized in mares. Yet, this species provides an interesting model for the study of gonadotropin-dependent gene expression in the ovary, considering the large size of the equine preovulatory follicle (40-45 mm in diameter), and the ability to precisely monitor follicular development by ultrasound imaging (44, 45). We have recently described the regulation of transcripts coding for key steroidogenic proteins and enzymes in equine follicles during the ovulatory process, including mRNAs for StAR, cytochrome P450 17α-hydroxylase/C17-20 lyase (P450$_{17\alpha}$), and cytochrome P450 aromatase (P450$_{AROM}$) (27, 34). To provide a more complete understanding of the control of equine follicular steroidogenesis, the present study reports the molecular cloning and characterization of equine P450$_{SCC}$ and 3ß-HSD, and the regulation and cellular localization of corresponding transcripts in a series of preovulatory follicles isolated between 0 and 39 h after an ovulatory dose of hCG. The equine P450$_{SCC}$ and 3ß-HSD mRNAs were found to encode 520 and 373 amino acid proteins, respectively, which is highly similar in length to corresponding enzymes in other mammalian species (3-7, 29, 37-41). The amino acid sequence of the equine P450$_{SCC}$ showed a high degree of conservation when compared to that of other mammalian homologs (71-79% identical to goat, sheep, cow, pig, human and rat P450$_{SCC}$), particularly within regions proposed to be involved in binding the substrate and a prosthetic heme group (3-7). The deduced amino acid sequence of equine 3ß-HSD was also highly similar to other species homologs, being 68-79% identical to rat (37), mouse (38), hamster (41), human (40), macaque (39) and cow (29) adrenal/gonadal-type 3ß-HSD. Additional studies will be needed to determine whether multiple isoforms of 3ß-HSD are present in the horse, as observed in numerous species (9-12).

To our knowledge, the overall regulation of P450$_{SCC}$ mRNA in equine follicles prior to ovulation is unique, and thus adds to the diversity of paradigms observed for the control of P450$_{SCC}$ expression in preovulatory follicles of other species (5, 19-21, 46-51). Prior to hCG treatment, P450$_{SCC}$ mRNA was relatively high in theca interna

and low in granulosa cells of equine follicles, which compares with observation in pigs (21, 50), but differs from reports in humans (19) and cows (20, 48) who express high levels of transcripts in granulosa cells prior to LH/hCG surge. This finding suggests that the equine theca interna plays a major role in follicular steroidogenesis prior to the gonadotropin rise, as implied previously from the theca cell-selective expression of StAR and P45017α transcripts in equine follicles at this stage (27, 34). Induction of the ovulatory/luteinization process with hCG leads to a down-regulation of follicular P450SCC mRNA, and a unique cellular redistribution of the transcript. Similar studies in other species revealed that a down-regulation of P450SCC also occurs in cows (20, 48) after the LH surge, whereas transcript levels remain unchanged in porcine (21) and ovine (46) follicles, and increase in rat follicles after the surge (47, 51). However, the cellular redistribution of P450SCC mRNA, defined as the disappearance of the transcript in theca interna and the concurrent increased expression in granulosa cells, is unprecedented in other species. This phenomenon could be related to the putative degeneration of the theca interna at the time of ovulation in mares, which is unique to this species and leads to the formation of a corpus luteum solely derived from granulosa cells (52). The timing of the loss of P450SCC mRNA in theca interna coincides with the disappearance of StAR and P45017α transcripts in this cell type (27, 34), providing further biochemical evidence for a putative demise of the equine theca interna prior to ovulation. The marked increase in P450SCC expression observed in the equine corpus luteum is in keeping with observations in other species (20, 46, 49, 51, 53-55).

The present study documents a novel pattern of 3ß-HSD mRNA expression in equine follicular cells during the ovulatory process. The presence 3ß-HSD transcript in equine granulosa cells and its absence or very low expression in theca interna contrast with findings in other species (19-21, 23, 24, 46, 56, 57). However, this pattern agrees with a previous report showing that 3ß-HSD activity was present in granulosa cells of large follicles isolated during estrus but absent in theca interna of all equine follicles tested (58). Thus, the equine theca interna presumably does not produce much

progesterone *in vivo*, although elevated expression of StAR (27) and P450scc mRNAs in this cell type suggests that it synthesizes large amounts of pregnenolone precursors. This model is further supported by studies *in vitro* showing that cultures of equine theca interna secrete negligible amounts of progesterone, and that gonadotropins have no effect on its secretion (33). The apparent lack of modulation of 3ß-HSD mRNA expression in equine granulosa cells during the ovulatory process contrasts with the down-regulation observed in cows (20). In other species such as the pig and the sheep, 3ß-HSD is not detectable in granulosa cells prior to ovulation, but is induced in the corpus luteum (21, 23, 46). The constant levels of 3ß-HSD mRNA in equine granulosa cells suggest a nonlimiting role for this enzyme during equine terminal follicular steroidogenesis. However, the present study does not exclude the likelihood that the 3ß-HSD expression undergoes regulatory processes at other developmental stages, as reported in other species (24, 46, 57, 59-61). The detection of two 3ß-HSD transcripts of comparable intensities was unexpected. Whereas the smaller transcript is in keeping with the size of the cloned cDNA, the precise nature of the larger transcript remains unknown but could represent a product derived from an alternative polyadenylation site, as observed for chicken 3ß-HSD (62), or a hybridization artifact. Likewise, the precise nature of a larger, albeit less abundant P450scc mRNA in some follicular samples remains unknown.

The molecular control of P450scc and 3ß-HSD gene expression in equine ovarian cells remains to be characterized. Several reports in other species have implicated the orphan nuclear receptor steroidogenic factor-1/adrenal 4-binding protein (SF-1/Ad4BP) in the transcriptional regulation of genes encoding steroidogenic enzymes, including P450scc (63-66) and 3ß-HSD (10, 67). Recent studies have demonstrated that the LH/hCG surge results in a pronounced down-regulation of SF-1 mRNA in rat granulosa cells (68, 69). Thus, the downregulation of P450scc mRNA in equine follicles after the hCG treatment could potentially result, at least in part, from a decrease in transcriptional activity due to a decline in SF-1. However, the fact that 3ß-

HSD mRNA levels were unaffected by hCG in equine follicles suggests that, although SF-1 likely plays a role in the control of equine steroidogenesis, additional cell type- and promoter-specific regulatory factors must be involved in the complex differential regulation of steroidogenic genes in theca and granulosa cells.

A working model for the control of equine follicular steroidogenesis is proposed based on results presented in this report and in previous studies (27, 32-34, 52, 58, 70) (Fig. 8). During the early follicular phase, the theca interna layer appears to be the site of very active steroidogenesis; the layer consists of plump polyhedral cells (32, 52) in which high levels of StAR (27), P450SCC (this study) and P45017α mRNAs (34) are either predominantly or exclusively expressed. Since the theca interna expresses very low or undetectable levels of 3ß-HSD mRNA (this study) or activity (58), and produces negligible amounts of progesterone *in vitro* (33), steroidogenesis likely proceeds primarily via the Δ5 pathway to generate the androgen dehydroepiandrosterone (DHEA) (Fig. 8). Estrogens would then be synthesized from DHEA by granulosa cells, which are the predominant, if not the only, follicular cells that express 3ß-HSD mRNA (this study) and activity (58), P450AROM mRNA (55) and protein (70), and estradiol synthetic capacity (33) (Fig. 8). At the end of the ovulatory process, morphological studies revealed that the equine theca interna undergoes a putative degenerative process (32, 52). Biochemically, this process is accompanied by an apparent loss of StAR (32), P450SCC (this study) and P45017α (34) in the theca interna layer, and an upregulation of StAR (32) and P450SCC mRNA (this study) in granulosa cells, thus putting all enzymes required for progesterone synthesis (i.e. StAR, P450SCC, and 3ß-HSD) in the same cell type (Fig. 8). These gonadotropin-dependent changes in enzyme expression would redefine the principal steroidogenic pathway from Δ5 to Δ4, with progesterone becoming the obligatory end product. An accessory role of the Δ4 pathway during the early follicular phase is not excluded; low-level expression of P450SCC in granulosa cells could lead to appreciable amounts of progesterone production in that tissue. Then, progesterone would be converted into androstenedione

after diffusion to the theca interna layer (Fig. 8). Although this model attempts to integrate current knowledge on the regulation of equine follicular steroidogenesis, its should not be viewed as the definitive paradigm but rather as a working model from which hypotheses can be generated and tested. Importantly, additional studies will be needed to establish a complete relationship between changes in transcripts, proteins and enzymatic activities, and to unravel the molecular basis for steroidogenic gene expression in equine ovarian cells.

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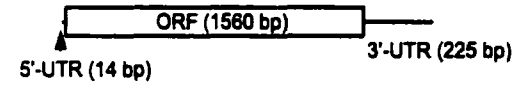
Fig. 1. Isolation and characterization of the nucleotide sequence of the equine P450SCC cDNA. *A*, Schematic representation of the isolated equine P450SCC cDNA clone. The cDNA is composed of a 5'-UTR of 14 bp, an ORF of 1560 bp, and a 3'-UTR of 225 bp. *B*, Complete nucleotide sequence of the P450SCC cDNA clone. The ORF is indicated by uppercase letters, the translation initiation (ATG) and stop (TGA) codons are highlighted in bold, the 5'-UTR and 3'-UTR are shown in lowercase letters, the polyadenylation signal is underlined, and numbers on the right refer to the last nucleotide on that line. The nucleotide sequence was submitted to GenBank (accession number AF031664).

A



B

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TACCATTTCT GGAGGGAGAA GGGCCCAAAG AAATTACACT ATCACCACTT     250
CCAGAATTTC CAGAAGTATG GCCCCATTTA CAGGGAGAAG CTTGGCAACG     300
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TTCGAGGGTC CCCATCCGGA ACGATTTCTC ATCCCACCCT GGACCGCCTA     400
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CCTGGAAGAA AGACCGGCTA GCTCTGAACC CGGAGGTGAT GGCTCTAGAG     500
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ccccatgacg gcaataaaca gctgaacttt gtgaagcaaa aaaaaaaaa      1799
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Fig. 2. Predicted amino acid sequence of equine P450SCC and comparison with known mammalian homologs. The deduced amino acid sequence of the equine (equ) P450SCC is aligned with the caprine (cap), ovine (ovi), bovine (bov), porcine (por), human (hum), and rat homologs. Identical residues are indicated by a printed period. The putative mitochondrial leader sequence cleavage site is indicated by an inverted arrowhead. The first three boxed regions represent proposed substrate binding regions A, B and C, whereas the fourth box represents the proposed heme-binding region. Note that the third box also encompasses the domain proposed to bind adreno-ferredoxin. Numbers on the right refer to the last amino acid residue on that line.

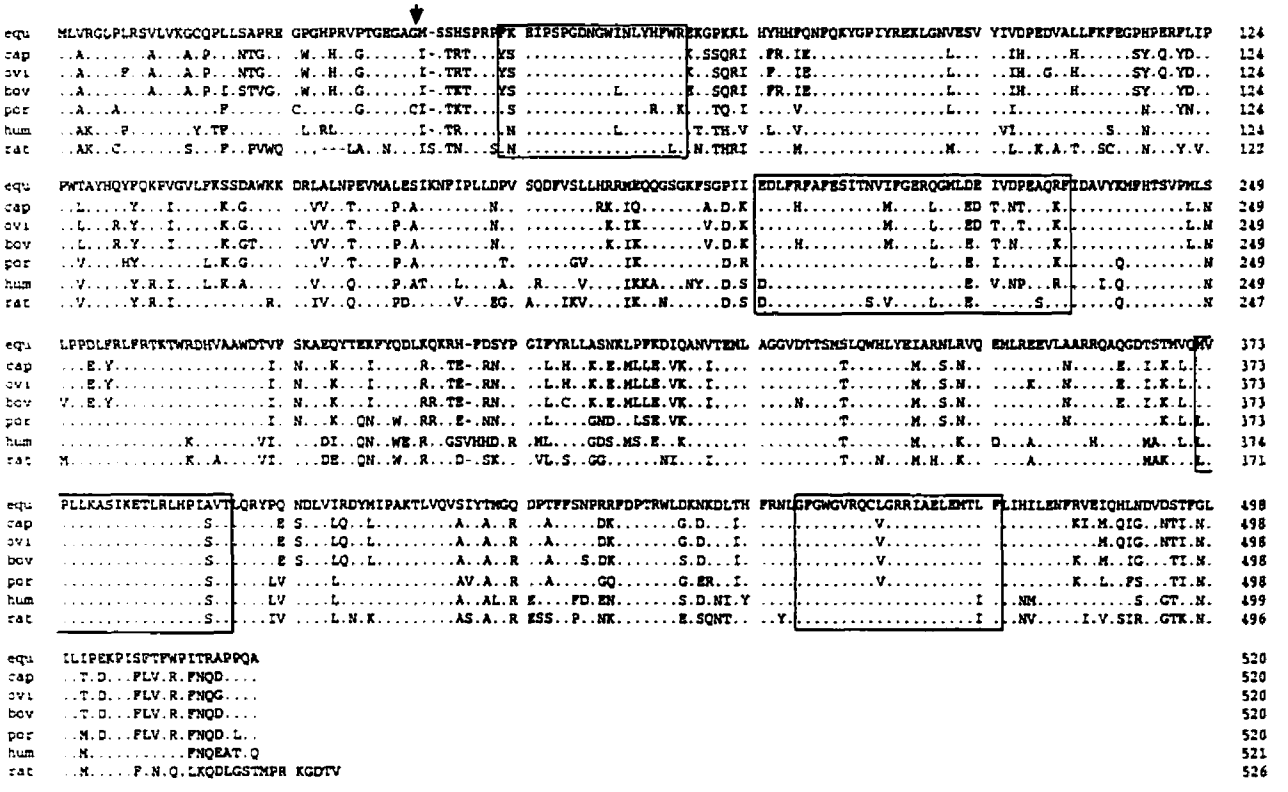


Fig. 3. Primer extension analyses of equine P450SCC and 3ß-HSD mRNAs. Two antisense oligonucleotides corresponding to regions located within the first 60 nucleotides of the cloned P450SCC and 3ß-HSD cDNAs were hybridized to RNA samples containing (corpus luteum [CL], day 8 of cycle) and not containing (spleen) P450SCC and 3ß-HSD, and reverse transcription was performed as described in *Materials and Methods*. Extension products were analyzed on a 6% polyacrylamide gel, and their size determined by comparison with the products of adjacent sequencing reactions. Results reveal a 95-nucleotide (*A*) and a 72-nucleotide (*B*) extension product corresponding to major transcription start sites of P450SCC and 3ß-HSD mRNA, respectively.

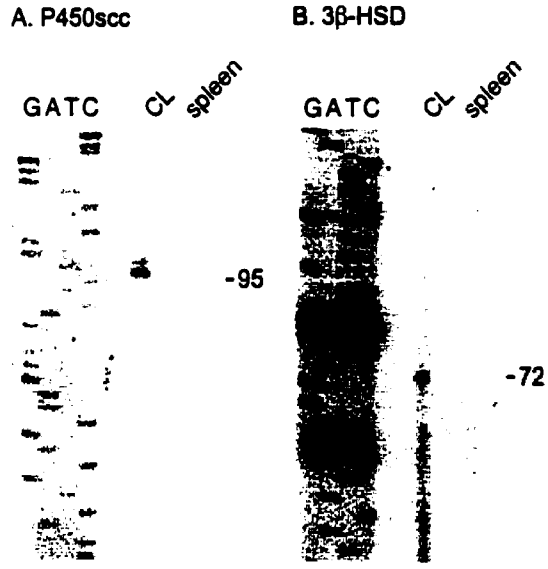
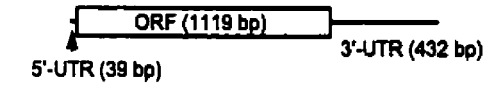

A. P450scc
B. 3β-HSD
GATC
CL
spleen
GATC
CL
spleen
-95
-72

Fig. 4. Isolation and characterization of the nucleotide sequence of the equine 3ß-HSD cDNA. *A*, Schematic representation of the isolated equine 3ß-HSD cDNA. The cDNA is composed of a 5'-UTR of 39 bp, an ORF of 1119 bp, and a 3'-UTR of 432 bp. *B*, Complete nucleotide sequence of the 3ß-HSD cDNA clone. The ORF is indicated by uppercase letters, the translation initiation (ATG) and stop (TGA) codons are highlighted in bold, the 5'-UTR and 3'-UTR are shown in lowercase letters, the polyadenylation signal is underlined, and numbers on the right refer to the last nucleotide on that line. The nucleotide sequence was submitted to GenBank (accession number AF031665).

A



B

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GCTTGCTGGT GGAGGAGAAG GAGGTACAGG AGATCAGAGC ACTGGACAAA    150
GTCTTCAGAC CAGAATTGCG GGAAGAGTTT TCCAAACTCC AGAGCAAGGT    200
CAAGCTGACC GTGCTGGAGG GAGACATTCT GGATGAGCAG TTCCTGAAGA    250
GAGCTTGCCA GGGCGCCTCG GCTGTCATTC ACACCGCCTC TATCATCGAC    300
GTCACGAACC TCTTTAACCC ACAGGTCACC ATGAATGTCA ATGTGGAAGG    350
TACCCAGCTC CTATTGGAGG CCTGTTCCCA GGCTAGCGTG CCAATCTTCA    400
TCTACACCAG CTCCGTAGCG GTGGCTGGGC CCAACTCCTA CAGGGAGATC    450
ATCCAGAATG GCCATGAAGA AGCACATCTC GAAACAAAAT GGTCCTCTCC    500
ATACCCATAC AGCAAAAAGC TTGCTGAGAA GGCTGTGCTG GCAGCTAATG    550
GGTTGCCTCT TAAAAATGGT GGCACCTTAT ACACTTGTGC CTTAAGGCCC    600
ATGTTTATCT ATGGGGAAGG AAGCCCAACC CTTTATTACC TTATGCATGA    650
GGGCCTGAAC AACAATGGCA TCCTGACACA CAACTGCAAG TTCTCCAGAG    700
CCAACCCAGT CTATGTTGGC AACATAGCCT GGGCCCACAT TATGGCCTTG    750
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CTACATCTCA GATGACACAC CTCCCCAAAG CTATGATGAC TTAACTTACA    850
CTTTGAGCAA AAAGTGGGGC TTCTGCCTTG ATTCCAGAAT GAGACTTCCC    900
ATATTTCTGA AGTACTGGCT TGCCTTCCTG CTGGAAATAG TGAGCTTCCT    950
GCTCAGTCCA ATTTACAAAT ATCGACCTCC CTTCGACCGC CACCTAGTGA   1000
CATGGCAAAA CAGTGTTTTT ACCTTCTCCT ATAAGAAAGC TCAGCGAGAT   1050
ATGGGATATG AGCCGCTCTT CAGCTGGGAG GAAGCCAAGA AGAGAACCAC   1100
TGAGTGGATC GACGCCCTGG TAGAACCGCA CCAGGAGGCC CTGAAAACAA   1150
AGACTCTCTG Atctacaggt gacatggatg cggatgttag gagatgtctg   1200
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gttttgcctg ttagttcccg ttcctttgtt aaatgcaaaa gcatttccta   1500
tcttttaaaa attcctatta cttcagacag ctcaatgaaa agaataataa   1550
atgttttact gcctaactgc ctaaaaaaaa aaaaaaaaaa             1590
```

Fig. 5. Predicted amino acid sequence of equine 3ß-HSD and comparison with known mammalian homologs. The deduced amino acid sequence of the equine (equ) 3ß-HSD is aligned with the murine (mur), rat, hamster (ham), human (hum), macaque (mac), and bovine (bov) homologs. Only primary adrenal/gonadal isoforms are represented. Boxed regions represent YXXXK motifs characteristic of short-chain alcohol dehydrogenase active sites. Double-overlined sequences are hydrophobic and may be involved in anchoring to membranes. The glycines involved in the formation of the hydrophobic cofactor-binding pocket are underlined. Numbers on the right refer to the last amino acid residue on that line.

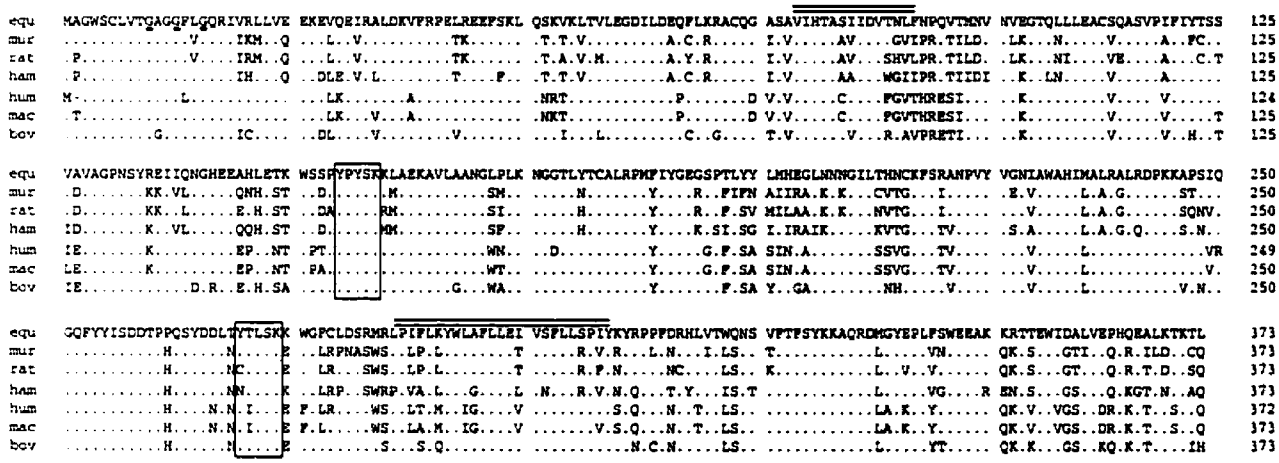


Fig. 6. Regulation of equine P450SCC and 3ß-HSD mRNAs in equine preovulatory follicles during hCG-induced ovulation. Preparations of follicular wall were obtained from preovulatory follicles isolated between 0 and 39 h after hCG, and corpora lutea (CL) were isolated on day 8 of the estrous cycle. Samples of total RNA (10 µg/lane) were analyzed by Northern blotting using an equine P450SCC cDNA probe (*A*), an equine 3ß-HSD cDNA probe (*B*), and the rat elongation factor Tu (*EFTu*) as a control gene for RNA loading (*C*). *Markers* on the right indicate the size of the transcripts. Filters in *panels A, B and C* were exposed to film at -70 C for 15, 24 and 13 h, respectively.

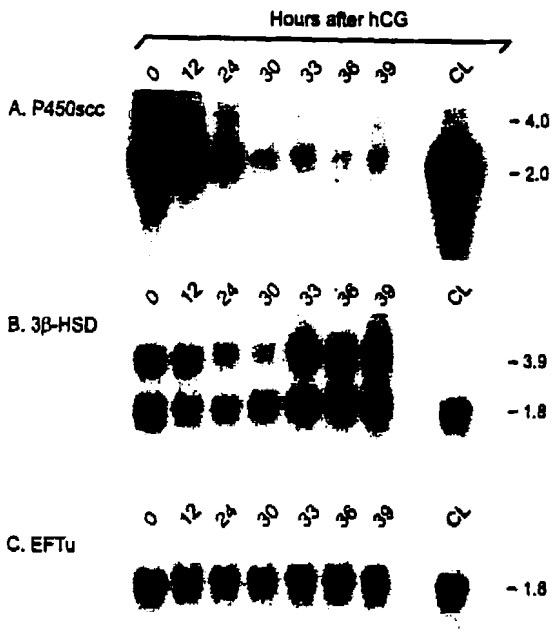

Hours after hCG
0
12
24
30
33
36
39
CL
A. P450scc
- 4.0
- 2.0
B. 3β-HSD
- 3.9
- 1.8
C. EFTu
- 1.8

Fig. 7. Relative changes of P450SCC and 3ß-HSD mRNA levels in equine follicle cells isolated between 0 and 39 h after hCG treatment. Samples (n=10 μg) of total RNA extracted from granulosa cells and theca interna were analyzed by Northern blotting with the equine P450SCC cDNA, the equine 3ß-HSD cDNA, and the rat elongation factor Tu (*EFTu*) cDNA as a control gene for RNA loading. After autoradiography (films not shown), the signal intensity was quantified by densitometric analysis, and data from steroidogenic transcripts were normalized with the control gene EFTu. Results are presented as a ratio P450SCC to EFTu ([P450SCC/EFTu] X 100) and a ratio of 3ß-HSD to EFTu ([3ß-HSD/EFTu] X 100) (mean ± SEM; n = 4 follicles [i.e. mares]/time point). Columns marked with an asterisk are significantly different ($P<0.05$) from 0 h post-hCG.

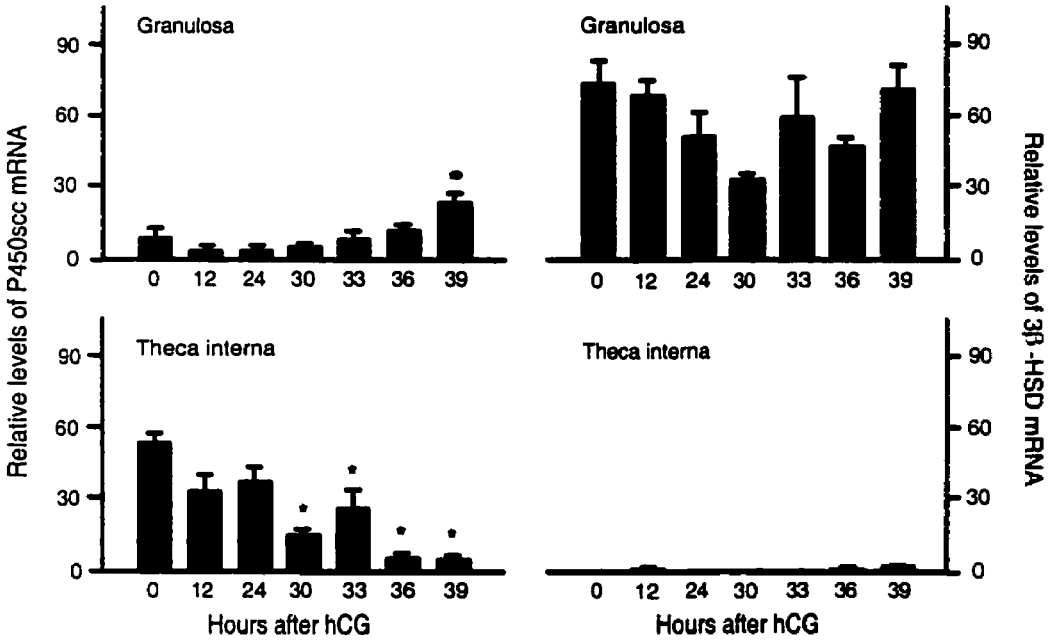
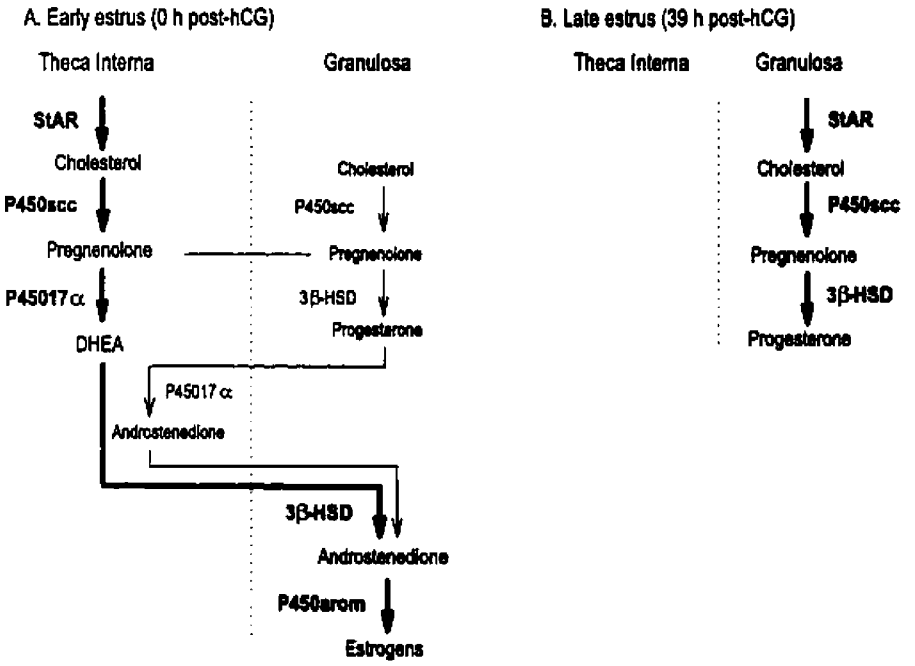

Granulosa
Granulosa
Theca interna
Theca interna
90
60
30
0
0 12 24 30 33 36 39
Hours after hCG
Relative levels of P450scc mRNA
Relative levels of 3β -HSD mRNA

Fig. 8. Proposed model for the regulation of equine follicular steroidogenesis during the early (0 h post-hCG, *panel A*), and late follicular phase (39 h post-hCG, *panel B*). The model is based on the regulation and cellular localization of transcripts involved in equine follicular steroidogenesis as reported herein and in previous reports (27, 34), as well as on the cellular localization of steroidogenic enzymes (70) or activities (58), the steroidogenic capacity of equine follicular cells *in vitro* (33), and the histology of equine follicular cells during the ovulatory process (32, 52). For more details see text.


A. Early estrus (0 h post-hCG)
Theca Interna
Granulosa
StAR
Cholesterol
P450scc
Pregnenolone
P45017α
DHEA
Cholesterol
P450scc
Pregnenolone
3β-HSD
Progesterone
P45017α
Androstenedione
3β-HSD
Androstenedione
P450arom
Estrogens
B. Late estrus (39 h post-hCG)
Theca Interna
Granulosa
StAR
Cholesterol
P450scc
Pregnenolone
3β-HSD
Progesterone

Summary of article #3

Dual regulation of promoter II- and promoter If-derived cytochrome P450 aromatase transcripts in equine granulosa cells during human chorionic gonadotropin-induced ovulation: a novel model for the study of aromatase promoter switching. **Derek Boerboom**, Abdurzag Kerban and Jean Sirois. Endocrinology 140(9):4133-4141 (1999).

Thesis author's contribution to the work: As the primary author, I was responsible for all aspects of the production of this article. Second authorship credits reflect technical contributions to animal procedures.

Summary:

- Two distinct P450arom cDNAs were isolated from an equine follicular cDNA library. One was derived from promoter If and encoded a well-conserved 503-amino acid protein, the other was derived from promoter II and encoded a truncated protein. A cDNA encoding P45017α was also cloned.
- Northern blot analyses were performed using preovulatory follicles obtained on a time-course after the administration of hCG. P450arom mRNA regulation was biphasic: levels were highest in granulosa cells at 0h post-hCG, decreased significantly during the ovulatory process at 12 and 24h, and increased again between 30–39h post-hCG and in corpora lutea.

- Probes used to detect specific P450arom mRNA variants revealed that promoter II usage accounts for the message present in follicles at 0h post-hCG and in corpora lutea, whereas promoter 1f is used exclusively during the ovulatory process.
- Levels of P45017α mRNA were high in theca interna at 0h, but significantly decreased in follicles at 36 and 39h post-hCG and in corpora lutea.
- Equine aromatase promoters II and 1f were cloned from a genomic library, and putative transcription start sites were identified by primer extension assays. Potential regulatory elements were identified by sequence analysis.

Work's contribution to the advancement of science:

- This study characterizes for the first time the complete structure of two distinct equine P450arom transcripts, as well as the promoters that direct their expression.
- It identifies novel patterns of expression and regulation of these transcripts in equine follicles prior to ovulation. The downregulation of P45017α may be related to the putative degeneration of the theca interna.
- It identifies a novel aromatase promoter-switching phenomenon in equine granulosa cells during follicular luteinization and provides a new model in which aromatase promoter switching is induced *in vivo*.
- Finally, it offers significant insight as to how the overall regulation of estradiol biosynthesis occurs throughout the ovulatory process.

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Dual Regulation of Promoter II- and Promoter 1f-Derived Cytochrome P450 Aromatase Transcripts in Equine Granulosa Cells during Human Chorionic Gonadotropin-Induced Ovulation: A Novel Model for the Study of Aromatase Promoter Switching*

DEREK BOERBOOM†, ABDURZAG KERBAN‡, AND JEAN SIROIS

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ABSTRACT

Estradiol biosynthesis is a key biochemical trait of developing follicles. To study its regulation in equine follicles, the objectives of this study were to clone and determine the structure of equine cytochrome P450 aromatase (P450AROM), and characterize the regulation of P450AROM and P450 17α-hydroxylase/C17–20 lyase (P45017α) messenger RNAs (mRNAs) *in vivo* in equine preovulatory follicles isolated during hCG-induced ovulation. Two distinct P450AROM complementary DNAs (cDNAs) were isolated from an equine preovulatory follicle cDNA library. One clone was 2682 bp in length and included 115 bp of 5′-untranslated region (UTR), 1509 bp of open reading frame encoding a well conserved 503-amino acid protein, and 1058 bp of 3′-UTR. Its 5′-most region represented the equine homolog of exon 1f, previously designated brain specific. The other cDNA clone encoded a truncated protein and contained a distinct 5′-UTR characteristic of transcripts derived from promoter II, previously identified as the predominant ovarian mRNA. Northern blot analyses were performed using preovulatory follicles obtained during estrus between 0–39 h after the administration of hCG and with corpora lutea isolated on day 8 of the estrous cycle (day 0 = day of ovulation). The results showed a biphasic regulation of P450AROM mRNA expression: levels were highest in follicles at 0 h post-hCG, decreased significantly during the ovulatory process at 12 and 24 h ($P < 0.05$), and increased again between 30–39 h post-hCG and in corpora lutea. When oligonucleotides specific for P450AROM mRNA variants were used as probes, a novel switching phenomenon was observed. Promoter II-derived transcripts accounted for the message present in follicles at 0 h post-hCG and in corpora lutea, whereas promoter 1f-derived mRNA was expressed exclusively during the ovulatory process (30–39 h post-hCG). Levels of P45017α mRNA were high in follicles at 0 h, but significantly decreased after hCG treatment ($P < 0.05$), with lowest levels in follicles at 36 and 39 h post-hCG and in corpora lutea. Northern blots performed on isolated cellular preparations revealed that P450AROM and P45017α transcripts were localized exclusively in granulosa cells and theca interna, respectively. Equine aromatase promoters II and 1f were cloned from a genomic library, and putative transcription start sites were characterized by primer extension assays. Sequence analyses identified distinct potential regulatory elements in each promoter. Thus, this study identifies a novel aromatase promoter-switching phenomenon in equine granulosa cells during follicular luteinization and provides a new model in which aromatase promoter switching is induced *in vivo*. (*Endocrinology* **140**: 4133–4141, 1999)

THE AROMATASE cytochrome P450 (P450AROM), a product of the CYP19 gene, catalyzes the final rate-limiting step in the biosynthesis of estrogens from androgens (1–4) and is expressed in the gonads and the brain of most vertebrate species (5–8). However, a more extensive tissue distribution of the enzyme has been reported in humans, including expression in the placenta, adipose tissue, liver, and skin (5–8). Placental expression of P450AROM has also been documented in cows (9, 10), pigs (11–13), and horses (9). A single CYP19 gene spanning more that 75 kb and containing nine coding exons (exons II–X) has been identified in humans (5, 14, 15), but there is evidence for multiple distinct, but closely related, aromatase genes in pigs (13, 16, 17).

The tissue-specific distribution of several aromatase transcripts has been linked in part to the use of different promoters (7, 9, 10, 12, 13, 18–21). In humans, these promoters direct the synthesis of distinct aromatase messenger RNA (mRNA) variants that differ only by their 5′-noncoding termini. At least nine 5′-untranslated first exons, and thus mRNAs variants, have been identified, including exons I.1, I.2, I.3, I.4, I.5, I.6, PII, 2a, and 1f (22). They are alternatively spliced into a common 5′-splice acceptor site found 38 bp upstream of the translation start site in exon 2 and generate transcripts that have distinct, but overlapping, tissue distribution (7).

Increasing evidence suggests that switching of aromatase expression from one mRNA variant to another may be a key regulatory mechanism in several physiological and pathological processes. Distinct aromatase transcripts are expressed in early *vs.* midpregnancy in porcine placenta (12, 13, 23) as well as in fetal *vs.* adult human liver (24). Several studies have shown that a similar switch occurs in healthy *vs.* cancerous human breast adipose tissue (24–29). It has been speculated that a switching mechanism may be involved in

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the ovulation/luteinization process (24). However, results from the study of Jenkins *et al.* (30) did not support this concept, as only promoter II-derived aromatase transcripts were detected in human follicles and corpora lutea.

One key function of P450AROM is to produce large amounts of estradiol in mammalian preovulatory follicles (31), and the obligatory role of estrogen synthesis in female reproduction was recently highlighted in mice by targeted disruption of the CYP19 gene (32). In contrast to that in other species, the molecular control of follicular steroidogenesis in mares has remained largely uncharacterized. Yet, the equine preovulatory follicle offers a good model for the study of ovarian gene expression because it has a relatively large size (40–50 mm in diameter), and its development can be precisely monitored *in vivo* by ultrasound imaging (33, 34). Therefore, the objectives of this study were to clone and determine the primary structure of equine P450AROM, characterize the regulation of P450AROM and P450 17α-hydroxylase/C17–20 lyase (P45017α) mRNAs in a series of equine preovulatory follicles isolated during hCG-induced ovulation, and determine the cellular localization of each transcript.

Materials and Methods

Materials

The equine genomic library and QuikHyb hybridization solution were purchased from Stratagene Cloning Systems (La Jolla, CA); the Prime-a-Gene labeling system and the DNA 5′-End Labeling System were obtained from Promega Corp. (Madison, WI); Biotrans nylon membranes (0.2 μm pore size) were purchased from ICN Pharmaceuticals, Inc. (Montreal, Canada); [α-^{32}P]deoxy (d)-ATP, [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{35}S]dATP were obtained from Mandel Scientific NEN Life Science Products (Mississauga, Canada); TRIzol total RNA isolation reagent, RNA ladder (0.24–9.5 kb), synthetic oligonucleotides, and culture media were purchased from Life Technologies (Gaithersburg, MD); T4 polynucleotide kinase and all sequencing reagents were purchased from Pharmacia Biotech (Baie D'Urfé, Canada); Kodak film X-Omat AR was obtained from Eastman Kodak Co. (Rochester, NY); electrophoretic reagents were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA); Lutalyse was obtained from UpJohn (Kalamazoo, MI); hCG was purchase from The Buttler Co. (Columbus, OH).

Cloning of equine cytochrome P450AROM and P45017α complementary DNAs (cDNAs)

The equine P450AROM and P45017α cDNAs were cloned from an expression library prepared from an equine preovulatory follicle isolated 36 h after the administration of an ovulatory dose of hCG (2500 IU), as previously described (35). Approximately 100,000 phage plaques were screened, and hybridization was performed at 55 C with QuikHyb hybridization solution (Stratagene). The probes, including a 5′, 1.0-kb *Eco*RI restriction fragment of the rat P450AROM cDNA (36) and a 2.0-kb *Xho*I restriction fragment of the bovine P45017α cDNA (37), were labeled with [α-^{32}P]dCTP using the Prime-a-Gene labeling system (Promega Corp.) to a final specific activity greater than 1×10^9 cpm/μg DNA. Positive clones were plaque purified through secondary and tertiary screening, and pBluescript phagemids containing the cloned DNA insert were excised *in vivo* with the Ex-Assist/SOLR system (Stratagene). DNA sequencing was performed by the Sanger dideoxy nucleotide chain termination method (38) using the T7 Sequencing Kit (Pharmacia Biotech), vector-based primers (T3 and T7), and custom oligonucleotide primers (Life Technologies, Inc.). Nucleotide and amino acid analyses were performed using MacDNASIS software (version 2.0, Hitachi, Hialeah, FL) and the FASTA program of Wisconsin Package (version 9.0, Genetics Computer Group, Madison, WI).

Isolation of equine preovulatory follicles and Northern blot analysis

Ovarian follicular development in Standardbred and Thoroughbred mares was monitored daily by ultrasonography (33). When the preovulatory follicle reached 35 mm in diameter during estrus, ovulation was induced with hCG (2500 IU, iv). The ovary bearing the presumptive preovulatory follicle was removed via colpotomy 0, 12, 24, 30, 33, 36, and 39 h post-hCG with a chain ecraseur (n = 4–5 follicles/time point) (35). Also, three corpora lutea were isolated on day 8 of the estrous cycle using the same approach. The recovered ovary was kept in ice-cold Eagles's MEM supplemented with penicillin (50 U/ml)-streptomycin (50 μg/ml; Life Technologies, Inc.), L-glutamine (2.0 mM; Life Technologies, Inc.), and nonessential amino acids (0.1 mM; Life Technologies, Inc.). Preovulatory follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) and isolated theca interna and granulosa cells, as described (35). All samples were stored at −70 C until RNA extraction. Animal procedures were approved by the institutional animal use and care committee.

RNA was extracted from equine tissues using TRIzol (Life Technologies, Inc.) and a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Fairlawn, NJ). For Northern analysis, RNA samples (10 μg) were denatured at 55 C for 15 min in denaturing buffer, electrophoresed on a 1.2% agarose, and transferred by capillarity to a nylon membrane, as previously described (35). A ladder of RNA standards was run with each gel, and ethidium bromide (10 μg) was added to each sample before electrophoresis to compare RNA loading and determine the migration of standards. Hybridization was performed using the QuikHyb solution (Stratagene) and the following cDNA probes: a 0.5-kb *Eco*RI fragment of the equine P450AROM cDNA (clone A17), a 2.0-kb *Eco*RI/*Hin*dIII fragment of the equine P45017α cDNA, and the rat elongation factor Tu cDNA (EFTu) as a control gene for RNA loading and transfer (39). Each cDNA was labeled using the Prime-a-Gene labeling system as described above, and stripping of hybridization signal was achieved by soaking filters in 0.1% SSC (standard saline citrate)-0.1% SDS for 30 min at 100 C.

To study the specific regulation of promoter II- and promoter 1f-derived aromatase transcripts in granulosa cells, two oligonucleotides complementary to unique sequences located at the 5′-end of each transcript were labeled using the DNA 5′-End Labeling System (Promega Corp.), and used as probes in Northern blot analysis. They included a 24-mer antisense oligonucleotide 5′-GTCTGCTGGTCAC TTCTAGTT-TCC-3′ complementary to nucleotides 50 and 73 in promoter 1f-derived transcript (clone A17; Fig. 1), and a 23-mer oligonucleotide 5′-CCAAAAGGTACAT CTAGGACTCC-3′ complementary to nucleotides 5 and 27 in promoter II-derived P450AROM transcript (clone A1; Fig. 1).

Cloning of equine aromatase promoter II and promoter 1f

DNA sequences located immediately upstream of aromatase exon II and exon 1f were cloned using an equine genomic library (Stratagene), following the manufacturer's protocol. To clone the equine aromatase promoter II, approximately 1×10^6 genomic phage plaques were screened using a random primed, 0.5-kb *Eco*RI cDNA fragment corresponding to the 5′-end of the equine aromatase promoter II-derived cDNA (clone A1, Fig. 1). To clone the aromatase promoter 1f, the same genomic phage plaques were screened with a radioactive DNA fragment generated by PCR and corresponding to the first 77 bases of the equine aromatase promoter 1f-derived cDNA (clone A17; Fig. 1). The latter probe was generated by inclusion of 0.5 mCi [α-^{32}P]dATP (6000 Ci/mmol) in a standard PCR reaction mixture (40) and by using clone A17 as template DNA, sense primer 5′-TTCCTAACAGCCGTGCATCAT-TAG-3′, and antisense primer 5′-CTGAGTCTGCTGGTCACTTCTAGT-3′. The DNA fragment was purified by electrophoresis on a 2% agarose gel. Six exon II- and three exon 1f-containing clones were isolated from the initial round and were purified through secondary and tertiary screening. After digestion with *Sac*I, Southern blot analyses were performed using oligonucleotide probes corresponding to the 5′-end of each equine aromatase transcript (same oligonucleotides described for Northern blots). Fragments yielding a positive signal were subcloned into the pBluescript plasmid vector (Stratagene) and sequenced using a T7 Sequencing Kit (Pharmacia Biotech) and custom oligonucleotide primers (Life Technologies, Inc.).

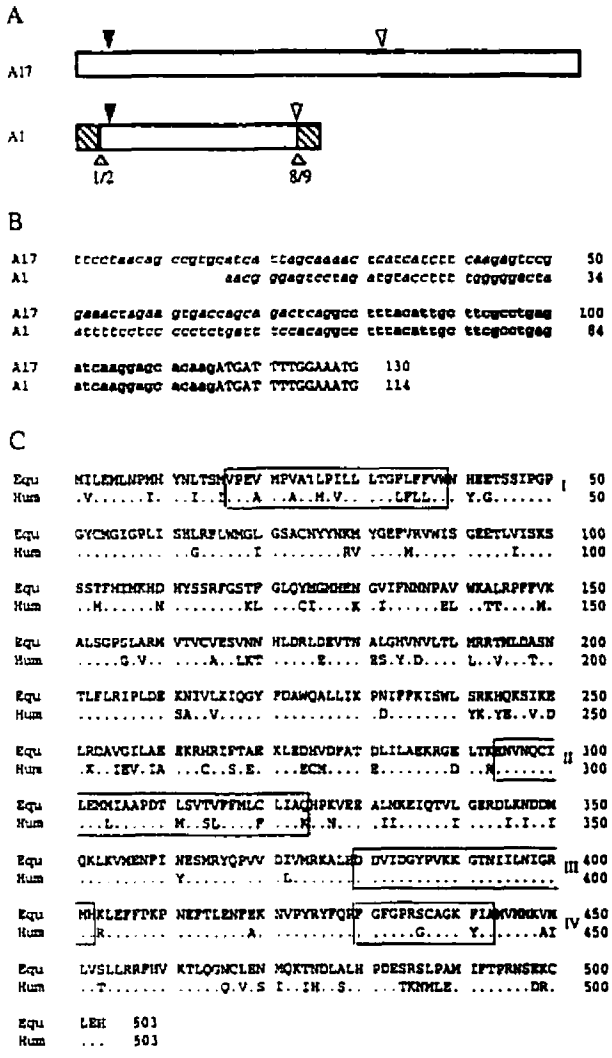

FIG. 1. Cloning and characterization of equine P450AROM. A, Schematic representation of two aromatase cDNA clones (A17 and A1) isolated by library screening. *Open* and *hatched boxes* represent regions with identical and divergent nucleotide sequences, respectively. *Black* and *white arrowheads* indicate start codons and stop codons, respectively. The putative exon 1/exon 2 (1/2) and exon 8/exon 9 (8/9) splice junctions of clone A1 are indicated. The complete nucleotide sequence of each clone has been deposited in GenBank (AF031521 for A17; AF031520 for A1). B, Nucleotide sequence of the 5'-end of clone A17 and A1. Nucleotides in the 5'-UTR are shown in *lowercase letters*, whereas nucleotides in the coding region are shown in *uppercase letters*. The divergent region of the 5'-UTR of clones A17 and A1 is *italicized*. C, Deduced amino acid sequence of equine (equ) P450AROM and comparison with the human (hum) homolog. Identical residues are indicated by a *printed period*. *Boxed regions* include a putative membrane-spanning domain (I), an I helix thought to serve as the substrate-binding pocket (II), a conserved region encompassing a putative cAMP-dependent protein kinase phosphorylation site (III), and the heme-binding region (IV).

Primer extension analysis

Primer extension analyses were performed in aqueous buffer, as previously described (35). To determine the putative transcription start site of the promoter II-derived transcript, the primer extension assay used RNA extracted from a corpus luteum (50 μg; day 8 of cycle), a tissue known to contain high levels of transcripts, and a 30-mer antisense oligonucleotide 5'-GGCGAAGCAATGTAAAGGCCTGTGGAA ATC-3' corresponding to the region located between +51 and +80 bp from the beginning of clone A1 (Fig. 1b). RNA isolated from spleen served as a negative control. The putative transcription start site of the promoter 1f-derived aromatase transcript was determined using RNA extracted from granulosa cells of a preovulatory follicles isolated 30 h post-hCG (30 μg) and a 30-mer antisense oligonucleotide (5'-GGCCTGAGTCTGCTGGTCACTTCTAGTTTC-3') corresponding to the region located between +51 and +80 bp from the beginning of clone A17 (Fig. 1b). RNA isolated from spleen and corpus luteum served as the negative control. The extension products were analyzed by electrophoresis on a 6% polyacrylamide-7 M urea gel, and the putative sites of transcription initiation were determined by comparisons with adjacent sequencing reactions that used the same oligonucleotides as primers and two corresponding aromatase genomic clones that contained these regions as templates.

Statistical analysis

Relative levels of P450AROM and P45017α mRNAs were quantified by densitometric analysis of autoradiogram bands using a computer-assisted image analysis system (Collage Macintosh program, Fotodyne, Inc., New Berlin, WI). The EFTu signal was also quantified and used to normalize results. Data were expressed as ratios of P450AROM to EFTu and P45017α to EFTu (n = 4 follicles/time point). One-way ANOVA was used to test the effect of time after hCG on relative levels of P450AROM and P45017α mRNAs. When ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple comparisons with the control (0 h post-hCG). Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC).

Results

Characterization of equine cytochromes P450AROM and P45017α cDNAs

Eighteen positive clones were originally purified after screening the equine expression library with the rat aromatase cDNA. Five of them, designated A1, A6, A12, A13, and A17, were selected for DNA sequencing, as others appeared to represent shorter fragments of the same cDNAs. Sequence analysis revealed that the clones corresponded to distinct aromatase transcripts, represented by clone A17 (similar to A12 and A6) and clone A1 (similar to A13; Fig. 1). Clone A17 was 2682 bp in length and included a 5'-untranslated region (UTR) of 115 bp, an open reading frame of 1509 bp, and a 3'-UTR of 1058 bp. The open reading frame encoded a 503-amino acid protein that included all conserved features characteristic of cytochrome P450AROM, such as a membrane-spanning region, an I helix, a heme-binding region, as well as a domain encompassing a putative cAMP-dependent protein kinase phosphorylation site (Fig. 1). The 5'-end of clone A17 (bases 1–77; Fig. 1B) was found to be the equine homolog of aromatase exon 1f, often designated as brain specific (21, 41–43). Clone A17 was designated as a promoter 1f-derived aromatase transcript. In contrast, the 5'-end of clone A1 (bases 1–61; Fig. 1B) represented the region immediately upstream of exon II. This latter region has previously been cloned by RT-PCR from equine tissues (9), and is known to be the primary mRNA species present in granulosa cells of several species (9, 18, 30, 36). Clone A1 was designated a promoter II-derived transcript. However, clone A1 was short and encoded a truncated 347-amino acid protein lacking important 3'-structural elements such as the heme-binding domain (data not shown). Database homology searches showed that homologous sequences ceased after the splice junction between exons 8 and 9, suggesting that the

clone may be a splice variant or an artifact of the cloning process.

Twelve P45017α clones were isolated from the equine cDNA library using a bovine homologous probe. Partial DNA sequencing of one selected clone showed that it was identical to an equine testicular P45017α cDNA previously characterized (data not shown) (44).

Regulation of equine P450AROM and P45017α mRNAs in preovulatory follicles

Changes in levels of aromatase mRNA during the ovulation-luteinization process were studied by Northern blots using a cDNA probe common to both transcripts. Results showed a biphasic pattern of aromatase expression after gonadotropin treatment (Fig. 2). High levels of aromatase mRNA were detected in walls of follicles isolated before hCG (0 h; Fig. 2A), but a marked drop in transcripts was observed 12 and 24 h post-hCG ($P < 0.05$). Then, aromatase levels increased again between 30 and 39 h post-hCG and were elevated in mature equine corpora lutea (Fig. 2A). To assess the cellular localization of the aromatase message within the follicle wall, Northern blots were performed on isolated preparations of granulosa cells and theca interna. Results showed that expression of aromatase transcripts was restricted to the granulosa cell layer and followed a pattern similar to that observed in follicle wall (Fig. 3). The size of the P450AROM mRNA was approximately 3.0 kb in most samples, with the exception of those isolated before hCG treatment (0 h post-hCG), where transcripts of about 4.0 and 1.2 kb were also observed. No aromatase transcripts were detected in theca interna (Fig. 3).

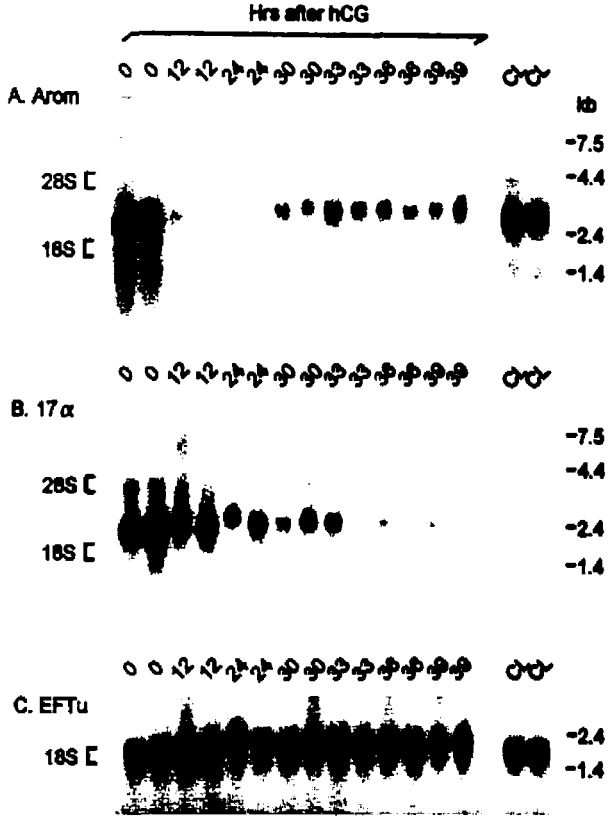

FIG. 2. Regulation of P450AROM and P45017α mRNA in equine preovulatory follicles. Preparations of follicle wall were obtained from preovulatory follicles isolated between 0–39 h after hCG, and two corpora lutea (CL) were isolated on day 8 of the estrous cycle. Samples of total RNA (10 μg/lane; two follicles per time point) were analyzed by Northern blotting using an equine P450AROM (A), an equine P45017α cDNA (B), and the rat elongation factor Tu (EFTu) as a control gene for RNA loading (C). *Brackets* on the *left* show the migration of 28S and 18S ribosomal bands, and markers on the *right* indicate the migration of RNA standards. Filters in A, B, and C were exposed to film at −70 C for 1.5, 4, and 2 h, respectively.

The concomitant regulation of P45017α mRNA in equine preovulatory follicles and corpora lutea was studied by Northern blots using the same membranes. Levels of P45017α mRNA were high in follicles at 0 h, but significantly decreased after hCG treatment ($P < 0.05$), with lowest levels in follicles at 36 and 39 h post-hCG and in corpora lutea (Fig. 2B). When Northern blots were performed with isolated cellular preparations, no P45017α mRNAs were detected in granulosa cells, except for a relatively weak signal in a few samples, probably resulting from contamination by theca interna cells (Fig. 3). In contrast, P45017α expression was high in theca interna of follicles isolated between 0–33 h post-hCG, but dropped markedly thereafter ($P < 0.05$; Fig. 3).

Differential expression of promoter II- and 1f-derived aromatase transcripts

The potential regulation of two distinct aromatase mRNAs in granulosa cells was studied using mRNA-specific, end-labeled oligonucleotide probes representing either promoter II- or promoter 1f-derived transcripts. The results suggested the presence of successive aromatase promoter-switching events during follicular luteinization and corpus luteum formation. Levels of promoter II-derived transcripts were high before hCG treatment (0 h), dropped drastically after hCG treatment, and remained very low before ovulation (Fig. 4A). Conversely, promoter 1f-derived mRNAs were undetectable at 0 h, but were induced between 30–39 h post-hCG (Fig. 4B). Interestingly, the process reversed itself after ovulation, as promoter 1f-derived mRNA disappeared and promoter II-derived transcripts reappeared in the corpus luteum (Fig. 4). The summation of promoter II- and promoter 1f-derived aromatase mRNAs seemed to represent the overall aromatase expression detected using a nonspecific probe (Fig. 3).

Cloning of aromatase promoters II and 1f, and characterization of putative transcription initiation sites

To determine the complete structure of the 5′-UTR of each aromatase transcript and identify potential regulatory elements involved in their expression, the 5′-flanking regions of exon II and exon 1f were cloned from an equine genomic library. Approximately 1.1 kb of each putative promoter was sequenced, and potential *cis*-acting elements were identified from a transcription factor database (Figs. 5 and 6). The equine aromatase promoter II contains a highly conserved adrenal 4 binding protein/steroidogenic factor-1 (SF-1) element involved in cAMP-dependent and -independent regulation in granulosa cells of other species (45–47) (Figs. 5 and

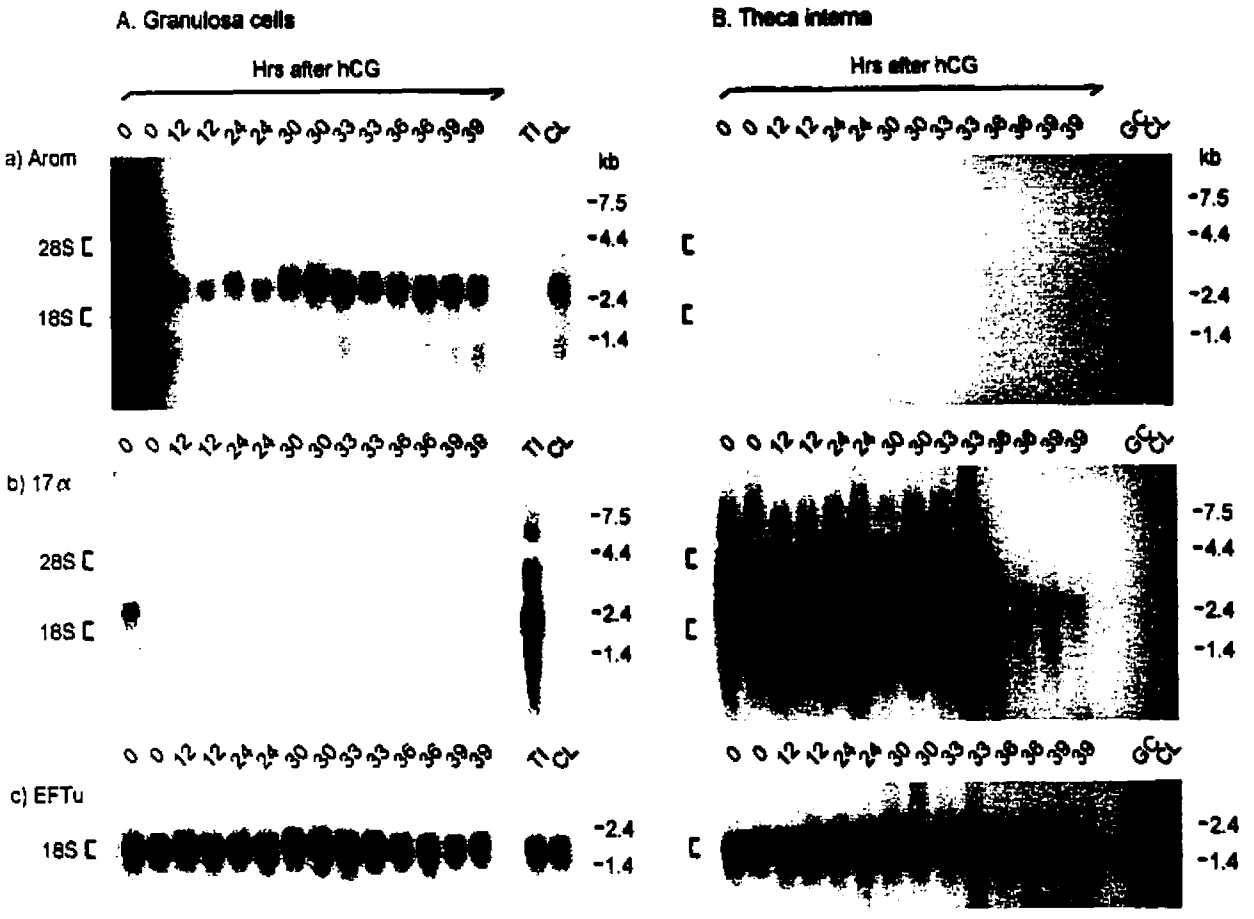

FIG. 3. Regulation of P450AROM and P45017α mRNA in granulosa cells and theca interna of equine preovulatory follicles. Isolated preparations of granulosa cells (A) and theca interna (B) were obtained from equine preovulatory follicles isolated between 0–39 h after hCG. Samples of total RNA (10 μg/lane; n = 2 follicles/time) were analyzed by Northern blotting using an equine P450AROM (a), an equine P45017α cDNA (b), and the rat elongation factor Tu (EFTu) as a control gene for RNA loading (c). In addition, preparations of theca interna (TI; 0 h) and of a corpus luteum (CL; day 8 of cycle) were included in A, whereas samples of granulosa cells (GC; 39 h) and of a corpus luteum (CL; day 8 of cycle) were included in B. *Brackets* on the *left* show the migration of 28S and 18S ribosomal bands, and markers on the *right* indicate the migration of RNA standards. Granulosa cell filters in a, b, and c were exposed to film at −70 C for 1, 4, and 2 h, respectively, whereas theca interna filters in a, b, and c were exposed for 1.5, 4, and 2 h, respectively.

7A). However, a cAMP response element (CRE)-like region known to bind cAMP-binding protein (CREB) in rat and human (45, 48), but not in bovine (49), ovarian tissues is not conserved in the horse (Fig. 7B). Alignment of the first 250 bases of the equine promoter 1f with the corresponding human and mouse regions shows a remarkable level of homology among species (90% and 96% identity between equine and murine, and equine and human sequences, respectively). This region features several perfectly conserved elements, notably overlapping c-*myc* and GRE consensus sites. Although genomic clones ranged in size from about 12–25 kb, no single clone was found to include both promoters, indicating that these equine aromatase promoters are separated by at least several thousand bases as reported in humans (7).

Primer extension analysis of promoter II-derived mRNA revealed a single putative transcription start site corresponding to a cytosine residue located 30 bp downstream of a consensus TATA box (Figs. 5 and 8). This cytosine residue was positioned 16 bp upstream of the first base of the aromatase cDNA clone A1 (Fig. 1), indicating that the total length of the 5′-UTR of promoter II-derived transcripts is 115 bp. Primer extension analysis of promoter 1f-derived transcripts identified a major putative transcription initiation site at a cytosine residue located 31 bp downstream of a consensus TATA box (Figs. 6 and 8). The criteria used to select this band as a major site was the presence of a strong signal with the granulosa cell RNA extract (which contains promoter 1f-derived mRNA) and the absence of a signal with the corpus luteum extract (which does not contain promoter 1f-derived mRNA; Fig. 4). The cytosine residue was located 16 bp upstream of the first base of the aromatase cDNA clone A17, giving an overall length of 93 bp for exon 1f and a total length of 131 bp for the 5′-UTR of promoter 1f-derived transcripts. Longer, but less abundant, extension products were observed with promoter 1f-derived transcripts (data not shown). These products were apparently transcribed down-

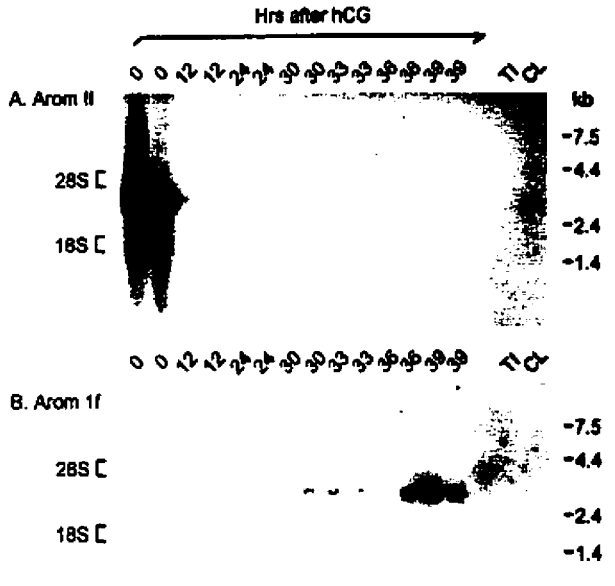

FIG. 4. Differential regulation of promoter II- and 1f-derived aromatase transcripts in granulosa cells after hCG treatment. Northern blot analysis was performed using RNA extracted from granulosa cells of equine preovulatory isolated between 0–39 h after hCG treatment (10 μg/lane; two follicles per time point). In addition, preparations of theca interna (TI; 0 h) and of a corpus luteum (CL; day 8 of cycle) were included in the same blot. Hybridization was performed using end-labeled antisense oligonucleotides specific for promoter II-derived (Arom II; A) and promoter 1f-derived (Arom 1f; B) transcripts, as described in *Materials and Methods*. *Brackets* on the *left* show migration of 28S and 18S ribosomal bands, and markers on the *right* indicate migration of RNA standards. Filters in A and B were exposed to film at −70 C for 4 days.

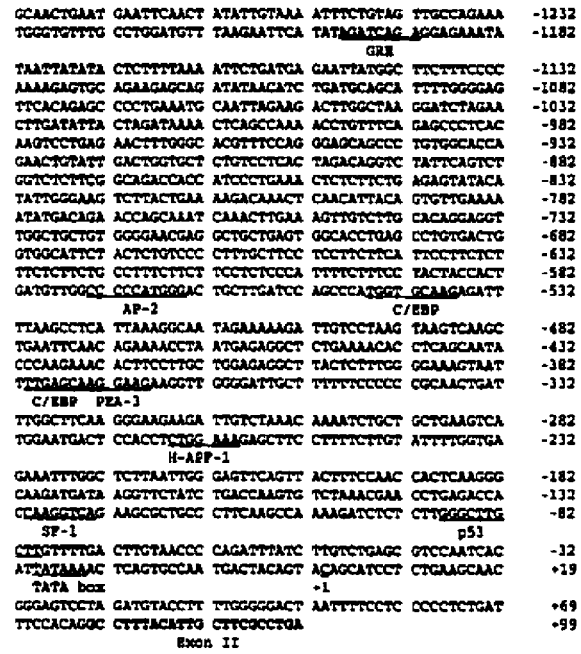

FIG. 5. Isolation and characterization of equine aromatase promoter II. A DNA fragment located immediately upstream of exon II was isolated from an equine genomic library. Nucleotide sequences are numbered according to the putative transcription initiation site (+1) shown in Fig. 7. Selected potential *cis*-acting promoter elements are *underlined*, whereas sequences representing exon II are in *boldface*. The nucleotide sequence has been deposited in GenBank (accession no. AF031893).

stream from two additional TATAA elements found in the promoter 1f at positions −87/−92 and −111/−116 (+1 = major putative transcription initiation site). These TATA boxes are also conserved in the mouse and human promoter 1f (21, 41), but their relative functional significance has not been determined.

Discussion

This study is the first to report an aromatase promoter-switching phenomenon in granulosa cells during follicular luteinization and provides a novel model in which aromatase promoter switching is induced *in vivo*. To date, promoter switching had been reported in distantly separated developmental (12, 23) or pathological (24–27, 50) stages *in vivo*, and in human mononuclear leukemic THP-1 cells and peripheral blood lymphocytes *in vitro* (51, 52). In the present study, a fine time-course dissection of the regulation of cytochrome P450AROM was realized in preovulatory follicles of mares, a species with a relatively long ovulatory process (36–48 h from hCG to ovulation) (53, 54). The cloning of two distinct equine follicular aromatase transcripts and studies on their expression after hCG-induced luteinization/ovulation revealed a dual regulation of promoter II- and promoter 1f-derived transcripts in granulosa cells. The down-regulation of promoter II-derived mRNAs with the accompanying induction of promoter 1f-derived mRNAs in granulosa cells after hCG treatment imply a first switching phenomenon, whereas the presence of promoter II-derived transcripts and the disappearance of promoter 1f-derived transcripts in corpora lutea suggest a second switching event. The use of alternate aromatase promoters was thought not to occur during the ovulatory process, as promoter II-derived mRNA was found to predominate in human follicles and corpus luteum (30). However, this latter study does not contradict our results, as it did not analyze samples from the time window in which switching occurred in equine granulosa cells. Further studies will be needed to determine whether aromatase promoter switching during follicular luteinization also occurs in humans or other animal species.

The elevated expression of promoter II-derived aromatase transcripts in equine follicles before hCG treatment is in keeping with previous reports showing that it is the predominant mRNA species in granulosa cells (18, 30, 36, 49). Also, the selective localization of aromatase mRNA in granulosa cells agrees with the immunohistochemical localization of the protein in equine follicles (55, 56), and the steroidogenic capacities of isolated equine follicular cells *in vitro* (57), thus resolving a previous controversy on the site of estrogen biosynthesis in the equine follicle (34). This study reports the cloning and characterization of the equine aromatase promoter II. Some of the molecular mechanisms involved in the regulation of this promoter have been studied in other species, and at least two *cis*-elements appear crucial for cAMP-

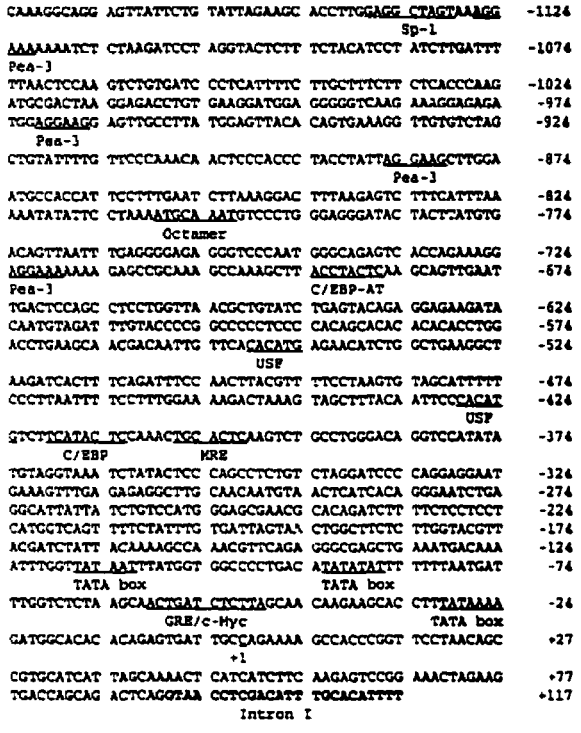

FIG. 6. Isolation and characterization of equine aromatase promoter 1f. A DNA fragment including exon 1f as well as its 5'-flanking sequences was isolated from an equine genomic library. Nucleotide sequences are numbered according to the putative transcription initiation site (+1) shown in Fig. 7. Selected potential *cis*-acting promoter elements are *underlined*, whereas intronic sequences 3' of exon 1f are in *boldface*. The nucleotide sequence has been deposited in GenBank (accession no. AF031894).

dependent and -independent expression in human and rat granulosa cells (45–48). A first element, a consensus SF-1-binding site conserved in all species, appears to mediate both constitutive and inducible aromatase transcription (45–47). A second element, a CRE-like element to which CREB can bind, appears required to achieve optimal transcriptional activity in rats and humans (44, 47). However, this latter element is poorly conserved in other species and contains a 1-bp deletion in the equine (this study), bovine (49), and porcine (16) promoters. In cattle, this deletion was initially thought to be responsible for the lack of aromatase expression in bovine luteal cells. However, a site-directed mutagenesis study designed to render the bovine CRE-like site identical to its human counterpart resulted only in partial restoration of cAMP-inducible promoter activity in luteal cells, suggesting that other elements are involved (49). The marked drop in promoter II-derived aromatase transcripts in equine follicles after hCG treatment agrees with a similar down-regulation in other species after the LH surge *in vivo* (36, 58–61). In rat granulosa cells, the decrease in promoter II activity and aromatase mRNA was associated with a drop in SF-1 and in the A kinase regulatory subunit type II (RIIβ), but not in CREB expression or binding activity (47, 60, 62).

The observed induction of promoter 1f-derived aromatase transcripts in equine granulosa cells is novel. Exon 1f-containing mRNA was originally cloned from brain tissues and has been described as brain specific (21, 41–43). However, several aromatase expression studies have shown a vast tissue distribution for many aromatase mRNA species (7, 10, 24), suggesting that the tissue specificity model for aromatase promoter usage may be oversimplified. This view is also supported by recent reports, including this one, that show the use of alternative aromatase promoters in a given tissue (10, 12, 20, 23–27). The molecular mechanisms involved in promoter 1f-derived aromatase expression have not been characterized, which contrasts with studies on promoter II. Although promoter 1f has previously been cloned in humans (41) and mice (21), no regulatory elements have been functionally identified. Potential *cis*-acting elements include the highly conserved, overlapping c-*myc* and glucocorticoid response element identified in horses (this study), humans (41), and mice (21). Recent studies have shown an increase in c-*myc* expression in rat granulosa cells during hCG-induced luteinization (62, 63). Also, a glucocorticoid response *cis*-element present in the human aromatase promoter I.4 is required (in conjunction with glucocorticoids) for cytokine-induced transcription in adipocytes via the Janus kinase/STAT (signal transducer and activator of transcription) pathway (64). Whether these pathways are involved in promoter 1f-derived aromatase expression in equine granulosa cells remains to be determined.

A. Promoter II Ad4BP/SF-1 element

horse	TGAGA--CCA	CCAAGGTCAG AAGCGCTGCC
cow	TGAGACTCTA	CCAAGGTCAG AAATGCTGCA
pig	TGAGACTCTG	CCAAGGTCAG AAATGCTGCA
human	TGAGACTCTA	CCAAGGTCAG AAATGCTGCA
rat	TGAG--TCTC	CCAAGGTCAT CCTTGTTTTG

B. Promoter II CRE-like element

horse	TAATTGGGAG	TTCA-GTTAC TTTCCAACCA
cow	TAATTGAGAA	CTCA-GTCAC TCT--ACCCA
pig	CAATTGGGAA	TTCA-GTCAT TCT--ACCCA
human	CAATTGGGAA	TGCACGTCAC TCT--ACCCA
rat	CAATTGAGTA	TGCACGTCAC TCT--ACCCA

FIG. 7. Homology analysis of putative aromatase *cis*-acting promoter elements. Selected equine putative aromatase promoter elements are aligned with corresponding sequences from all species homologs characterized to date. A, Adrenal 4 binding protein (Ad4BP)/SF-1 element in aromatase promoter II (−130/−123; see Fig. 5). B, CRE-like sequence in aromatase promoter II (−208/−201; see Fig. 5).

This study provides a first characterization of the regulation and cellular localization of cytochrome P45017α transcripts in equine preovulatory follicles. Selective expression of P45017α mRNA in the theca interna layer is similar to reports in other species (58, 59, 61, 65) and supports studies *in vitro* showing that secretion of androgens was observed in cultures of equine theca interna cells, but not in those of granulosa cells (57). No major changes were observed in levels of P45017α mRNA in theca interna between 0 and 33 h post-hCG, but a marked drop occurred thereafter. Comparable loss of P45017α mRNA has been observed in bovine (58), porcine (61), and rat (66) theca interna after the LH surge. Interestingly, the time course of P45017α mRNA disappearance (between 33–36 h post-hCG) was distinct from that of promoter II-derived aromatase transcripts in granu-

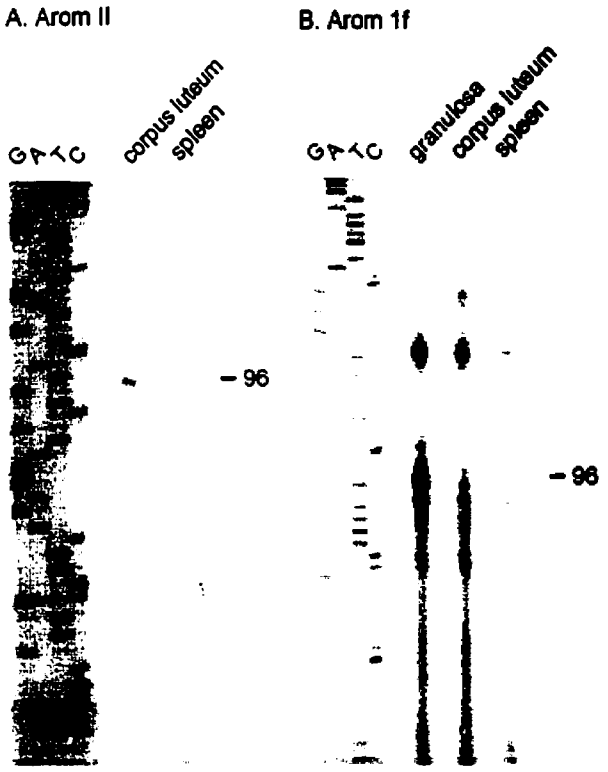

FIG. 8. Putative transcription initiation sites of promoter II- and promoter 1f-derived aromatase transcripts. Primer extension analyses were performed using antisense oligonucleotides complementary to 5'-UTR regions of promoter II- and promoter 1f-derived transcripts (A and B, respectively). Primers were hybridized to RNA samples containing promoter II-derived transcripts (corpus luteum; A) and promoter 1f-derived mRNA (granulosa cells 30 h post-hCG; B), and extension reactions were analyzed on a 6% polyacrylamide gel, as described in *Materials and Methods*. Results revealed 96-nucleotide extension products corresponding to the putative transcription initiation sites of promoter II- and promoter 1f-derived aromatase mRNA.

losa cells (between 0–12 h post-hCG), suggesting the presence of separate down-regulatory mechanisms in each follicular cell type. However, the decrease in P45017α transcript in theca interna coincides precisely with the loss of steroidogenic acute regulatory protein mRNA in theca interna (67). This apparent reduction in thecal steroidogenic capacity could relate to the reported demise of the layer at the time of ovulation in mares (34, 68). Despite high levels of aromatase transcripts in the equine corpus luteum, estrogen biosynthesis is very limited during the luteal phase (34). Insufficient luteal P45017α expression, and thus aromatizable androgen substrates, has been proposed to be rate-limiting in luteal estrogen synthesis (69).

In summary, this study reports the cloning and characterization of two equine aromatase transcripts, as well as approximately 1 kb of genomic sequences putatively involved in their transcription. This study provides a first characterization of the regulation and cellular localization of the P450AROM and P45017α mRNAs during the ovulatory process, with results indicating that the classic two-cell (theca interna/granulosa cells), two-gonadotropin (LH/FSH) model for estradiol production is operative in the mare. Most importantly, we report the presence of a novel aromatase promoter-switching phenomenon in equine granulosa cells during follicular luteinization, characterized by a down-regulation of promoter II- and an up-regulation of promoter 1f-derived transcripts after hCG treatment. This phenomenon apparently reverses itself after ovulation, as promoter II-derived mRNAs become elevated, whereas those derived from promoter If disappear in the corpus luteum. Considering the potential role of aromatase promoter switching in various physiological and pathological processes and the uncharacterized nature of its molecular control, we propose that the equine preovulatory follicle provides a valuable model system to study this phenomenon.

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Summary of article #4

Expression and regulation of transcripts encoding two members of the NR5A subfamily of orphan nuclear receptors, steroidogenic factor-1 and NR5A2, in equine ovarian cells during the ovulatory process. **Derek Boerboom**, Nicolas Pilon, Ramin Behdjani, David W. Silversides and Jean Sirois. Endocrinology (in press).

Thesis author's contribution to the work: As the primary author, I was responsible for all aspects of the production of this article. Secondary authorship credits reflect contributions to experimental design.

Summary:

- The equine SF-1 and NR5A2 primary transcripts were isolated in fragments by a combination of cloning processes. They were found to encode proteins that are 60% identical to each other, with nearly identical DNA-binding domains that reflect the identical DNA-binding specificities of their human homologs.
- Northern analysis established a clear relationship between the expression of SF-1 and that of the steroidogenic genes in all tissues except granulosa cells and CL, in which very low levels of SF-1 mRNA often contrast with high levels of StAR, 3β-HSD, P450scc and P450arom expression. Conversely, a role for NR5A2 in regulating steroidogenic genes in these tissues is suggested by its level of expression, which far surpasses that of SF-1.

- The regulation of SF-1 and NR5A2 mRNA throughout the ovulatory process was examined using an RT-PCR/Southern blotting technique on ovarian follicular RNA isolated on a time-course after the administration of hCG. In granulosa cells, approximately 3-fold decreases in SF-1 and NR5A2 mRNA levels were attained by 30h post-hCG. Different regulatory patterns were observed in theca interna, as SF-1 mRNA levels were high until 33 hours post-hCG and dropped significantly thereafter. NR5A2 appears not to be expressed in theca interna.

Work's contribution to the advancement of science:

- This study reports the novel cloning of equine SF-1 and NR5A2.
- It demonstrates a regulatory pattern of SF-1 in theca interna that apparently mirrors the regulation patterns of StAR, P450scc and P450$17\alpha$, suggesting a direct link to their transcriptional regulation.
- It documents for the first time the expression of NR5A2 in granulosa cells and in the CL. Its high level of expression in these tissues, combined with its presumed DNA binding specificity, suggest that it could function as a counterpart of SF-1 to regulate the transcription of steroidogenic genes. This partially resolves a paradox, as the proposed transcriptional regulation by SF-1 of all steroidogenic genes in all ovarian cell types could difficultly account for their differential mRNA regulatory patterns.
- Finally, it provides novel insight into the control of ovarian steroidogenesis, permitting refinements to the working model presented in article #2.

Expression and Regulation of Transcripts Encoding Two Members of the NR5A Subfamily of Orphan Nuclear Receptors, Steroidogenic Factor-1 and NR5A2, in Equine Ovarian Cells During the Ovulatory Process*

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Key Words: SF-1, NR5A1a, NR5A2, orphan nuclear receptor, messenger RNA, granulosa cells, theca interna, follicle, ovary, horse, steroidogenesis

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Running title

Regulation of SF-1 and NR5A2 mRNAs in equine follicles

Abstract

Steroidogenic factor-1 (SF-1, NR5A1a) is a member of the NR5A nuclear receptor subfamily and has been implicated as a key transcriptional regulator of all ovarian steroidogenic genes *in vitro*. To establish links between the expression of SF-1 and that of the steroidogenic genes *in vivo*, the objectives of this study were to clone equine SF-1 and examine the regulation of its mRNA in follicular cells during human chorionic gonadotropin (hCG)-induced ovulation. The equine SF-1 primary transcript was cloned by a combination of reverse transcription-polymerase chain reaction (RT-PCR) techniques. Results showed that the transcript was composed of a 5'-untranslated region (UTR) of 161 bp, an open reading frame (ORF) of 1386 bp that encodes a highly-conserved 461-amino acid protein, and a 3'-UTR of 518 bp. The cloning of SF-1 also led to the unexpected and serendipitous isolation of the highly-related orphan nuclear receptor NR5A2, which was shown to include a 5'-UTR of 243 bp, an ORF of 1488 bp and a 3'-UTR of 1358 bp. The NR5A2 ORF encodes a 495-amino acid protein that is 60% identical to SF-1, including 99% similar DNA-binding domains. Northern blot analysis revealed that SF-1 and NR5A2 were expressed in all major steroidogenic tissues, with the exception that NR5A2 was not present in the adrenal. Interestingly, NR5A2 was found to be by far the major NR5A subfamily member expressed in the preovulatory follicle and the corpus luteum. Using a semi-quantitative RT-PCR/Southern blotting approach, the regulation of SF-1 and NR5A2 mRNAs *in vivo* was studied in equine follicular cells obtained from preovulatory follicles isolated between 0 and 39 h post-hCG. Results showed that the theca interna was the predominant site of SF-1

mRNA expression in the follicle, and that hCG caused a significant decrease in SF-1 levels between 12-39 h in theca interna and between 24-39 h post-hCG in granulosa cells ($P < 0.05$). In contrast, the granulosa cell layer was the predominant, if not the sole, site of NR5A2 mRNA expression in the follicle. Importantly, NR5A2 was much more highly expressed in granulosa cells than SF-1. The administration of hCG caused a significant decrease in NR5A2 transcripts in granulosa cells at 30, 36 and 39 h post-hCG ($P < 0.05$). Thus, this study is the first to report the concomitant regulation of SF-1 in theca interna and granulosa cells throughout the ovulation/luteinization process, and to demonstrate the novel expression and hormonal regulation of NR5A2 in ovarian cells. Based on the marked expression of NR5A2 in equine granulosa and luteal cells and on mounting evidence of a functional redundancy between SF-1 and NR5A2 in other species, it is proposed that NR5A2 may play a key role in the regulation of gonadal steroidogenic gene expression.

Introduction

The recently-defined nuclear receptor subfamily NR5A includes three highly-related orphan type receptors, so named for their lack of a known ligand (1). One member of this subfamily is steroidogenic factor-1 (SF-1, NR5A1a), also known as adrenal 4-binding protein (Ad4BP) (2). SF-1 was originally isolated as a transcription factor capable of binding discrete regulatory elements present in the promoters of various steroid hydroxylases (3). Embryological expression studies (4) and a gene knockout analysis (5) have subsequently demonstrated a critical role for SF-1 in the development of the steroidogenic organs, a role that has been extended to all levels of the hypothalamic-pituitary-gonadal axis (6, 7, 8). Further insight into potential roles of SF-1 has been obtained by the identification of numerous genes whose transcriptional activity it appears to modulate. In addition to the cytochrome P450 steroid hydroxylases (3), the list of SF-1 target genes now includes 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) (9), steroidogenic acute regulatory protein (StAR) (3), ACTH receptor (10, 11), Mullerian inhibitory substance (12, 13), LH β-subunit (14, 15), GnRH receptor (16, 17), oxytocin (18, 19), Dax-1 (20, 21) and several others (22-30). Considering the roles of these genes in various metabolic and developmental processes, it is evident that the functions of SF-1 extend well beyond those that originally prompted its discovery. Several studies on the regulation of SF-1 activity have focused on post-translational mechanisms, including phosphorylation (31), potential ligands (32-34) and associated proteins (12, 35-41). However, few studies have identified physiological processes that modulate SF-1 activity at the transcriptional level. GnRH has been reported to up-regulate

SF-1 mRNA levels in the pituitary (42), while a transient down-regulation of SF-1 mRNA has been observed in ovarian cells following the LH surge (43-45).

Another member of the NR5A orphan nuclear receptor subfamily is NR5A2 (1), that has been previously termed hB1F (46), LRH-1 (GenBank accession number M81385), PHR-1 (47), xFF1rA (48), FTF (49) and CPF (50). NR5A2 and SF-1 have been found to share a high degree of structural similarity, notably within regions refered to as the hybrid P box, the A box and the T box. As these structures are directly or indirectly implicated in determining and interacting with the binding site, it was proposed that both receptors share identical DNA binding mechanisms and specificities (51). This has subsequently been demonstrated by several groups (27, 46, 48-50), and both nuclear receptors were found to transactivate at least one common promoter (27). Interestingly, NR5A2 has also been shown to transactivate a hepatic steroid hydroxylase gene (50). While these data suggest a functional redundancy between the receptors, no overlap in their tissue distributions has been reported, and no role for NR5A2 in processes such as gonadal steroidogenesis has been proposed.

A recent series of studies examined the regulation and cellular distribution of transcripts encoding various steroidogenic proteins and enzymes throughout the equine ovulatory process (52-54). It was shown that hCG triggers a marked down-regulation of StAR (54), P450scc (53) and P45017α (52) mRNA in theca interna prior to ovulation. Different mRNA regulatory processes were observed within the granulosa cell layer, in which P450arom expression was abrogated by administration of hCG (52), while StAR (54) and P450scc (53) were induced and 3β-HSD expression did not vary

(53). Considering the divergent regulation of steroidogenic transcripts during the equine ovulatory process, it is likely that factors other than SF-1 are involved, or that co-activators and co-repressors enter into play to activate and silence transcription in a time-, tissue- and gene-specific manner. The objectives of this study were to clone equine SF-1 and to characterize the regulation of its transcript in theca interna and granulosa cells following hCG administration to identify possible links between the regulation of SF-1 mRNA and those of the various steroidogenic genes. As a serendipitous finding, this paper also reports the cloning of equine NR5A2 and its novel expression in granulosa and luteal cells.

Materials and methods

Materials

Human CG was purchased from The Buttler Co. (Columbus, OH); Biotrans nylon membranes (0.2 μm) were purchased from ICN Pharmaceuticals (Montreal, Canada); [α-^{32}P]dCTP and [α-^{35}S]dATP were obtained from Mandel Scientific-New England Nuclear Life Science Products (Mississauga, Canada); QuikHyb hybridization solution and ExAssist/SOLR system were purchased from Stratagene Cloning Systems (La Jolla, CA); TRIzol total RNA isolation reagent, RNA ladder [0.24-9.5 kilobases (kb)], 1-kb ladder, synthetic oligonucleotides, 5'-RACE System for Rapid Amplification of cDNA Ends, SuperScript II and culture media were obtained from Life Technologies (Gaithersburg, MD); Prime-a-Gene labeling system, Access RT-PCR kit, pGEM-T easy Vector System I and AMV reverse transcriptase were purchased from Promega (Madison, WI); Kodak X-Omat AR film was obtained from Eastman Kodak (Rochester, NY); electrophoretic reagents were purchased from Bio-Rad Laboratories (Richmond, CA); Taq DNA polymerase, RNAguard and all sequencing reagents were obtained from Pharmacia Biotech (Baie D'Urfé, Canada).

Isolation of equine tissues and RNA extraction

Equine preovulatory follicles and corpora lutea were isolated at specific stages of the estrous cycle from Standardbred and Thoroughbred mares as previously described (55). Briefly, follicular development was monitored by real-time ultrasonography during estrus. When preovulatory follicles reached 35 mm in diameter, the ovulatory process was induced by

injection of hCG (2500 IU, iv) and unilateral ovariectomies were performed via colpotomy using a chain ecraseur at 0, 12, 24, 30, 33, 36 or 39 h post-hCG (n=4/time point), as described (55). Corpora lutea (CL) were isolated by the same method on day 8 of the estrous cycle (n=3; day 0 = day of ovulation). Follicles were dissected into preparations of follicle wall (theca interna with attached granulosa cells) or further dissected into separate isolates of granulosa cells and theca interna. Male gonadal tissues were obtained from the large animal hospital of the Faculté de Médecine Vétérinaire (Université de Montréal) following a routine castration procedure, and other tissues were obtained at a local slaughterhouse. All animal procedures were approved by the institutional animal use and care committee. Total RNA was isolated from tissues using TRIzol reagent (Life Technologies) according to manufacturer's instructions using a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific).

Cloning of the equine SF-1 transcript

The equine SF-1 transcript was isolated in fragments using a 5-step cloning strategy (Fig. 1). First, a reverse transcription-polymerase chain reaction (RT-PCR) technique was performed using 5 μg of total RNA isolated from adrenal gland, corpus luteum and follicle wall (Fig. 1Ba). Reverse transcription reactions were done with poly-dT oligonucleotides and either SuperScript II (Life Technologies) or StrataScript RNase H^- reverse transcriptase (Stratagene) essentially under the manufacturer's recommended conditions. These reactions were pooled and used as a template in a PCR reaction that included primers designed by sequence alignments of known SF-1 species homologues (Fig. 1C). Amplification was performed as

previously described (56) using Taq polymerase (Pharmacia) and an Omnigene TR3 SM5 thermal cycler (Hybaid Limited) for 40 cycles of 94 C for 45 seconds, 58 C for 1 minute and 72 C for 90 seconds. Following electrophoresis on a 1.2 % TAE-agarose gel, the DNA fragment was excised and ligated into the pGEM-T easy vector (Promega) according to the manufacturer's instructions. DNA sequencing was performed using the T7 Sequencing kit (Pharmacia Biotech) with vector-based (Sp6 and T7) and custom oligonucleotide primers (Life Technologies, Inc.). A 545 bp equine SF-1 sequence was generated and submitted to GenBank (accession number AF168796).

The 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 kit (Life Technologies) was employed to obtain transcript sequences upstream of the RT-PCR product (Fig 1Bb). Reverse transcription was performed as directed using 5 µg of total RNA from granulosa cells. Following TdT tailing, nested PCR reactions were performed with appropriate oligonucleotides (Fig. 1C) and Taq DNA polymerase (Pharmacia), using 35 (first reaction) or 30 (second reaction) cycles of 94 C for 30 seconds, 55 C for 1 minute and 72 C for 2 minutes, with a final 7-minute extension step at 72 C. The largest 5'-RACE products were isolated and sequenced. A 3'-RACE technique was used to obtain sequences downstream of the SF-1 PCR product (Fig. 1Bc). Reverse transcription was performed using 5 µg of RNA from corpus luteum, to which 500 ng of poly-dT oligonucleotide (Fig. 1C) was added and the mixture heated to 70 C for 5 minutes. RNAguard (39 units, Pharmacia), AMV-RT (10 units, Promega) and dNTPs (200 µM each final) were added and the reaction incubated at 42 C for 1 h. Nested PCR reactions were performed using appropriate oligonucleotides (Fig. 1C) and 40 cycles of

94 C for 1 minute, 60 C for 1 minute and 72 C for 4 minutes. As the 3'-end of the 3'-RACE product contained non-homologous sequences, RT-PCR was used to clone the balance of the coding region (Fig. 1Bd). Oligonucleotide primers were designed from the 3'-RACE product and 3'-UTR sequence alignments of all known SF-1 species homologues (Fig. 1C). The RT-PCR reaction was performed using the Access RT-PCR kit (Promega) as described below, except 40 amplification cycles were used and 100 ng of granulosa cell total RNA served as the template. A final 3'-RACE was performed to isolate the remainder of the 3'-UTR (Fig. 1Be). The procedure was done as described above, except reverse transcription was performed with 4 μg of preovulatory granulosa cell RNA, appropriate primers (Fig. 1C) and the cycling conditions were 94 C for 30 seconds, 55 C for 1 minute and 72 C for 4 minutes.

Cloning of the equine NR5A2 and GAPDH transcripts

The 5' SF-1 RT-PCR cloning product (Fig. 1Ba) was used to screen an equine cDNA library prepared from a 36h post-hCG preovulatory follicle (55) with the intent to isolate a full-length SF-1 cDNA clone. The probe was labeled to a specific activity of greater than 10^8 cpm/μg of input DNA by means of the Prime-a-Gene labeling kit (Promega) following manufacturer's instructions. Approximately 100,000 phage plaques were screened, and hybridization was performed using QuikHyb solution (Stratagene). Primary screening yielded seven weak positive clones that required a one-week exposure at -70 C to X-OMAT AR film (Eastman Kodak Co.) to be clearly identified, and only one clone was successfully purified through secondary and tertiary rounds of screening. The ExAssist/SOLR system (Stratagene) was used for *in vivo* excision, producing the cDNA clone inserted in the

pBluescript vector, and sequencing was performed as described above. Comparison with available GenBank sequence data revealed that the clone was the equine homologue of NR5A2 (1). As the NR5A2 cDNA clone was incomplete at both the 5'- and 3'-end (Fig. 2Ba), the RACE procedures described for SF-1 were repeated. The conditions used for 5'- and 3'-RACE were the same as for SF-1, except for the use of gene-specific oligonucleotides (Fig. 2C) and a temperature of 58 C for the annealing step in the 3'-RACE PCR reactions. Whereas the 5'-RACE was successful (Fig. 2Bb), an improperly spliced 3'-RACE product was obtained for NR5A2 (Fig. 2Bc), requiring that an additional RT-PCR cloning procedure be performed to obtain the downstream coding regions (Fig. 2Bd). The reaction was performed as for SF-1, except that 100 ng of total RNA from a corpus luteum was used as a template. A final 3'-RACE procedure under the same conditions as for SF-1 permitted the isolation of the remaining portion of the 3'-UTR (Fig. 2Be).

To generate an internal standard for RNA loading and amplification, an equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment was isolated by RT-PCR. Reaction conditions were the same as those that generated the original SF-1 cDNA fragment, except the oligonucleotides 5'-AGGTC CACCA CCCTG TTGCT GTA-3' and 5'-CCTTC ATTGA CCTCA ACTAC ATGGT-3' were used and the annealing step of the PCR reaction was carried out at 55 C. The resulting 828 bp product was sequenced and submitted to GenBank with accession number AF157626.

Northern analysis

RNA samples (10 μg) were processed, electrophoresed on 1.2% formaldehyde-agarose gels and transferred to nylon membranes as

previously described (55). A ladder of RNA standards was run with each gel, and ethidium bromide (10 μg) was added to each sample before electrophoresis to compare RNA loading and determine the migration of standards. Hybridization was performed using QuikHyb solution (Stratagene) and several equine cDNA probes, including SF-1, NR5A2, P450arom (52), P450scc (53), P45017α (52), 3β-HSD (53), StAR (54) and GAPDH. Each cDNA was labeled using the Prime-a-Gene labeling system as described above, and stripping of hybridization signal between successive rounds of probing was achieved by soaking filters in 0.1% SSC-0.1% SDS for 15 min at 90-95 C. Following autoradiography at -70 C, a computer-assisted image analysis system was used to capture and digitize the images (Collage Macintosh program, Fotodyne, New Berlin, WI).

Semi-quantitative RT-PCR and Southern analysis

The Access RT-PCR System (Promega) was used for semi-quantitative analysis of SF-1, NR5A2 and GAPDH levels in theca interna and granulosa cells isolated between 0-39 h after hCG treatment. Reactions were performed as directed by the manufacturer, using the oligonucleotide pairs 5'-CCCGA GCTCA TCCTG CAGCT G-3' and 5'-CTGGC GGTCC AGCTG CAGCG-3' for SF-1, 5'-AGAAA GCGTT GTCCC TACTG TCG-3' and 5'-TCTGG CTCAC ACTTC AAAAG TTCC-3' for NR5A2 and 5'-ATCAC CATCT TCCAG GAGCG AGA-3' and 5'-GTCTT CTGGG TGGCA GTGAT GG-3' for GAPDH. These reactions resulted in the generation of 429, 539 and 341 bp products, respectively. Each reaction was performed using 100 ng of total RNA, and cycling conditions were one cycle of 48 C for 45 minutes and 94 C for 2 minutes, followed by a variable number of cycles of 94 C for 30 seconds, 55 C

for 1 minute and 68 C for 2 minutes. The number of cycles used was optimized for each gene in preliminary experiments to fall within the linear range of PCR amplification, and included 16, 10 and 10 cycles for SF-1, NR5A2 and GAPDH, respectively. Following PCR amplification, samples were electrophoresed on 2% TAE-agarose gels and transferred to nylon membranes as previously described (56). The membranes were probed with the corresponding radiolabeled cDNA fragment as described in Northern analysis. After autoradiography, films were scanned using an IBM Flatbed Scanner and Corel Photo-Paint version 6.00 software (Corel Corporation). Signal strength was quantified by density analysis of the digital images using NIH image software version 1.61 (NIH, Bethesda, MA).

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG on levels of SF-1, NR5A2 and GAPDH transcript levels in theca interna and granulosa cells. When ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple comparisons with the control (0 h post-hCG). SF-1 and NR5A2 levels were normalized with GAPDH, and results are expressed as means ± SEM (n = 4 follicles [i.e. mares]/time point). No difference was observed in GAPDH levels at any time point between 0 and 39 h post-hCG ($P < 0.05$). Statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC).

Results

Cloning and characterization of cDNAs encoding equine SF-1 and NR5A2

To clone the equine SF-1 primary transcript, reverse transcriptions of steroidogenic tissue RNA were pooled and amplified by PCR using oligonucleotides designed by sequence alignment of known SF-1 species homologs. The resulting cDNA fragment (Fig. 1Ba) was then employed as a probe to isolate a clone from a equine follicular expression library (55). As no SF-1 clones were obtained, 5'- and 3'-RACE were used to isolate the balance of the SF-1 transcript. While the 5'-RACE reaction produced a cDNA fragment encompassing all upstream coding regions as well as a considerable amount of 5'-UTR (Fig. 1Bb), the 3'-RACE experiment generated a truncated product (Fig. 1Bc). This latter product consisted of a few hundred bases of coding sequences followed by a consensus splice junction and non-homologous sequences, and most likely resulted from inappropriate priming of intronic sequences. An RT-PCR cloning strategy was therefore employed to obtain all remaining downstream coding sequences (Fig. 1Bd). Following this, the 3'-RACE protocol was successfully applied, generating a fragment representing all the remaining 3'-UTR (Fig. 1Be). The deduced equine SF-1 transcript includes a 5'-UTR of 161 bp, an open reading frame (ORF) of 1386 bp that encodes a highly-conserved 461-amino acid protein, and a 3'-UTR of 518 bp (Figs. 1A and 3A).

While attempts to clone SF-1 from a follicular cDNA library failed, they resulted in the unexpected isolation of the highly-related orphan nuclear receptor NR5A2. The single cDNA clone obtained was incomplete at its 5'-end and contained a consensus splice junction followed by non-coding

sequences at its 3'-end (Fig. 2Ba). A combination of 5'- and 3'-RACE was performed to further characterize the NR5A2 transcript. Whereas all relevant 5' sequence information was obtained (Fig. 2Bb), the 3'-end remained incomplete (Fig. 2Bc). To conclude the isolation of the NR5A2 primary transcript, an RT-PCR product encompassing sequences downstream of the first 3'-RACE product was generated (Fig. 2Bd), and was followed by a second 3'-RACE (Fig. 2Be). The complete deduced NR5A2 transcript includes a 5'-UTR of 243 bp, an ORF of 1488 bp that encodes a highly conserved 495-amino acid protein, and a 3'-UTR of 1358 bp (Figs. 2A and 3B).

When translated, the deduced primary transcripts were found to encode orphan nuclear receptors that are more closely related to each other than to any other known members of the nuclear receptor family. When their sequences are aligned, an overall homology of approximately 60% is observed, most of which is clustered within the putative DNA- and ligand-binding domains (Fig. 4A). Direct comparison of the DNA-binding domains of SF-1 and NR5A2 to each other and to their human homologs reveals a 90% or higher degree of identity, and a 99% or higher degree of similarity (Fig. 4B). This includes the near-perfect duplication of the hybrid P box, A box and T box regions, which are critical determinants of DNA-binding specificity (51). In addition, a serine residue located in the AF-1 domain of SF-1 whose phosphorylation has recently been implicated in mediating cofactor recruitment (31) is also present in NR5A2 (Fig. 4A).

Tissue distribution of equine SF-1 and NR5A2

The isolation of an NR5A2 cDNA from an ovarian follicular cDNA library was unexpected, as previous studies have reported the expression of

NR5A2 in adult tissues as being limited to liver and pancreas (46, 49, 50). An RNA blot was prepared from different equine steroidogenic and non-steroidogenic tissues and probed with the NR5A2 cDNA, as well as with SF-1 to compare distribution patterns. Results clearly show a single 4.6 kb NR5A2 transcript that is very highly expressed in female gonadal tissues and to lesser degree in testis (Fig. 5). Overexposures showed low mRNA levels in liver, uterus, thymus and the head of the epididymus (data not shown), but no transcript was detected in the adrenal gland. Conversely, SF-1 mRNA was detected in equine adrenals as well as in other major steroidogenic organs, albeit to considerably lower levels than those of NR5A2 in preovulatory follicles and corpora lutea (Fig. 5). Low levels of SF-1 were detected in the spleen when the blot was overexposed (data not shown). The same membrane was reprobed with equine cDNAs encoding several steroidogenic enzymes. Results show a relationship between the expression of SF-1 and that of the steroidogenic enzymes with a few notable exceptions, such as the lack of aromatase signal in the adrenal gland (Fig. 5). Also, an apparent discrepancy occurred during the transition from the preovulatory follicle to the corpus luteum (CL), during which a drop in SF-1 mRNA levels was associated with a marked increase in StAR and P450scc transcripts. In contrast, NR5A2 expression remained at relatively high levels in the preovulatory follicle and the corpus luteum.

Expression and regulation of equine SF-1 and NR5A2 mRNA in granulosa cells and theca interna during the ovulatory process

To further examine the regulation of the NR5A nuclear receptors in follicular cells, SF-1 and NR5A2 expression was studied in granulosa cells and

theca interna throughout the equine ovulatory process (i.e. between 0 and 39 h post-hCG). Results obtained using a semi-quantitative RT-PCR/Southern blotting technique showed that the theca interna was the predominant site of SF-1 expression in the follicle wall (Fig. 6). Levels of SF-1 mRNA in theca interna were high prior to hCG treatment (0 h), and subsequently decreased in a biphasic manner; as an initial two-fold drop induced within 12 h ($P < 0.05$) was followed by another significant decrease between 33 and 36 h post-hCG ($P < 0.05$) (Fig. 6B). In granulosa cells, a significant decrease in SF-1 mRNA levels was also observed after hCG treatment (Fig. 6A). Levels observed at 24 h were approximately 2.5-fold lower than at 0 h ($P < 0.05$), and did not vary significantly thereafter (Fig. 6A).

When NR5A2 expression was examined in follicular cells, results clearly showed that the granulosa cell layer was the predominant, if not the sole, site of NR5A2 expression (Fig. 7). Importantly, when considering the number of amplification cycles used for SF-1 (16 cycles, Fig. 6) and NR5A2 (10 cycles, Fig. 7), it becomes apparent that NR5A2 is much more highly expressed in granulosa cells than SF-1. The administration of hCG caused a significant decrease in NR5A2 transcript levels at 30, 36 and 39 h ($P < 0.05$, Fig. 7A). The absence of an effect at 33 h post-hCG was attributed to the heterogenous results obtained at this time point, which resulted from two mares that apparently did not respond to hCG (note high SEM at 33 h post-hCG, Fig. 7A). Contrary to granulosa cells, extremely low levels of NR5A2 were observed in theca interna prior to hCG (Fig. 7B). Although a modest increase in NR5A2 transcripts was observed at 36 and 39 h post-hCG, the physiological nature of this finding remains to be verified.

Discussion

The equine preovulatory follicle has recently been used as a model to examine the effects of hCG on the expression of several steroidogenic genes, including StAR (54), P450scc (53), 3β-HSD (53), P45017α (52) and P450arom (52). In response to this stimulus, each of these transcripts was found to undergo a different regulatory process, and their cellular localization within the follicle wall varied in a gene-specific and time-dependent fashion (52-54). Using other model systems, the orphan nuclear receptor SF-1 has been proposed as a common transcriptional regulator of all these key ovarian steroidogenic genes (3). However, the precise mechanisms by which SF-1 could generate diverse mRNA regulatory patterns in ovarian cells in response to hCG remain to be elucidated. To determine if the regulation of SF-1 mRNA represents an important level of control in the equine ovary, this study reports the cloning of equine SF-1 and the regulation of its transcript in the follicle wall compartments during hCG-induced ovulation and luteinization. Unexpectedly, our cloning efforts also led to the serendipitous isolation of a cDNA encoding the orphan nuclear receptor NR5A2, a member of the NR5A subfamily that also includes SF-1 (1).

The present study is the first to document the concomitant regulation of SF-1 in both granulosa cells and theca interna throughout the ovulation/luteinization process *in vivo*. Although our data indicate that SF-1 transcripts were present in both follicular cell types, the equine theca interna appeared as the predominant site of SF-1 mRNA expression, as previously observed by *in situ* analysis in murine follicles (57). The expression and regulation of thecal SF-1 mRNA before and following hCG administration

closely paralleled those of the equine thecal steroidogenic genes StAR, P450scc and P45017α (52-54). Most notably, a marked decrease in the mRNA levels of all these genes was observed a few hours before ovulation (i.e. at 36 h and 39 h post-hCG). As previously pointed out for thecal steroidogenic genes (52-54), the down-regulation of SF-1 with the approach of ovulation coincides with the proposed degeneration of the thecal layer, a process unique to the equine follicle that leads to the formation of a corpus luteum derived solely from granulosa cells (58, 59). This finding could provide a putative mechanism for the transcriptional down-regulation of steroidogenic genes in thecal cells, in which the level of SF-1 expression represents a key rate-limiting factor. It is also tempting to propose that the decrease in SF-1 mRNA could represent a consequence of degenerative (and likely apoptotic) signaling processes that presumably occur in the equine theca interna prior to ovulation. In granulosa cells, the down-regulation of equine SF-1 mRNA by hCG is in agreement with similar reports in rats that demonstrated losses of SF-1 transcripts, protein and DNA-binding activity in response to gonadotropin (43-45). This down-regulation of SF-1 in rat granulosa cells has been correlated with the LH/hCG-induced abrogation of P450arom expression (43-45). The same relationship is not as clear in equine follicles, as the near complete loss of P450arom mRNA (12 h post-hCG; 52) occurred prior to the first significant drop in SF-1 transcript (24 h post-hCG; this study). Also, transcripts for other steroidogenic genes such as StAR and P450scc were shown to be induced in granulosa cells after hCG treatment *in vivo* (53, 54), whereas 3β-HSD levels were not found to vary in equine granulosa cells following hCG (53). Thus, mechanisms other than regulation of SF-1 mRNA must come into play to ensure gene-specific control of steroidogenesis in

equine granulosa cells, and are likely to include post-transcriptional mechanisms such as the regulation of SF-1 translation, phosphorylation and association with cofactors.

This report is also the first to demonstrate the expression of NR5A2 in gonadal tissues, as well as hormonal regulation of its mRNA in ovarian cells. NR5A2 expression has previously been localized in liver and pancreas, but not in reproductive organs (46, 49, 50). Interestingly, results from the present study indicate that follicular NR5A2 expression is primarily, if not solely, localized to the granulosa cell layer. Even more unforeseen, levels of NR5A2 mRNA far surpassed those of SF-1, thus making it the predominant NR5A subfamily receptor present in granulosa cells. NR5A2 was also the predominant NR5A nuclear receptor mRNA present in the corpus luteum. Considering that the equine corpus luteum is thought to be derived solely from granulosa cells (58, 59), our results suggest that NR5A2 could function as an important transcriptional regulator in these cells at various stages of differentiation (i.e. in both unluteinized and luteinized cells). For example, the elevated expression of NR5A2 transcripts in equine granulosa cells and the corpus luteum closely parallels that of promoter II-derived P450arom mRNA (52). The very high levels of expression of other steroidogenic genes such as StAR and P450scc in the equine corpus luteum (53, 54) also coincide well with those of NR5A2. Although these evidences remain circumstantial, the potential role of another nuclear receptor closely related to SF-1 involved in the transcription of steroidogenic genes should be considered. In contrast to granulosa and luteal cells, the expression of NR5A2 transcripts in theca interna was extremely low. The modest increase in thecal mRNA levels observed in the hours just prior to ovulation (i.e at 36 and 39 h post-hCG;

ovulation occurs between 39 and 42 h post-hCG in this model [60, 61]) should be interpreted with caution. In fact, we believe that this finding could be artefactual since the complete separation of granulosa cells from the theca interna becomes increasingly difficult between 30 and 39 h post-hCG in mares as a result of the copious synthesis of mucosubstances by granulosa cells (58). The presence of only a few contaminating granulosa cells in the theca interna preparation would likely be sufficient to generate the low levels of NR5A2 transcript observed by RT-PCR. *In situ* hybridization or immunohistochemistry analyses will be required to resolve this issue.

The predominant expression of NR5A2 over SF-1 (NR5A1a [1]) in granulosa cells and the corpus luteum raises the obvious question of its role in the ovary. Equine SF-1 and NR5A2 share important structural features, such as 99% similar DNA binding domains featuring nearly identical hybrid P box, A box and T box elements. The A box is of particular interest as it appears to dictate the overall DNA-binding specificity of the receptor by contacting DNA regions 5' of the hexamer half-site (51). It therefore seems likely that both equine SF-1 and NR5A2 will be found to share the same DNA-binding properties, as demonstrated for their human homologs (46, 48-50). Another key structural element that is very similar in equine SF-1 and NR5A2 is the putative ligand-binding domain, which for SF-1 has been suggested to bind a small molecule ligand (33) or to mediate protein-protein interactions that modulate transcriptional activity (12, 35, 36, 38). The conserved phosphorylatable serine residue in the AF-1 domain of NR5A2 could also be involved in recruiting transcriptional cofactors such as GRIP1 and SMRT, as reported for in SF-1 (31). Interestingly, human SF-1 and NR5A2 (known as FTF [49]) have recently been shown to transactivate at least one promoter in

common (27). Thus, based on the marked expression of NR5A2 in granulosa/luteal cells, on the mounting evidence of a potential functional redundancy between SF-1 and NR5A2 and on the demonstrated ability of the human homolog of equine NR5A2 to transactivate a hepatic steroid hydroxylase gene (cholesterol 7α-hydroxylase; 50), we believe that NR5A2 may play a key role in the regulation of gonadal steroidogenesis.

In summary, this study reports the cloning and characterization of two members of the equine NR5A nuclear receptor subfamily, and the regulation and cellular localization of their transcripts in equine follicles during hCG-induced ovulation *in vivo*. The most significant finding of this study resides in the novel localization and elevated expression of NR5A2 transcript in gonadal cells, and in its potential implication in the control of ovarian steroidogenesis. Future studies will be required to demonstrate that NR5A2 can transactivate classic steroidogenic target genes in granulosa cells, and that gonadal expression of this nuclear receptor is conserved in other species.

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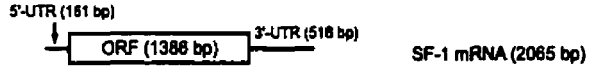
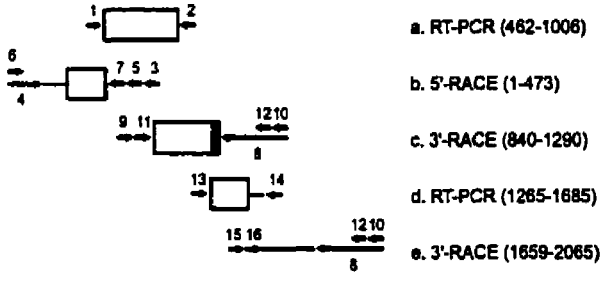
Fig. 1. Cloning strategy for equine SF-1. A, Schematic representation of the deduced SF-1 primary transcript. *Lines* indicate untranslated regions (UTRs), the *open box* designates the open reading frame (ORF). The sizes of the complete transcript as well as each structural element are indicated in base pairs (bp). The nucleotide sequence of the deduced SF-1 transcript has been deposited in GenBank with accession number AF203911. B, Cloned cDNA fragments. Each fragment is schematically represented, with its identity indicated on the right along with its position in the deduced transcript sequence in parentheses. The *gray box* represents intron sequences that are excluded from the deduced transcript. *Arrows* indicate the position and orientation of the oligonucleotides employed in the cloning processes, with numbers indicating their identity. C, Oligonucleotides used in the various cloning procedures. All primers were employed only in PCR reactions except those noted with a *single asterisk*, which were used for reverse transcriptions, and the one noted with a *double asterisk*, which was used for both reverse transcription and PCR. Oligonucleotides 4 and 6 are components of the 5'-RACE System for Rapid Amplification of cDNA Ends kit (Life Technologies).

A



B

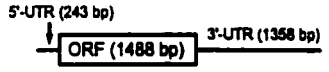


C

1. 5'-GAATTCCAGCTCCTTGAAGACCATGCAC-3'
2. 5'-GAATTCAACAAGTTTGGGCCCATGTACAAG-3'
3.* 5'-CTGTCTCCAGCTTGAAGC-3'
4. Abridged anchor primer
5. 5'-TCCAGCTTGAAGCCATTGGCTC-3'
6. Abridged universal amplification primer
7. 5'-TGTGCCTTCTTTTGCTGCTTCAG-3'
8.* 5'-GTACCGGATCCTCTAGAGAGCTCGTCGACCTCGAGG AATTCAAGCTTTTTTTTTTTTTTTTTTT-3'
9. 5'-GCCTGAGCCCAAGGGCCTGGC-3'
10. 5'-GTACCGGATCCTCTAGAGAGCTC-3'
11. 5'-ATCCTGCAGCTGTTGCAGCTG-3'
12. 5'-GTCGACCTCGAGGAATTCAAGCTT-3'
13. 5'-TGTCTGCCTCAAGTTCCTCATCC-3'
14.** 5'-CCCTCACCCACCTTCCCAAAC-3'
15. 5'-TCCAGATGGTTGATTCTATCGTGC-3'
16. 5'-CTGCCCCTGAGCTCTCTGAAGC-3'

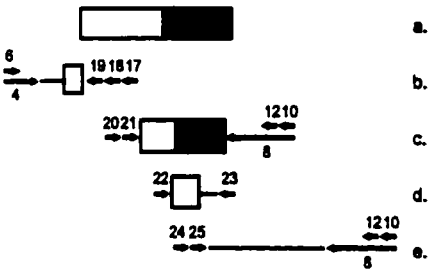
Fig. 2. Cloning strategy for equine NR5A2. A, Schematic representation of the deduced NR5A2 primary transcript. *Lines* indicate untranslated regions (UTRs), the *open box* designates the open reading frame (ORF). The sizes of the complete transcript as well as each structural element are indicated in base pairs (bp). The nucleotide sequence of the deduced NR5A2 transcript has been deposited in GenBank with accession number AF203913. B, Cloned cDNA fragments. Each fragment is schematically represented, with its identity indicated on the right along with its position in the deduced transcript sequence in parentheses. The *gray boxes* represent intron sequences that are excluded from the deduced transcript. *Arrows* indicate the position and orientation of the oligonucleotides employed in the cloning processes, with numbers indicating their identity. C, Oligonucleotides used in the various cloning procedures. All are primers were employed only in PCR reactions except the one noted with a *single asterisk*, which was used for reverse transcription, and the one noted with a *double asterisk*, which was used for both reverse transcription and PCR. Sequences of oligonucleotides 4, 6, 8, 10 and 12 are reported in Fig. 1.

A



NR5A2 mRNA (3089 bp)

B



a. cDNA clone (429-1335)

b. 5'-RACE (1-463)

c. 3'-RACE (1093-1477)

d. RT-PCR (1423-1927)

e. 3'-RACE (1819-3089)

C

17.* 5'-GACAACGCTTTCTCTGTG-3'
18. 5'-GCAGTTCTGGTTTTCTATACATGTG-3'
19. 5'-ACCTTTTATTATTTTGGACTGTTCG C-3'
20. 5'-CCATCAAATCCGAGTACCCAG-3'
21 5'-CAAGCCAACCGCAGCAAGCATG-3'
22. 5'-TCTCTGCAGTTTGATCAACGAGAG-3'
23.** 5'-CACAGTTTGCAATACAAATACCCTG-3'
24. 5'-GGGAGGGGAG AAGAACAGGA GG-3'
25. 5'-AAAAATACTCTGAACTGCTCCAAGC-3'

Fig. 3. Primary structure of equine SF-1 and NR5A2 cDNAs. A, Complete nucleotide sequence of equine SF-1, as deduced from the cloned fragments described in Fig. 1. B, Complete nucleotide sequence of the equine NR5A2, as deduced from the cloned fragments described in Fig. 2. For each transcript, the ORF is indicated by *uppercase letters*, the translation initiation (ATG) and stop (TAA) codons are highlighted in bold, the 5'-UTR and 3'-UTR are shown in *lowercase letters*, and numbers on the left refer to the first nucleotide on that line.

A. SF-1

```
   1 actgtccgcc tgccgccagc cgtggcgtga aggagttcct gtgtgcccac
  51 ggtcgccact accctgcctg gcccaccgcg gcctccctc ggaccccggg
 101 tgcccactgt ccaccctcat ccagcgtgca gctcgccttc tgctccgcgg
 151 acgccgcggg cATGGACTAT TCGTACGACG AGGACCTGGA CGAGCTGTGC
 201 CCCGTGTGTG GGGACAAGGT GTCCGGCTAC CACTACGGGC TGCTCACATG
 251 CGAGAGCTGC AAGGGCTTCT TCAAGCGCAC GGTGCAGAAC AACAAGCACT
 301 ACACGTGCAC CGAGAGCCAG AGCTGCAAGA TCGACAAGAC GCTGCGCAAG
 351 CGCTGTCCCT TCTGCCGCTT CCAGAAGTGC CTGACGGTGG GGATGCGCCT
 401 GGAAGCCGTG CGTGCTGACC GCATGCGGGG TGGCCGGAAC AAGTTTGGGC
 451 CCATGTACAA GCGGGACCGG GCCCTGAAGC AGCAAAAGAA GGCACAGATT
 501 CGAGCCAATG GCTTCAAGCT GGAGACAGGC CCCCCAATGG GGGTACCCCC
 551 TCCGCCCCCT CCCCCACCGG ACTACATGCT GCCCCCTGGC CTGCATGTGC
 601 CTGAGCCCAA GGGCCTGGCC TCTGGTCCAC CTGCTGGGCC ACTGGGCGAC
 651 TTTGGGGCCC CAGCCCTGCC CATGGCCGTG CCCAGCACCA ACGGGCCGCT
 701 GGCTGGCTAC CTCTATCCTG CCTTCCCTGG CCGTGCCATC AAGTCTGAGT
 751 ACCCGGAGCC CTACGCCAGC CCTCCGCAGC CTGGGCCACC CTATGGCTAC
 801 CCAGAGCCCT TCTCCGGGGG GCCTGGCGTG CCCGAGCTCA TCCTGCAGCT
 851 GTTGCAGCTG GAGCCGGACG AGGACCAGGT TCGGGCGCGC ATCATCGGCT
 901 GCCTGCAGGA ACCAGCCAAA GGCCGCCCCG ACCAGCCTGC GTCCTTCAAC
 951 CTCCTGTGCA GGATGGCCGA CCAGACCTTC ATCTCCATCG TGGACTGGGC
1001 ACGCAGGTGC ATGGTCTTCA AGGAGTTGGA GGTGGCCGAC CAGATGACCC
1051 TGCTGCAGAA CTGCTGGAGC GAGCTGCTGG TGTTCGACCA TATCTACCGC
1101 CAGGTCCAGC ACGGCAAGGA GGGCAGCACC CTGCTGGTCA CCGGGCAGGA
1151 GGTGGAGCTG ACCACGGTGG CGGCCCAGGC CGGCTCACTG CTGCATGGGC
1201 TGGTGCTGCG GGCCCAGGAG CTGGTGCTGC AGATGCACGC GCTGCAGCTG
1251 GACCGCCAGG AGTTTGTCTG CCTCAAGTTC CTCATCCTCT TCAGCCTCGA
1301 TGTGAAGTTC TTGAATAACC ACAGCCTGGT GAAGGATGCT CAGGAGAAGG
1351 CCAATACCGC CCTGCTCGAT TACACCCTGT GCCACTACCC GCACTGCGGG
1401 GACAAGTTCC AGCAGCTGCT GCTGTGCCTG GTGGAGGTGC GGGCACTGAG
1451 CATGCAGGCC AAGGAATACC TGTACCACAA GCACCTGGGC AACGAGATGC
1501 CCCGCAACAA CCTGCTCATC GAGATGCTGC AAGCCAAGCA GACTTGAgcc
1551 tgggctgggt ggggtcgggc cggggccggg ggaggctcac agccacccct
1601 gctgccctcc agatggttga ttctatcgtg cccacccagg agccccaccc
1651 tgtagccccc gccctgagc tctctgaagc cctgtgtttg ggaaggtggg
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1751 gcactagcct gccactcaga gtgccccaag gaggcagctg ctacccactc
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1951 gagcgggtca gctgggagag aagagagggt ccctagccct ccgtcacagc
2001 gcccaggagg gaagccctct gttttgtaaa ctaggaataa actgagtttg
2051 caaaactgga aaaaa
```

B. NR5A2

```
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  51 agactggctt gtaactttgc gagttcttgg attttttttt ttttttttt
 101 ttttactttt tcttaacttt cactaagggt tactatagtc tgatgtgttc
 151 tccccaaggc tagaaaaatt gacaagctgt acttttttct gtgctcaatg
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 301 CCTATTGTGT CTCAGTTTAA AATGGTGAAT TACTCCTATG ATGAGGACCT
 351 GGAGGAGCTC TGTCCGGTGT GTGGAGATAA AGTGTCTGGG TACCACTACG
 401 GTCTCCTCAC CTGTGAAAGC TGCAAGGGGT TTTTTAAGCG AACAGTCCAA
 451 AATAATAAAA GGTACACATG TATAGAAAAC CAGAACTGCC AAATTGACAA
 501 AACACAGAGA AAGCGTTGTC CCTACTGTCG ATTTCAAAAA TGTCTAAGTG
 551 TTGGAATGAA GCTAGAAGCG GTAAGAGCCG ACCGAATGCG TGGAGGGAGG
 601 AATAAGTTTG GGCCAATGTA CAAGAGGGAC AGGGCCCTGA AGCAACAGAA
 651 GAAAGCCCTC ATTCGAGCGA ATGGACTTAA GCTGGAAGCC ATGTCTCAGG
 701 TGATCCAGGC AATGCCCTCT GAGCTGAGCA TCTCCTCTGC CATCCAGAAC
 751 ATCCATTCTG CCTCCAAAGG CCTACCTCTG AACCACGCTG CCTTGCCTCC
 801 CACGGACTAT GACAGAAGTC CCTTTGTAAC GTCCCCCATT AGCATGACGA
 851 TGCCACCTCA TGGCAGCCTG CAAGGTTACC AAACCTACAG CCACTTTCCC
 901 AGCCGAGCCA TCAAATCCGA GTACCCAGAC CCCTACACCA GCTCACCAGA
 951 GTCAATAATG GGCTATTCCT ACATGGATGG TTACCAGACC AGCTCCCCGG
1001 CGAGCATCCC TCATCTGATA CTGGAACTTT TGAAGTGTGA GCCAGATGAG
1051 CCTCAAGTCC AGGCCAAAAT CATGGCCTAT TTGCAGCAAG AGCAAGCCAA
1101 CCGCAGCAAG CATGAAAAGC TGAGCACGTT TGGGCTAATG TGCAAAATGG
1151 CCGATCAGAC CCTCTTCTCC ATTGTCGAGT GGGCCAGGAG TAGCATCTTC
1201 TTCCGAGAGC TCAAGGTGGA TGACCAAATG AAGCTGCTGC AGAACTGCTG
1251 GAGTGAGCTC TTAATTCTCG ACCACATTTA CCGACAAGTG GTACATGGAA
1301 AGGAAGGGTC CATCTTCCTG GTTACCGGGC AACAAGTGGA CTATTCCATC
1351 ATTGCCTCCC AGGCTGGGGC CACCCTCAAC AATCTCATGA GTCATGCACA
1401 GGAGTTAGTG GCAAAGCTTC GTTCTCTGCA GTTTGATCAA CGAGAGTTTG
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1551 GGACTACACC ATGTGCAACT ACCCACAGCA AACAGAGAAA TTTGGGCAGC
1601 TCCTTCTTCG ACTACCTGAA ATCCGGGCCA TCAGCATGCA GGCTGAGGAG
1651 TACCTCTACT ACAAGCACCT GAACGGGGAC GTGCCCTACA ATAACCTCCT
1701 CATTGAAATG TTGCATGCAA AGAGAGCTTA Agtcacaacc cgcaggagcc
1751 ctgctttcaa aacaaaaaga gattggtggg ggtgggaggg gagaagaaca
1801 ggaggaaaga aagaaaacaa aaatactctg aactgctcca agcaacacta
1851 attaaaaact tggtttaaag atattgaatt taaaaaggca taataatcca
1901 atacttagta gcaaataagt gatgtatcag ggtatttgta ttgcaactgt
1951 gaatcgaagt cttcacatcc ccagaggagt ccatgcaaag gacactgtaa
2001 tggagtggac tgaactcgcc ggggaatacc agtgccgcgt ccgaacggga
2051 atggacgaaa cgattcttgt atatttaaac tgatctccgc tgtgaagaaa
2101 tttagcaact gatctgtgtt attaattagg ctctgacagc gggggatttg
2151 agcttacaga attcctccac ggtaaagcgg aacggaaaca attctccaga
2201 tccatcagct ggacctataa tagcctgtcc ctcttccttt cgaggaccca
2251 gcaccttctg tcctgtgatc gcggaatctg tgctaaggac ttgtgctgtg
2301 ccacacccac tcgtagctcc accaaattac gaaaagccta attttgaatg
2351 tctgtgtctt agacttgcaa acagctaata agagcagtct attaatctgt
2401 tagcttgcca ttttaaatat gttctgggtt ggtttgtcat gtgttcacaa
2451 tgttaaaaaa agcaggcagt atcccttttc tgaccttcta gaagcgttaa
2501 ttaatattag ggaaatgact acaaactttc aaagcaacgc tccatagttc
2551 aagcaagcca gcccttgttt ctgctactgt tactgaaatg cggctttggc
2601 attgctggat ttcataaaaa ataaaacatg aaccatattt tgctaggctg
2651 tcagatagtc acagttctaa gtagttaaaa accaaaccaa agcatgctaa
2701 gctatgcaaa aagacgggaa aggatgagct gataaattga gtgactcgag
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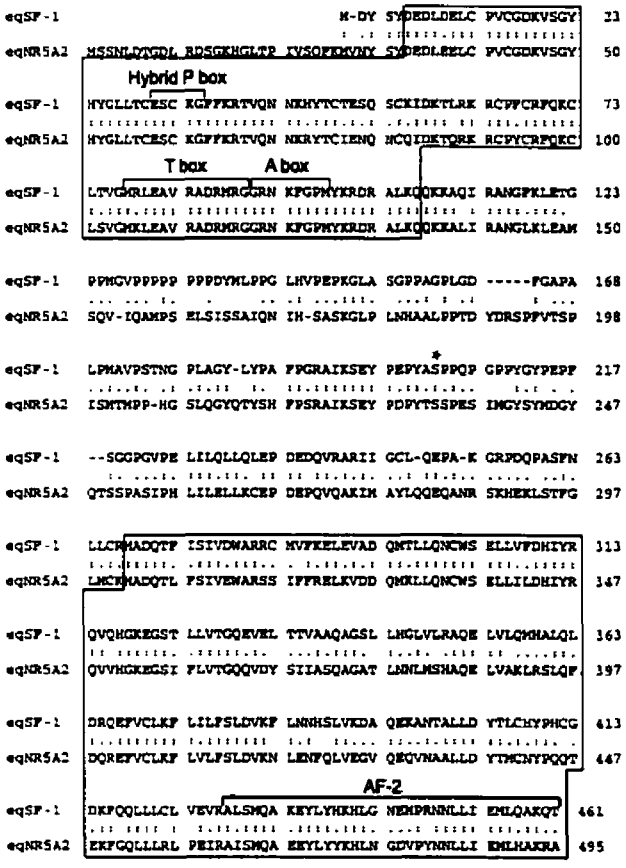
2851 gaagggaaag gaagagagtg atactgacct ttttaagtca tagaccaaag
2901 tctgctacag aacaaatatt ggaggacaaa gaattgcaaa caagttctcc
2951 aggagacact atcagtatta ttaacatgca gtgccacaga tatggagatc
3001 ttgccttatt tcacaattct aaaaggtagc tgtgcagatg tggatcaaca
3051 tttatttcaa ataaagtatt aataaagtcc aataaaaaa
```

Fig. 4. Predicted amino acid sequences of equine SF-1 and NR5A2. A, Alignment of the equine SF-1 and NR5A2 proteins. Identical residues are linked with a *colon*, similar residues with a *printed period*. Gaps in protein sequences created to optimize alignment are indicated with *hyphens* . Numbers designate the sequence position of the last residue in that row. The first boxed region encompasses the DNA-binding domain, the second box represents the putative ligand-binding domain. Sequences representing the hybrid P box, T box, A box and activation function-2 (AF-2) regions are *overlined*. A conserved, phosphorylatable serine residue required for maximal SF-1-mediated transcription is indicated with an *asterisk* . Sequence analysis and alignment was performed using MacDNASIS software version 2.0 (Hitachi, Hialeah, FL). B, Quantification of the homology between SF-1 and NR5A2 within the highly-conserved domains. Equine SF-1 and NR5A2 domains are compared to each other and to their human homologs. Eq= equine, hu= human, id= identity, si= similarity, aa= amino acids.

A


Hybrid P box
T box
A box
AF-2

B

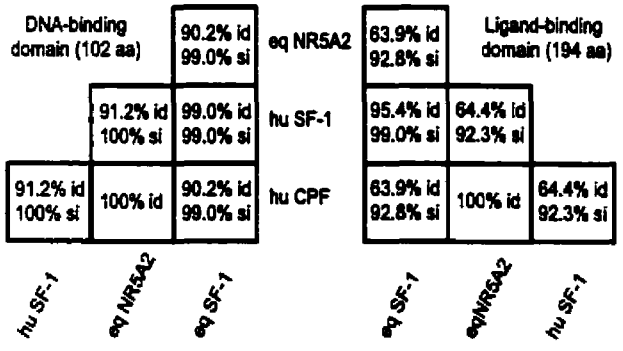

DNA-binding domain (102 aa)
Ligand-binding domain (194 aa)
90.2% id 99.0% si
eq NR5A2
63.9% id 92.8% si
91.2% id 100% si
99.0% id 99.0% si
hu SF-1
95.4% id 99.0% si
64.4% id 92.3% si
91.2% id 100% si
100% id
90.2% id 99.0% si
hu CPF
63.9% id 92.8% si
100% id
64.4% id 92.3% si
hu SF-1
eq NR5A2
eq SF-1
eq SF-1
eqNR5A2
hu SF-1

Fig. 5. Expression of SF-1 and NR5A2 mRNAs in equine tissues. Samples of total RNA (10 μg/lane) extracted from various equine tissues were analyzed by Northern blotting using labeled cDNA probes, as described in *Materials and Methods* (follicle wall = theca interna with attached granulosa cells of a preovulatory follicle isolated prior to hCG treatment). The same membrane was probed and stripped successively to produce the images shown. The cDNA probes used, along with exposure times to film (in hours) were as follows: SF-1 (21 h), NR5A2 (16 h), StAR (16.5 h), P450scc (1 h), P45017α (14.5 h), 3β-HSD (2 h), P450arom (1 h) and GAPDH (93 h). Apparent molecular weights are expressed in kilobases (kb).

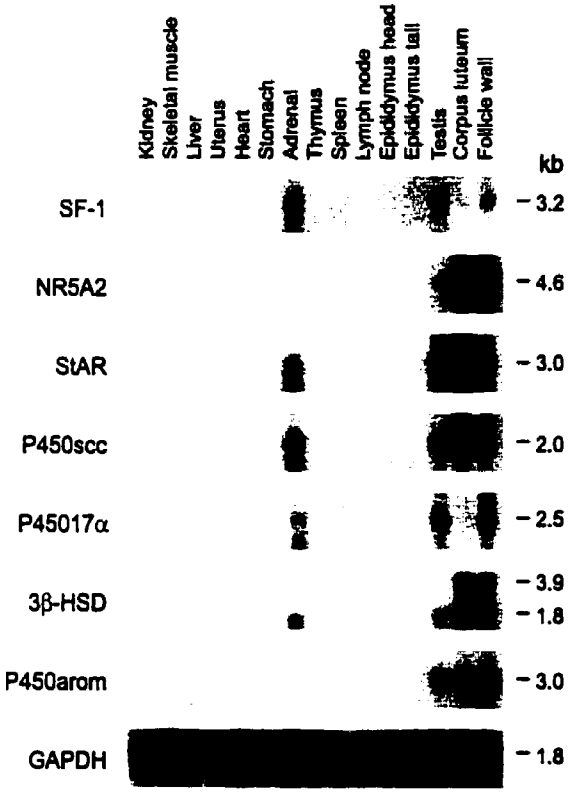

Kidney
Skeletal muscle
Liver
Uterus
Heart
Stomach
Adrenal
Thymus
Spleen
Lymph node
Epididymus head
Epididymus tail
Testis
Corpus luteum
Follicle wall
kb
SF-1
NR5A2
StAR
P450scc
P45017α
3β-HSD
P450arom
GAPDH
3.2
4.6
3.0
2.0
2.5
3.9
1.8
3.0
1.8

Fig. 6. Regulation of SF-1 mRNA by hCG in equine follicular cells during the ovulatory process. Preparations of granulosa cells (A) and theca interna (B) were isolated from equine preovulatory follicles obtained 0, 12, 24, 30, 33, 36 and 39 h post-hCG, and samples (100 ng) of total RNA were analyzed by a semi-quantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. After autoradiography, the SF-1 signal intensity was quantified by densitometric analysis and normalized to the control gene GAPDH. Results are presented as a signal ratio of SF-1 to GAPDH (mean ± SEM; n = 4 samples [i.e. mares] per time point). No significant difference of GAPDH mRNA levels was detected between 0-39 h post-hCG. *Bars* marked with a *single asterisk* are significantly different from 0 h post-hCG, whereas those marked with a *double asterisk* are significantly different from the 33 h time point ($P < 0.05$). *Inserts* show representative results of SF-1 mRNA levels from one sample per time point. Numbers of PCR cycles for each gene were within the linear range of amplification, and represented 16 and 10 cycles for SF-1 and GAPDH, respectively.

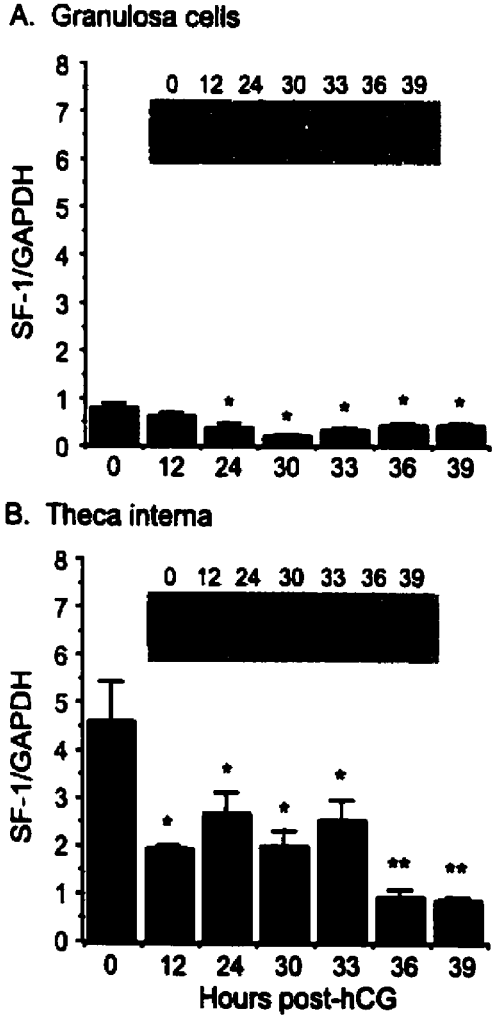
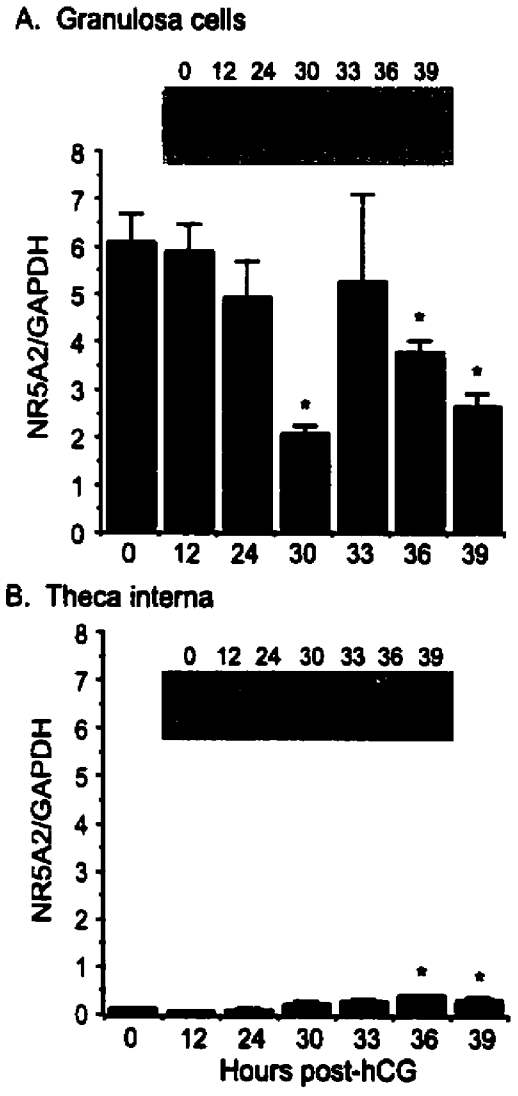

A. Granulosa cells
0 12 24 30 33 36 39
SF-1/GAPDH
0 1 2 3 4 5 6 7 8
0 12 24 30 33 36 39
B. Theca interna
0 12 24 30 33 36 39
SF-1/GAPDH
0 1 2 3 4 5 6 7 8
0 12 24 30 33 36 39
Hours post-hCG

Fig. 7. Regulation of NR5A2 mRNA by hCG in equine follicular cells during the ovulatory process. Preparations of granulosa cells (A) and theca interna (B) were isolated from equine preovulatory follicles obtained 0, 12, 24, 30, 33, 36 and 39 h post-hCG, and samples (100 ng) of total RNA were analyzed by a semi-quantitative RT-PCR/Southern blotting technique, as described in *Materials and Methods*. After autoradiography, the NR5A2 signal intensity was quantified by densitometric analysis and normalized to the control gene GAPDH. Results are presented as a signal ratio of NR5A2 to GAPDH (mean ± SEM; n = 4 samples [i.e. mares] per time point). No significant difference of GAPDH mRNA levels was detected between 0-39 h post-hCG. *Bars* marked with an *asterisk* are significantly different from 0 h post-hCG ($P < 0.05$). *Inserts* show representative results of NR5A2 mRNA levels from one sample per time point. Numbers of PCR cycles for each gene were within the linear range of amplification, and represented 10 cycles for NR5A2 and GAPDH.


A. Granulosa cells
0 12 24 30 33 36 39
NR5A2/GAPDH
0 1 2 3 4 5 6 7 8
0 12 24 30 33 36 39
B. Theca interna
0 12 24 30 33 36 39
NR5A2/GAPDH
0 1 2 3 4 5 6 7 8
0 12 24 30 33 36 39
Hours post-hCG

GENERAL DISCUSSION

A primary objective of the work presented in this thesis was to establish the mare as a model for the study of the molecular control of ovarian gene expression in monoovulatory species. The mare was chosen due to several characteristics of its ovarian physiology that are well suited for the required analyses. Notably, the large size of the equine preovulatory follicle facilitates the isolation of the different cellular components of the follicle wall, and provides samples of sufficient size to perform multiple quantitative assays. Also, precise ultrasound imaging techniques are available to monitor follicular development *in vivo*, and the mare's processes of follicular recruitment, selection, and dominance are similar to those of other monoovulatory species (6, 340). Despite these advantages, the equine model for the study of ovarian gene expression has remained as yet largely uncharacterized. This thesis therefore reports a series of cloning and mRNA regulation studies that elucidate some of the gene regulation events induced by the LH surge in the equine ovary. The conclusions drawn from these results are grouped hereafter in four main sections.

The regulation of PGHS-2 mRNA in equine preovulatory follicles supports its putative role as a determinant of the mammalian ovulatory clock

To study the genes involved in ovarian prostaglandin biosynthesis, the first article presented herein characterized the primary structure of the equine

PGHS-2 gene, transcript and protein. The coding regions and the intron/exon structure of the equine gene were found to be highly conserved with respect to their species homologs (79-81). A few variations among species were observed however, most notably in the length of the transcript 3'-UTRs. Genomic sequence analysis indicated that our equine cDNA clone and the published human transcript were most likely reverse transcribed from internal poly(A) sequences in the 3'-UTR rather than the poly(A) tail. Isolation of the complete equine and human PGHS-2 3'-UTRs will therefore require additional experimental procedures, such as 3'-RACE. Another interesting structural feature of the equine PGHS-2 3'-UTR is the presence of numerous Shaw-Kamen's sequences, a motif known to confer instability to immediate early gene mRNAs (84, 85). Our sequence analysis demonstrated that a group of five Shaw-Kamen's repeats is consistently found immediately downstream of the termination codon in all PGHS-2 species homologs characterized thus far (78, 82, 83, 341), suggesting their greater relative importance in mediating mRNA degradation. Mutational analysis will however be required to confirm this hypothesis.

In order to determine the regulation of PGHS-2 mRNA during the ovulatory process, Northern blotting was performed using RNA extracted from a series of equine preovulatory follicles isolated between 0–39 h after hCG treatment. A unique timecourse of PGHS-2 mRNA induction was observed in granulosa cells as it was first detected at 30 h post-hCG, contrasting with other cell types in which it is typically induced within 1 h by various agonists (78, 82, 342, 343). Although PGHS-2 is considered to be an early response gene in most

cell systems, the greatly delayed induction that we observed suggests that it does not serve this role in equine granulosa cells. This is further supported by another study showing that PGHS-2 induction by gonadotropins in rat granulosa cells is dependent on protein synthesis (344). Most significantly, the delayed induction of equine PGHS-2 occurs approximately 10 h prior to ovulation, which is the same timespan that has been previously reported in cows and rats despite the very different overall lengths of their ovulatory processes (28 h and 12-14 h, respectively). Cumulatively, these data therefore suggest that PGHS-2 induction is a determinant of the mammalian ovulatory clock (345). The length of the equine ovulatory process, with the accompanying extended delay before PGHS-2 induction, indicates that the mare represents an important model to study the transcriptional regulation of PGHS-2 in species with long ovulatory processes.

Insight into the mechanisms underlying the species-specific length of the mammalian ovulatory process has been provided by a recent study examining the transcriptional regulation of PGHS-2 in bovine granulosa cells following the administration of hCG (79). This study suggests that PGHS-2 induction occurs upon the alleviation of two negative regulatory processes; the binding of C/EBPβ to a CCAT box element and the binding of mini-USF-2 to an E-box element. This is supported by the demonstration that a transient decrease in C/EBPβ protein levels and mini-USF-2 binding activity occur in bovine granulosa cells in a manner that is temporally coincident with the induction of PGHS-2. Conversely, similar studies in rats showed no evidence of ovarian mini-USF proteins (103). We can therefore postulate that species with long

ovulatory processes such as the cow and the mare could conceivably delay the induction of PGHS-2 until the appropriate moment by specific transcriptional repression mechanisms, perhaps involving C/EBPβ and mini-USF-2 in both species. On the other hand, species with short ovulatory processes such as the rat may have different or no such mechanisms. Comparative analyses of the transcriptional mechanisms involved in ovarian PGHS-2 induction in additional species with long and short ovulatory processes will ultimately be required to elucidate the fundamental working of the mammalian ovulatory clock.

The cellular distribution and hormonal regulation of steroidogenic mRNAs define the biochemical pathways that direct equine ovarian steroidogenesis

Another main objective of this work was to study the hormonal regulation of genes involved in ovarian steroidogenesis. Articles two and three and Annex I therefore report the cloning and characterization of equine transcripts encoding StAR, P450scc, 3β-HSD, P45017α and P450arom and examined their regulation during the ovulatory process.

Annex I reports the cloning of two distinct equine StAR transcripts. These were found to encode a protein that lacks the amino-terminal motif for mitochondrial two-step cleavage identified in murine StAR (140), which has recently been proposed to be the mechanism by which StAR proteins are ultimately inactivated (125). Considering that the human, bovine, and porcine StAR proteins also lack this motif (127, 137, 138), it could be speculated that proteolysis occurs at a different site in these species, that a different inactivation

mechanism may be involved, or that proteolytic cleavage may not in fact be required at any step of StAR action or inactivation. Elucidation of the true function of StAR cleavage has been difficult, as amino-terminal truncated proteins remain functional but are not specifically targeted to mitochondria (141, 142). Proof of the proteolytic inactivation hypothesis will ultimately require a demonstration of the inactive state of the truncated StAR proteins present within the mitochondria.

The key finding presented in Annex I is the reciprocal regulation of StAR transcripts by gonadotropins in each cellular compartment, the net result of which is the systematic redistribution of StAR expression from the theca interna to the granulosa cells. While the pattern of induction of StAR mRNA in equine granulosa cells compares with that observed in an immature rat model after hCG administration (30, 31), the concomitant loss of StAR expression in theca interna represents a novel finding. Although the biological significance of the loss of thecal StAR transcripts remains to be precisely established, we believe that it could be a consequence of a putative degenerative process of the equine theca interna (4, 346). In contrast to other species, the theca interna in mares undergoes a process resembling apoptosis with the approach of ovulation, and apparently does not contribute to the formation of the corpus luteum (4). The redistribution of StAR expression could therefore represent the means by which the steroidogeneic potential of the follicle is transferred from the degenerating theca interna to the luteinizing granulosa cell layer. This would promote the luteinization-induced shift of the predominant steroid hormone product from estradiol-17β to progesterone, as StAR expression is transferred from the

androgen-synthetic theca interna to the luteinizing granulosa cells that will eventually form the corpus luteum. In support of this, article two also demonstrates a significant rise in follicular fluid concentrations of progesterone that parallels the increase in steady state levels of StAR mRNA in granulosa cells. The induction of StAR expression in granulosa cells may thus be considered a molecular marker of the onset of follicular luteinization.

Importantly, article two demonstrates that a similar regulatory process also occurs with P450scc. Prior to hCG treatment, P450scc mRNA was found to be relatively high in theca interna and low in granulosa cells of equine follicles, as has been observed in pigs (39, 347), but unlike humans (348) and cows (32, 40) who express high levels of P450scc transcripts in granulosa cells prior to the LH/hCG surge. Induction of the ovulatory/luteinization process with hCG was then shown to cause a down-regulation of follicular P450scc mRNA, and a cellular redistribution of the transcript to the luteinizing granulosa cells that is unprecedented in other species. This provides further evidence that the mare transfers the steroidogeneic potential of the follicle from the degenerating, androgen-synthetic theca interna to the progesterone-synthetic luteinizing granulosa cells during the hours preceding ovulation.

Article two also documents a novel pattern of 3β-HSD mRNA expression in equine follicular cells during the ovulatory process. The presence of 3β-HSD transcript in equine granulosa cells and its complete absence in theca interna contrast with findings in other species (32, 33, 39, 41, 209, 348-350). Thus, the equine theca interna presumably does not produce progesterone *in vivo*, although elevated expression of StAR and P450scc mRNAs in this cell type

suggests that it synthesizes large amounts of pregnenolone precursors. This is further supported by studies *in vitro* showing that cultures of equine theca interna secrete negligible amounts of progesterone, and that gonadotropins have no effect on its secretion (7). The apparent lack of modulation of 3β-HSD mRNA expression prompts us to propose that it represents an unregulated, non-rate-limiting step in equine ovarian steroidogenesis.

Article three provides a characterization of the regulation and cellular localization of cytochrome P45017α transcripts in equine preovulatory follicles. Expression of P45017α mRNA was found to be confined to the theca interna as reported in other species (32, 34, 39, 221), and this supports studies *in vitro* showing that secretion of androgens was limited to equine theca interna cells (7). A marked drop in P45017α mRNA levels was then shown to occur in theca interna 33 h post-hCG, precisely as observed for StAR and P450scc, however this was not accompanied by the redistribution of P45017α expression to the granulosa cell layer. As this presumably leads to the loss of follicular androgen synthesis, the loss of P45017α expression likely represents the means by which estradiol-17β synthesis is ultimately down-regulated, and this can therefore be directly linked to the preovulatory degeneration of the theca interna.

The hCG-induced regulation of P450arom mRNA was also examined in article three. In agreement with prior immunohistochemical and biochemical analyses (7, 124, 351), an elevated level of promoter II-derived P450arom mRNA was detected selectively in granulosa cells before hCG treatment, thus resolving a previous controversy on the site of estrogen biosynthesis in the equine follicle (346). Following the administration of hCG, P450arom expression was sharply

downregulated as has been observed other species (32, 34, 39, 44, 275), and this event likely represents the immediate cause of the luteinization-associated reduction of estrogen biosynthesis. P450arom mRNA levels were then shown to re-increase notably in the CL, reflecting the limited but functionally significant estrogen-synthetic potential of the ovary during the luteal phase (346).

Taken together, our steroidogenic gene mRNA regulation data allow us to formulate a working model for the control of equine follicular steroidogenesis that is presented in article two. During the early follicular phase, steroidogenesis proceeds essentially via the Δ5 pathway, with the androgen dehydroepiandrosterone (DHEA) being synthesized in thecal cells in which StAR, P450scc and P45017α are either predominantly or exclusively expressed. DHEA then diffuses to the granulosa cell layer, where it serves as a substrate for estrogen synthesis by 3β-HSD and P450arom, in agreement with the classic two-cell, two-gonadotropin model of ovarian steroidogenesis. Following the LH/hCG surge, P450arom expression is sharply downregulated, which is the likely primary event that results in the luteinization-associated reduction of estrogen biosynthesis. The subsequent loss of P45017α in the degenerating theca interna then results in the abrogation of the androgen-synthetic potential of the follicle, further enhancing the drop in estrogen production by vastly reducing P450arom substrate availability. As additional consequences of the degeneration of the theca interna, StAR and P450scc are redistributed to granulosa cells, thereby consolidating the expression of the enzymes required for progesterone synthesis (i.e. StAR, P450scc, and 3β-HSD) in the cells that will form the corpus luteum. These gonadotropin-dependent changes in enzyme

expression would redefine the principal steroidogenic pathway from Δ5 to Δ4, with progesterone becoming the predominant and obligatory end product. The induction of P450arom expression in the late follicular phase and in the CL would explain the low but functionally important level of estrogen synthesis by these tissues during the luteal phase (346), although the lack of P45017α expression at these stages suggests that circulating androgens would be the only available source of substrate. Low levels of P450scc expression in the granulosa cells of follicles before the LH rise would suggest a certain degree of alternative usage of the Δ4 pathway for estradiol biosynthesis. In this manner, resultant pregnenolone could be converted immediately to progesterone by 3β-HSD that is expressed in the same cell type, which could subsequently diffuse to the theca interna for conversion into androgen by P45017α. The lack of StAR expression in granulosa cells at this stage would however severely limit the availability of cholesterol substrate molecules for P450scc. Furthermore, should the catalytic properties of equine P45017α resemble those of humans, cows and sheep (123), progesterone would represent a poor substrate for conversion to androgens, further reinforcing the functional blockage of the Δ4 pathway that is implied by the cellular distribution of the steroidogenic enzymes within the follicle wall.

The alternate use of two P450arom promoters during the ovulatory process provides an inducible model system to study P450arom promoter switching

Article three also reports the cloning of two distinct equine follicular P450arom cDNAs derived from transcripts that arise from the alternative use of

two distinct promoters, II and 1f. This was unexpected given that promoter II-derived mRNA was shown to be the only form expressed in human follicles and corpus luteum (252), whereas promoter 1f-derived mRNA was originally cloned from brain tissues and has been described as brain-specific (254, 266, 352, 353). However, several recent studies have shown a vast tissue distribution for many P450arom mRNA species as well as the use of alternative P450arom promoters within a given tissue (247, 256-260, 264, 265, 354). This strongly suggests that the traditional model describing each promoter as being tissue-specific is in fact a misleading oversimplification.

Analysis of the relative contribution of each promoter to ovarian P450arom expression revealed a novel promoter switching phenomenon. The down-regulation of promoter II-derived mRNAs after hCG treatment was found to be followed by an induction of promoter 1f-derived mRNAs later in the ovulatory process, implying a first switching phenomenon. The presence of promoter II-derived transcripts and the disappearance of promoter 1f-derived transcripts in the CL suggests that a second switching event occurs at an undetermined moment following ovulation. The use of alternate P450arom promoters was thought not to occur during the ovulatory process, as promoter II-derived mRNA was found to predominate in human follicles and corpus luteum (252). Our results do not contradict this however, as our analysis included samples representing a more minute dissection of the ovulatory process than previous studies. Also, it remains possible that P450arom promoter switching during follicular luteinization is a process unique to mares. To date, P450arom promoter switching had been reported only in distantly

separated developmental (256, 265) and pathological (245, 257–260) stages *in vivo*, and in human mononuclear leukemic THP-1 cells and peripheral blood lymphocytes *in vitro* (251, 355). Considering this and proposed role of P450arom promoter switching in consequential processes such as carcinogenesis, we propose that the equine preovulatory follicle represents a valuable model system to study this phenomenon, particularly given the inducible nature of the promoter switch by a known agonist (i. e. LH/hCG).

To provide insight into the transcriptional mechanisms involved in ovarian P450arom promoter switching, article three also presents the cloning and preliminary characterization of equine promoters II and 1f. Sequence analysis led to the identification of a site identical to that present in the human, rat and bovine promoters that binds SF-1 and has been shown to be responsible for both basal and inducible P450arom promoter activity (275-277). The CRE-like element present in the human and rat promoters that binds cAMP response element binding protein (CREB) and acts in synergy with SF-1 to promote inducible P450arom expression (276-278) is not conserved however in equine promoter II. The corresponding region features a 1 bp deletion similar to that identified in the cow and pig that is believed to render the element non-functional (279-281). The mechanisms reponsible for the observed regulation of P450arom promoter II in response to hCG remain largely conjectural. In rat granulosa cells, the decrease in promoter II activity was associated with concomitant drops in SF-1 mRNA and binding activity (44, 276, 336). The modest, gradual drop that we observe in equine SF-1 mRNA levels in response to hCG however seem unlikely to account for the marked and rapid down-

regulation of promoter II-derived P450arom mRNA (article four). We therefore speculate that additional post-transcriptional mechanisms regulate SF-1 activity in response to hCG. These may include one or several processes identified in the regulation of other SF-1 target genes, including ligand binding (327), protein-protein interactions (301, 328-330) and serine phosphorylation (276, 291, 297, 332). In addition, studies in eutopic endometrial cells have shown that COUP-TF can compete with SF-1 for its binding site, thereby silencing P450arom expression (282). COUP-TF-mediated silencing of transcriptional activity has also been demonstrated for the P45017α gene (227, 234), but its function in regulation P450arom promoter II in granulosa cells remains unknown. Unlike promoter II, the molecular mechanisms involved in promoter 1f-derived P450arom expression have not been characterized. Potential cis-acting elements identified by sequence analysis in article four include highly conserved, overlapping c-myc and glucocorticoid response elements (GRE) also identified in the human (254) and mouse promoters (266). Recent studies have shown an increase in c-myc expression in rat granulosa cells during hCG-induced luteinization (336, 356), which would provide a simple mechanism for promoter 1f induction. We can also speculate that the GRE may be regulated by the androgen receptor, which binds the same element. The relevance of this mechanism relates to the extended delay between the down-regulation of P450arom mRNA (which occurs at 0-12 h post-hCG) and P45017α mRNA (at 30-33 h post-hCG), which allows for the possibility of a transient excess in androgen production. These androgens could then activate P450arom promoter 1f via the androgen receptor binding the GRE, thereby up-regulating P450 arom

activity late in the ovulatory process. This could serve as a limiting mechanism to prevent the accumulation of excess androgens in the follicular fluid. Alternatively, P450arom promoter 1f activation during the ovulatory process may serve no useful function whatsoever. Gene knockout analyses that target specific P450arom promoters will ultimately be required to elucidate the specific biological functions of each type of transcript.

Putative roles of SF-1 and SF-2 in the gene regulation of equine ovarian steroidogenesis

In apparent contrast with the varied steroidogenic gene mRNA regulatory patterns presented in articles two and three and in Annex I, SF-1 has been proposed to be a common transcriptional regulator of all the key ovarian steroidogenic genes (155, 156, 158, 185, 188, 210, 234, 276, 277, 286, 297). To identify possible links between the regulation of specific steroidogenic genes and that of SF-1, article four reports the cloning of equine SF-1 and the regulation of its mRNA in the follicle wall compartments by hCG. In addition to a conserved SF-1 transcript, our cloning efforts also produced a cDNA encoding NR5A2, a member of the same nuclear receptor subfamily as SF-1 (283). These were found to share many structural features, such as 99% similar DNA binding domains that have been shown to confer identical DNA-binding properties to their human homologs (357-360). For this and other reasons, we suspect that NR5A2 may play a role in the regulation of gonadal steroidogenesis, and have decided to name it SF-2.

Regulation studies similar to those performed for PGHS-2 and the steroidogenic genes demonstrated an approximately threefold down-regulation of SF-1 mRNA by hCG in granulosa cells by 30 h post-hCG. The poor resemblance between this SF-1 mRNA regulation pattern and that of any of the steroidogenic genes led us to conclude that it is unlikely that the regulation of SF-1 at the mRNA level represents a key mechanism governing steroidogenesis in granulosa cells. Again, post-transcriptional mechanisms such as the regulation of SF-1 translation, phosphorylation and association with cofactors would have to come into play to ensure proper gene-specific regulation. Conversely, SF-1 mRNA levels in the theca interna before and following hCG administration were found to closely parallel those of the thecal steroidogenic genes StAR, P450scc and P45017α. This allows for a simple mechanism for the transcriptional regulation of steroidogenic genes in thecal cells, in which the level of SF-1 expression is the key rate-limiting factor for all target genes. As the down-regulation of SF-1 with the approach of ovulation coincides with the apparent degeneration of the thecal layer in the mare, we speculate that the regulation of SF-1 mRNA may be linked to apoptotic signalling processes. Therefore, the LH-induced passage from the estrogen-synthetic preovulatory follicle to the progesterone-synthetic corpus luteum could be an apoptosis-dependant process, at least in the mare.

Article four also demonstrates for the first time the expression of SF-2 in gonadal tissues, as well as hormonal regulation of its mRNA. Similarly to SF-1, SF-2 mRNA levels dropped 3-fold by 30 h post-hCG in granulosa cells. Unlike SF-1 however, granulosa cells and the granulosa cell-derived CL represent the

sole sites of SF-2 expression within the ovary. Furthermore, Northern and semi-quantitative RT-PCR analyses demonstrated that SF-2 expression levels in these tissues greatly surpass those of SF-1, making it by far the major NR5A subfamily receptor present in granulosa cells at all stages of luteinization. Given the proposed functional redundancy between SF-1 and SF-2, we propose that SF-2 could function as a major transcriptional regulator of steroidogenic genes in these cells. In apparent agreement with this, several discrepancies were noted between the expression of SF-1 and that of different steroidogenic genes. Most notably, a sizeable induction of StAR and P450scc was observed when their expression in the CL was compared to late-stage ovulatory follicles, despite a marked decrease in SF-1 expression. While this could be explained by mechanisms such as SF-1 protein stabilization or increased coactivator activity, SF-2 activity may also be invoked given its abundance in luteal cells.

Careful examination of the studies that have identified SF-1 as a transactivator of steroidogenic gene promoters reveals that supershift analyses were typically performed using polyclonal antisera that have yet to be shown to distinguish between SF-1 and SF-2 (158, 185, 187, 277). Our SF-2 structure and expression data therefore argue that the identity of the transcription factor that binds steroidogenic gene promoters in granulosa and luteal cells is not conclusively established, and SF-2 may well have been systematically mistaken for SF-1 in earlier studies. Demonstration of this will first require assays showing that SF-2 can also transactivate established SF-1 ovarian target genes. Subsequently, the synthesis of antibodies that can distinguish between SF-1 and SF-2 will be required in order to perform supershift analyses that will

demonstrate the true identity of the transcription factor that regulates each promoter.

In conclusion, the data presented in this thesis represent significant advancements in our understanding of the gene regulation events induced by the LH surge. Notably, they illustrate the molecular mechanisms underlying the preovulatory accumulation of prostaglandins in the follicular fluid and the changeover of the predominant ovarian hormone product from estradiol-17β to progesterone. In addition, they identify a P450arom promoter switching phenomenon in granulosa cells, thereby providing a novel inducible model for the study of this potentially crucial process. Finally, they report the expression of the NR5A subfamily orphan nuclear receptor SF-2 in ovarian cells, whose characteristics and expression patterns indicate that it may be a key regulator of ovarian steroidogenesis.

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Human Chorionic Gonadotropin Induces an Inverse Regulation of Steroidogenic Acute Regulatory Protein Messenger Ribonucleic Acid in Theca Interna and Granulosa Cells of Equine Preovulatory Follicles*

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ABSTRACT

The time- and gonadotropin-dependent regulation of steroidogenic acute regulatory protein (StAR) has not been characterized *in vivo* in preovulatory follicles of large monoovulatory species or sexually mature animals. The objectives of this study were to clone equine StAR and describe the regulation of its messenger RNA (mRNA) in equine follicles after the administration of an ovulatory dose of hCG. The screening of an equine follicle complementary DNA (cDNA) library with a mouse StAR cDNA probe revealed two forms of equine StAR that differ only in the length of their 3'-untranslated region (3'-UTR); a long form of 2918 bp and a short form of 1599 bp. The StAR long form cDNA contains a 5'-UTR of 117 bp, an open reading frame (ORF) of 855 bp, and a 3'-UTR of 1946 bp. Primer extension analysis showed that the cDNA clone lacked the first 10 bp of the primary transcript, giving a total of 127 bp for the complete StAR 5'-UTR. The ORF encodes a 285-amino acid protein that is 86–90% identical to StAR of other species characterized to date. The regulation of StAR mRNA *in vivo* was studied in equine preovulatory follicles isolated during estrus at 0, 12, 24, 30, 33, 36, and 39 h (n = 4–5 follicles/time point) after an ovulatory dose of hCG. Results from Northern blots showed no significant changes in StAR mRNA levels after hCG treatment when analyses were performed on intact follicle wall (theca interna with attached granulosa cells). However, Northern blots performed on isolated follicle cells revealed an unexpected regulation of StAR mRNA. In granulosa cells, StAR transcripts were undetectable at 0 h but were significantly increased at 30 h post-hCG, and this induction was associated with a rise in follicular fluid concentrations of progesterone ($P < 0.05$). In contrast, StAR mRNA levels were high in theca interna at 0 h, remained unchanged until 33 h post-hCG, and dropped dramatically thereafter ($P < 0.05$). Thus, this study describes the primary structure of equine StAR, documents the regulation of StAR mRNA *in vivo* in preovulatory follicles of a large monoovulatory species, and identifies a novel inverse regulation of StAR transcripts in theca interna and granulosa cells of equine follicles before ovulation. (*Endocrinology* **140**: 667–674, 1999)

THE BIOSYNTHESIS of all steroid hormones begins in mitochondria with the conversion of cholesterol to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme complex (P450scc) (1, 2). Adequate delivery of hydrophobic cholesterol to the inner mitochondrial membrane, where resides P-450scc, is a key rate-limiting step in the acute regulation of steroidogenesis (3–5). Although the mechanism of intracellular transport of cholesterol to the mitochondrion remains unresolved, its translocation from the outer to the inner mitochondrial membrane appears to involve a protein originally described by Orme-Johnson and collaborators (6, 7) and recently purified, cloned, and named steroidogenic acute regulatory protein (StAR) by Clark *et al.* (8). StAR is a phosphoprotein synthesized in the cytosol as a short-lived 37-kDa precursor that is processed into more stable 30-kDa proteins after mitochondrial import (9–11). Interestingly, the 37-kDa precursor protein is believed to represent the active form of StAR involved in moving cholesterol across mitochondrial membranes, whereas the role, if any, of the 30-kDa proteins remains unknown (12, 13). The deduced amino acid sequence of the StAR protein has been characterized in mouse (8), human (14), cow (15), rat (16–19), sheep (20), pig (21), and hamster (22).

The critical role of StAR in steroid hormone synthesis has been clearly demonstrated using various models, including a biochemically defined *in vitro* system (11), cultures of intact cells (14, 23), and a targeted gene disruption approach to generate StAR knockout mice (24). Moreover, the finding that mutations within the StAR gene are responsible in humans for congenital lipoid adrenal hyperplasia, an autosomal recessive disease in which the synthesis of all adrenal and gonadal steroid is severely impaired, further underscores the importance of the protein (25, 26).

Results from recent studies have documented the pattern of expression and regulation of StAR in ovarian cells during various physiological processes. High levels of StAR messenger RNA (mRNA) and protein were observed in corpora luteum of sheep (20), cows (15, 21, 27), rats (18, 28), humans (29, 30), and pigs (31). Luteal StAR transcripts were increased by LH and GH in hypophysectomized sheep (20) and by 17β-estradiol in rabbits (32). In contrast, regression of the

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corpus luteum is accompanied by a marked decrease in StAR expression (20, 27, 28, 30). StAR is also regulated in a gonadotropin-dependent and stage-specific manner during follicular development (17, 29, 31, 33). Experiments *in vitro* showed that gonadotropins and activators of the protein kinase A pathway up-regulate StAR expression in granulosa cells (16, 27, 29, 33–36), whereas $PGF_{2\alpha}$ and phorbol 12-myristate 13-acetate appeared to be negative regulators of StAR expression *in vivo* and *in vitro* (20, 28, 29, 33). The equine CG/hCG-treated immature rat model was used to study the control of StAR expression *in vivo* in preovulatory follicles (17, 33). However, the regulation of follicular StAR in a more physiological system using sexually mature animals has not been characterized, and in-depth studies in large monoovulatory species are lacking. Therefore, the general objective of this study was to use the equine preovulatory follicle as a model to study the cell-specific and time-dependent regulation of StAR by gonadotropins *in vivo*. The specific objectives were to clone and characterize equine StAR, describe the regulation of its mRNA in preovulatory follicles after the administration of an ovulatory dose of gonadotropins, and determine the contribution of theca interna and granulosa cells to follicular StAR expression.

Materials and Methods

Materials

Lutalyse was purchased from UpJohn (Kalamazoo, MI); hCG was obtained from The Buttler Co. (Columbus, OH); Torbugesic was purchased from Fort Dodge Laboratories, Inc. (Fort Dodge, IA); Rompun was obtained from Haver (Bayvet Division, Shawnee, KS); Dormosedan was purchased from SmithKline Beecham, Animal Health (West Chester, PA); RNAsin, Prime-a-Gene labeling system, DNA 5'-End Labeling System, and AMV reverse transcriptase were obtained from Promega Corp. (Madison, WI); Biotrans nylon membranes (0.2 μm) were purchased from ICN Pharmaceuticals, Inc. (Montreal, Canada); [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{35}S]dATP were obtained from Mandel Scientific New England Nuclear Life Science Products (Mississauga, Canada); TRIzol total RNA isolation reagent, RNA ladder (0.24–9.5 kb), synthetic oligonucleotides, and culture media were purchased from Life Technologies (Gaithersburg, MD); QuikHyb hybridization solution was obtained from Stratagene Cloning Systems (La Jolla, CA); T4 polynucleotide kinase and all sequencing reagents were purchased from Pharmacia Biotech (Baie D'Urfe, Canada); Kodak film X-Omat AR was obtained from Eastman Kodak Co. (Rochester, NY); electrophoretic reagents were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA).

Cloning and sequencing of equine StAR

To clone the equine StAR complementary DNA (cDNA), an expression library prepared with equine follicle mRNA (37) was screened with a mouse StAR cDNA (8). The probe was labeled with [α-^{32}P]deoxy-CTP using the Prime-a-Gene labeling system (Promega Corp.) to a final specific activity greater than 1×10^9 cpm/μg DNA. Approximately 100,000 phage plaques were screened, and hybridization was performed at 55 C with QuikHyb hybridization solution (Stratagene). Positive clones were plaque purified through secondary and tertiary screening, and pBluescript phagemids containing the cloned DNA insert were excised *in vivo* with the Ex-Assist/SOLR system (Stratagene). DNA sequencing was performed by the Sanger dideoxynucleotide chain termination method (38) using the T7 sequencing kit (Pharmacia Biotech) and vector-based primers (T3 and T7), and specific primers synthesized as internal StAR sequences were obtained. Nucleotide and amino acid analyses were performed using the FASTA program of Wisconsin Package version 9.0 (Genetics Computer Group, Madison, WI) and MacDNASIS software version 2.0 (Hitachi, Hialeah, FL).

Primer extension analysis

Primer extension analysis was performed in aqueous buffer as previously described (37, 39). The reaction used total RNA extracted with TRIzol (Life Technologies) from a corpus luteum isolated on day 8 of the estrous cycle and from spleen (negative control), and a 30-mer antisense oligonucleotide 5'-GGCTCCGAGGCAGTGCTGGAGGAG-3' corresponding to 46–75 bp of the longest StAR cDNA clone (Fig. 1). The extension product was analyzed by electrophoresis on a 6% polyacrylamide-7 M urea gel, and its size was determined by comparison with the products of an unrelated sequencing reaction run in adjacent lanes.

Isolation and dissection of equine preovulatory follicles

Standardbred and Thoroughbred mares were teased daily with a pony stallion for detection of estrus, and ovarian follicular development was monitored daily by transrectal real-time ultrasonography (40). Ovulation was induced during estrus with hCG (2500 IU, iv) when the preovulatory follicle reached 35 mm in diameter. Ovariectomy was performed via colpotomy 0, 12, 24, 30, 33, 36, and 39 h post-hCG with a chain ecraseur (n = 4–6 follicles/time point) (41). During the procedure, neuroleptanalgesia was induced with a combination of xylazine (Rompun; 0.65 mg/kg, iv), butorphanol (Torbugesic; 0.005 mg/kg, iv), and detomidine (Dormosedan; 0.02 mg/kg, iv), as described previously (42). The recovered ovary was kept in ice-cold Eagles's MEM supplemented with penicillin (50 U/ml)-streptomycin (50 μg/ml; Life Technologies), L-glutamine (2.0 mM; Life Technologies), and nonessential amino acids (0.1 mM; Life Technologies). Each preovulatory follicle was dissected into three cellular preparations, as previously described (42, 43). They included pieces of follicle wall (theca interna with attached granulosa cells) and isolated preparations of theca interna and granulosa cells. All samples were stored at −70 C until RNA extraction. Animal procedures were approved by the animal research committee of the University of Montreal.

RNA extraction and Northern blot analysis

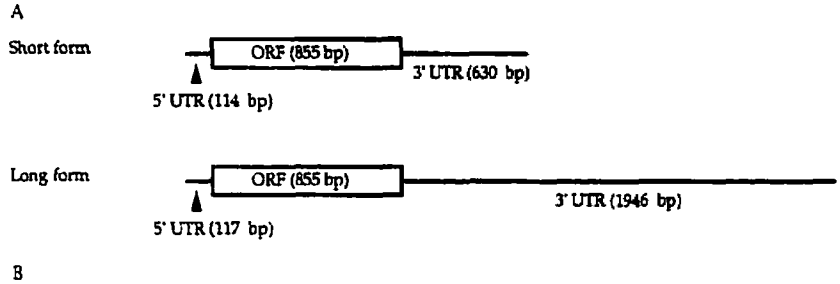
Total RNA was extracted with TRIzol (Life Technologies) using a Kinematica PT 1200C Polytron homogenizer (Fisher Scientific International, Inc., Pittsburgh, PA) from equine tissues. For Northern analysis, RNA samples (10 μg) were denatured at 55 C for 15 min in 50% deionized formamide-6% formaldehyde, electrophoresed in a 1% formaldehyde-agarose gel, and transferred onto a nylon membrane as previously described (37). A ladder of RNA standards was run with each gel, and ethidium bromide (10 μg) was added to each sample before electrophoresis to compare RNA loading and determine the migration of standards. The membrane was first hybridized to the ^{32}P-labeled equine StAR cDNA probe using QuikHyb solution (Stratagene, La Jolla, CA). After stripping the radioactivity with 0.1% SSC-0.1% SDS for 30 min at 100 C, the same blot was subsequently hybridized with a rat elongation factor Tu (EFTu) cDNA as a control gene for RNA loading and transfer (44).

Progesterone RIA

Nonextracted aliquots of follicular fluid were assayed for progesterone by a specific RIA (45). The sensitivity of the assay was 7.29 pg/assay tube, and the intra- and interassay coefficients of variations were 11.4% and 18.6%, respectively.

Statistical analysis

One-way ANOVA was used to test the effect of time after hCG administration on relative StAR mRNA levels and concentrations of progesterone in follicular fluids. When ANOVAs indicated significant differences ($P < 0.05$), Dunnett's test was used for multiple comparisons with the control (0 h post-hCG). Data were transformed to logarithms before analysis when heterogeneity of variance was observed with the Hartley test. Statistical analyses were performed using software from SAS Institute, Inc. (Cary, NC). Relative levels of StAR mRNA were quantified by determining the optical density of the StAR band on autoradiograms with a computer-assisted image analysis system (Collage Macintosh program, Fotodyne, Inc., New Berlin, WI). The EFTu signal was also quantified and used to normalize results. For each



B

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1     ggagccagag gcagatcctg acaggctcag gaggtctgga agaagcttcc cttgagagta ggagcaatag cagcagcagc aacagcactg ctacatttgc
101   acccgcggct ggaaacgATG CTCCTCGCGA CGTTTAAGCT GTGCGCTGGG AGCTCCTACA GACATGTGCG CAATATGAAG GGGCTGAGGC ACCAAGCTGC
201   CTTGGCCATT GGCCAGGAGC TGAACTGGCG GGCACCTGGG GGCCCGACCC AGAGTGGGTG GATCAACCAG GTTCGGCGTC AGAGCTCTCT GCTTGGCTCT
301   CAGCTAGAAG ACACTCTCTA CAGCGACCAG GAGCTGGCCT ATATCCAGCA GGGAGAGGAG GCAATGCAGA AGGCCCTGGG CATCCTCCGC AACCAGGAGG
401   GCTGGAAGGA GGAGAACCAG CAGGCAAACG GGGACAAAGT GCTGAGTAAA GTGGTCCCAG ACGTGGGCAA GGTATTCCGG CTGGAGGTGG AGGTGGACCA
501   GCCCATGGAG AGGCTTTATG AAGAGCTTGT GGAACGCATG GAGGCCATGG GAGAGTGGAA CCCAAATGTC AACGAGATCA AGGTCCTGCA GAAGATTGGA
601   AAAGATACCG TCATCACCCA TGAGTTGGCT GCAGAATCAG CAGGAAACCT TGTGGGGCCC CGAGACTTTG TGAGTGTGCG TTGTGCCAAG CGCCGAGGCT
701   CCACCTGTGT GTTGGCTGGC ATGGCCACAC AGTTTGAGGA GATGCCCGAG CAGAAAGGTG TCATCAGAGC TGAGCATGGT CCCACTTGTA TGGTGCTCCA
801   TCCCCTGGCT GGAAGTCCCT CAAAGACCAA ACTCACTTGG CTGCTCAGCA TTGACCTCAA GGGATGGCTG CCAAAGACTA TCATCAACCA GGTCCTTTCG
901   CAGACCCAGG TGGATTTTGC CAACCATCTG CGCAAGCGCC TGGAGTCCAG CCCTGCTCCT GAAGCCAGGT GTTAAAgacc aacttgctgc tcccaactgt
1001  tcctagctgc attggcttgc atgctcatca ggaaaatccc tgccagaagc ctcaacattt gtttaagatc tttatctgag gatagtgtga tgggtggtga
1101  gtatgtttag agtatggtaa taggacttag actggtaaaa ttttactacc aagaaaatgg ggacaggact cttagaagaa acttctagct tctttcactga
1201  ttagctacga aatgaaggtt aagggtccca aaatatttgt aaaacttttc cctgggtcta atgtctacct gaaaacatct taaaaatgct actggctgat
1301  acgggggtgca gaggtgctaa atacaaggac tgggacctca tgctttacgg gctcaagagc tccattctct gcaggcagtg tgtatggaca ttcagggtct
1401  tacaaagggg ctccgggaac cctcccctcc tacaccaagt aggtagagag ctgctctcact ggacaagcaa ctcccacag caggtgcctg ctaaaagcct
1501  agtccaagtt ttcttgatga aaaagtacaa aactaattat tagactgctt cccttacttc ttagacgagc aagtcagaat aaagaatcat aactaacaga
1601  caaacttcag cctgtgcatg ttttaaaatt ctgctcttag aaaaaaaaaa tcttagtaaat tgcaactgca tactaaagga gtcctggaca aagatttaa
1701  ttaaactaaa tcccctagct cattaaaagg aaaaactact gggtagttac gtcattctgac tagaatcagt taccagaatc tttcataaaa atttaatttc
1801  cacgatatgc acaagccttc atcagttctt aaacttctaca actggtaaca tttcaagatg aagattatga ctgaggatct ttatttacct aattaaaagg
1901  aaggacagta ccaaaatatga gagaacgaac ggcattacta agattaaaac aaattttact ttcccgtagt gattgtttag acctaaaaat ggaactgaat
2001  ccttagttca gatcttatta tatcagcttc cagcttggct tgggctgcca ctttatctgc ttagtaggtc agtgtttttac tatttgagg agatggctgg
2101  aagaaggggt aatatgggtgt tagcaaatcc cttttcaaga ttcagaggct ccttgccttg aaaatataaa tcgggatcgg attattttgt taaaatgtgc
2201  aagtaggatg aaaaaccaac attaccactt gaagctttte cattttagtt ctaagagcat tatggaaaac atttgtgaaac atagctctag cttattaaac
2301  aaattagcag tagtgggaat gctcatgttt actgaggtat tgaaacttta caattggtag aggtattatt atttattctg attgtttgtt agaatagtct
2401  attttcggca tagatcaaac tgtttatctt tccaggagcc tcagtgtgag agagagagct gtagaggtgt ttctagaaca gaattaccat ccactcccag
2501  ctaagaaaca tgytcacttg tttgtagatg ggtaaagcaa tacatactct ggttttcaaaa gtaaagaaat ggtcttttca cacacaccat gyttttgagc
2601  tcaattataa tttgcctttct tcaaactacg cactgtgtct ttgggtgtac tggagagata ttttcccact tgtttaattt catattggaa taaaatcagt
2701  tgtcactgat gatctgttta aagaaaccat tttatacagg actgtatggg gcgtggatat tctttgagcc cttcaaatga actcctcaaa agacagacac
2801  ttcgaatacg aagtataaag aggcaaaate attattcctg taacctctaa tttatgaaag ccaaatgtac aaaaacctaa ttttatcatt caaattaaat
2901  gagtgtacaa gttaaaaa
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FIG. 1. Primary structure of equine StAR cDNA. A, Schematic representation of two forms of equine StAR; the short and the long form differ in the lengths of their 3'-UTR. B, Complete nucleotide sequence of the equine StAR long form obtained from clone 1-2 as described in *Materials and Methods*. The ORF is indicated by *uppercase letters*, the translation initiation (ATG) and stop (TAA) codons are highlighted in *bold*, the 5'-UTR and 3'-UTR are shown in *lowercase letters*, and numbers on the *left* refer to the first nucleotide on that line. The first (c) and the last (c) nucleotide of the equine StAR short form cDNA are *underlined* and in *boldface*. Nucleotide sequences were submitted to GenBank (accession no. AF031696 and AF031697).

cellular preparation, data were expressed as ratios of StAR mRNA to EFTu and are presented as the mean ± SEM (n = 4 follicles/time point).

Results

Characterization of the equine StAR cDNA

Twelve positive clones were isolated from an equine follicle cDNA library after an initial screening of approximately 100,000 phage plaques. DNA sequencing analyses revealed that the clones represent two forms of equine StAR, a short form composed of 1599 bp (clone 10-1) and a long form of 2918 bp (clone 1-2). The short and long clones had 5'-untranslated regions (5'-UTR) of 114 and 117 bp, respectively, and a common open reading frame (ORF) of 855 bp (Fig. 1). However, they differed in the lengths of their 3'-UTR, corresponding to 630 and 1946 bp in the short and long forms, respectively (Fig. 1).

The coding region of equine StAR cDNA encodes a 285-amino acid protein, which is identical in length to human (14), pig (21), and bovine StAR (15, 21), but is one amino acid longer than those of the mouse (8), rat (17–19), and hamster (22) proteins (Fig. 2). Comparison across species indicates that the amino acid sequence of equine StAR is highly similar to that of other mammalian homologs, being 90%, 89%, 88%, 87%, 87%, 86%, and 88% identical to the human, porcine, bovine, murine, rat, hamster, and ovine StAR. Computer analysis of the StAR protein sequence using Prosite PC/Gene (Oxford Molecular Group, Inc., Oxford, UK) identified several potential phosphorylation sites, including two cAMP- and cGMP-dependent protein kinase (Ser^{56} and Ser^{195}), three protein kinase C (Thr^{5}, Ser^{13}, Ser^{186}), and four casein kinase II (Ser^{61}, Ser^{69}, Thr^{204}, Thr^{263}) phosphorylation sites. Also, a putative mitochondrial transit peptide was predicted in positions 1–55.

Length of the StAR 5'-UTR

Primer extension analysis was used to determine the full length of the StAR 5'-UTR. One major extension product was produced with RNA extracted from a corpus luteum, whereas none was generated when negative control spleen RNA was used (Fig. 3). The size of the extension product, as determined by comparisons with an unrelated sequencing reaction run in adjacent lanes, was 85 nucleotides. Therefore, our longest StAR cDNA clone (clone 1-2) appears to lack 10 nucleotides of the primary transcript, suggesting a full-length 5'-UTR of 127 nucleotides.

```
                B       B
equ  MLLATPKLCA GSSYRHVRNM KGLRHQAALA IGQELMWRAP GAPTQSGMIM   50
hum  .......... ......M... ....Q..... .S.......L ....P.T...   50
pig  .......... .......... .......... L.........L ....SGS...   50
bov  .......... .......S.. ...QQ..... ..........L ...APAA...   50
mou  .F........ ......M... .......... ......W..L .DSSPG-.MG   49
rat  .F........ ......M... .......V.. ......R..L .D.SPG-.MG   49
ham  .......... ......L... .......... ..........L .DSSPG-..S   49
ovi

               A     C     C
equ  QVRRQSSLLG SQLEDTLYSD QELAYIQQGE KAMQKALGIL KNQEGMKEDN   100
hum  ....R..... .R..E..... .....L.... .......... S......K.S   100
pig  ....R..... ......F... .D........ ....R..D.. S......K.S   100
bov  ....RG.... .....P.... ....H..... ....R..... KD.....K.S   100
mou  ....R..... ....A..... ...S...... V......... N......K.S   99
rat  ....R..... ....A..... ...S...... .......... N......K.S   99
ham  ....R..... ....AA...E ...S...... V......... S......K..   99
ovi                                                .R..

equ  QQAHGDKVLS KVVPDVGKVF RLEVEVDQPK ERLYEELVER KEAMGEMDFN   150
hum  ..D.....M. .......... ....V..... .......... ..........   150
pig  R.E...E... ..I....... ....V..... .......... .........S   150
bov  R.....E... ..I....... ....V..... .......... ..........   150
mou  ..E...E... .M........ ....V..... D.......D. ..........   149
rat  ..E...E... ....G..... ...LVL.... D.......D. ..........   149
ham  ..E...E... .......... ....V..... D...A...D. ..........   149
ovi  R.....E... ..I....... ....V..... .......... .........S
                                                 B       A
equ  VKEIKVLQKI GKDTVITHEL AAESAGNLVG PRDFVSVRCA KRRGSTCVLA   200
hum  .......... ....F..... ...A...... .......... ..........   200
pig  .....I.... .......... ...A...... .......G.T .....V....   200
bov  .......... .......... ...V...... .........T .....M....   200
mou  ........R. .......... ..AA...... .........T ..........   199
rat  .......K.. .......... R.AA...... .........T ..........   199
ham  ........K. .......... ..AA...... .......... ..........   199
ovi  .......... ....I..... ...A...... .....R...T .....M....
          C
equ  GMATQFEEMP EQKGVIRAEH GPTCKVLHPL AGSPSKTKLT WLLSIDLKGM   250
hum  ....D.GN.. .......... .......... .......... ..........   250
pig  ....D.G... .......... .......... .......... ..........   250
bov  ....LY.... Q......... .......... .......... ..........   250
mou  ....H.G... ..S....... .......... .......... ..........   249
rat  ....H.G... ..S....... .......... .......... ..........   249
ham  ....H.G... ..S......Q .......... ........F. ..........   249
ovi  .T.TLY.... Q.......

                  C
equ  LPRTIINQVL SQTQVDPANH LRKRLESSPA PEARC    285
hum  ...S...... .......... .......H.. S....    285
pig  .......... .......... .......R.. L....    285
bov  .......... .......... .......C.. L....    285
mou  .......... ....IE.... ......A... S..Q.    284
rat  .......... ....IE..S. .......... S..Q.    284
ham  ...S...... ....ME.... .......... S....    284
ovi
```

FIG. 2. Deduced amino acid sequence of equine (equ) StAR and comparison with human (hum), pig, bovine (bov), mouse (mou), rat, hamster (ham), and ovine (ovi) homologs. Ovine StAR has not been fully characterized, and only a partial sequence is shown. Identical residues are indicated by a *printed period*. Potential phosphorylation sites for cAMP- and cGMP-dependent protein kinase (A), protein kinase C (B), and casein kinase II (C) are highlighted in ***bold uppercase letters***.

Inverse regulation of StAR mRNA in granulosa cells and theca interna

To study potential changes in StAR mRNA levels during equine follicular luteinization, preovulatory follicles were isolated between 0–39 h post-hCG and Northern blot analyses were performed on RNA extracted from intact follicle wall (theca interna with attached granulosa cells). StAR transcripts of approximately 3.0 kb were detected in walls of follicles isolated at 0 h, and administration of hCG had no significant effect on relative transcript levels (Fig. 4A). However, higher levels of StAR mRNA were observed in two corpora lutea obtained on day 8 of the estrous cycle (Fig. 4A).

To determine the relative contribution of each steroidogenic cell type in follicular StAR mRNA expression, Northern blots were prepared from isolated preparations of granulosa cells and theca interna. Results revealed an unexpected reciprocal regulation of StAR transcripts by hCG in each cell

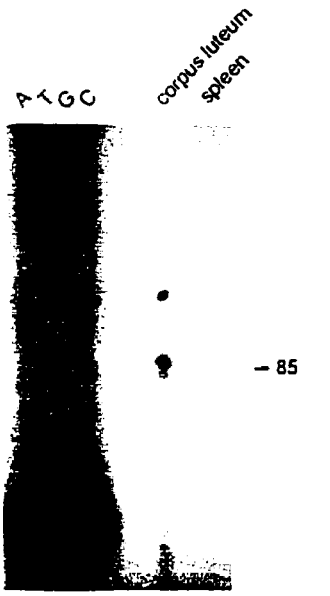

FIG. 3. Primer extension analysis of equine StAR mRNA. A labeled 30-mer antisense oligonucleotide complementary to the region from 46–75 bp of the StAR long form cDNA (Fig. 1) was hybridized to RNA samples containing (corpus luteum) and not containing (spleen) StAR, and primer extension was performed as described in *Materials and Methods*. Reactions were analyzed on a 6% polyacrylamide gel, and the size of the extended product was determined by comparison with the products of an unrelated sequencing reaction shown on the *left*. The results show a 85-nucleotide extension product corresponding to a major transcription initiation site. No extension product was detected with RNA isolated from spleen (negative control).

type. In granulosa cells, StAR was undetectable or present at very low levels between 0–12 h post-hCG, but the transcript increased between 24–39 h (Fig. 5A). In contrast, levels of StAR mRNA in theca interna were high before hCG injection (0 h), remained relatively constant until 33 h post-hCG, and then dropped dramatically at 36 and 39 h post-hCG (Fig. 6A). Uniform RNA loading in all Northern blots was confirmed by hybridization with a cDNA encoding the rat elongation factor Tu (Figs. 4B, 5B, and 6B).

To provide a quantitative estimate of the relative changes in StAR mRNA during the gonadotropin-induced luteinization process, Northern blots were prepared from follicle wall, theca interna, and granulosa cells of four separate preovulatory follicles isolated at each time point between 0–39 h post-hCG, and results were quantified by densitometric analyses. No significant changes were observed in the relative levels of StAR transcripts in follicle wall (Fig. 7A). However, this apparent constant expression of follicular StAR concealed a significant increase in message levels in the granulosa cell layer, first detected at 30 h post-hCG (Fig. 7B), and a significant drop in StAR transcripts in the theca interna layer at 36 and 39 h post-hCG (Fig. 7C).

Follicular fluid concentrations of progesterone

Concentrations of progesterone were measured in follicular fluid of preovulatory follicles isolated between 0–39 h post-hCG to assess whether the regulation of StAR mRNA in

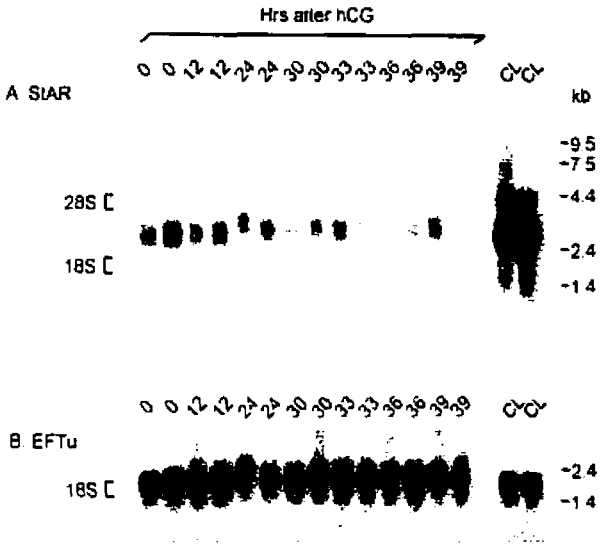

FIG. 4. Regulation of StAR mRNA by hCG in equine preovulatory follicles. Preparations of follicle wall (theca interna with attached granulosa cells) were obtained from preovulatory follicles isolated 0, 12, 24, 30, 33, 36, and 39 h after hCG treatment, as described in *Materials and Methods*. In addition, two corpora lutea (CL) were isolated on day 8 of the estrous cycle. Samples of total RNA (10 μg/lane; two follicles per time point) were analyzed by Northern blotting using a ^{32}P-labeled equine StAR cDNA probe (A). The same blot was stripped of radioactivity and hybridized with a cDNA encoding the rat EFTu as a control gene for RNA loading (B). *Brackets* on the *left* show migration of 28S and 18S ribosomal bands, and markers on the *right* indicate the migration of RNA standards. Filters in A and B were exposed to film at −70 C for 4 and 2 h, respectively.

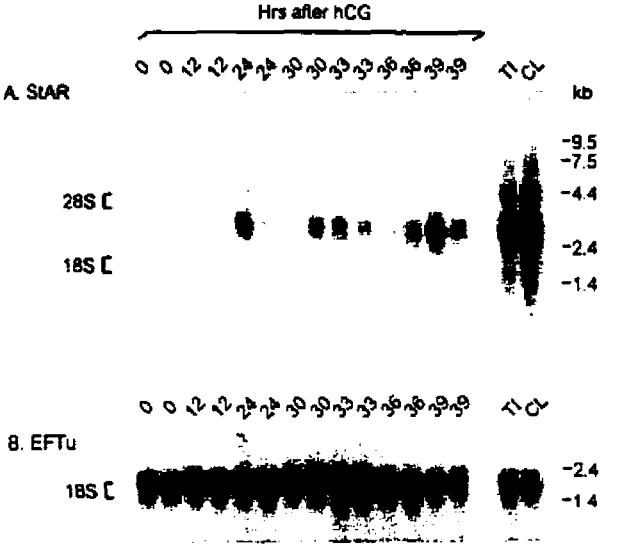

FIG. 5. Regulation of StAR mRNA by hCG in granulosa cells of equine preovulatory follicles. Isolated preparations of granulosa cells were obtained from equine preovulatory follicles isolated between 0–39 h after hCG treatment, as described in *Materials and Methods*. In addition, preparations of theca interna (TI; 0 h) and corpus luteum (CL; day 8 of cycle) were isolated. Samples of total RNA (10 μg/lane; n = 2 follicles/time) were analyzed by Northern blotting using a ^{32}P-labeled equine StAR cDNA probe (A). The same blots were stripped of radioactivity and hybridized with a cDNA encoding the rat EFTu as a control gene for RNA loading (B). *Brackets* on the *left* show the migration of 28S and 18S ribosomal bands, and markers on the *right* indicate the migration of RNA standards. Filters in A and B were exposed to film at −70 C for 6 and 2 h, respectively.

granulosa and theca interna related to changes in progesterone biosynthesis. Progesterone levels were relatively low in follicles isolated before gonadotropin treatment (0 h post-hCG; 53.6 ± 21.1 ng/ml) and remained unchanged at 12 and 24 h (Fig. 8). However, follicular fluid concentrations of progesterone were significantly increased at 30 h and reached maximal levels at 39 h post-hCG (783.0 ± 246.1 ng/ml).

Discussion

Previous reports have used the equine CG/hCG-primed immature rat model to study the regulation of StAR in follicles *in vivo* (17, 33) and cultures of granulosa cells to investigate *in vitro* some of the molecular mechanisms of StAR expression (16, 27, 29, 33–36, 46). This study is the first to document the precise time-course and cell-specific regulation of StAR mRNA *in vivo* in preovulatory follicles of a large monoovulatory species and sexually mature animals. The mare was selected as an animal model for several reasons: the preovulatory follicle reaches a relatively large size (40–45 mm in diameter), its development can be precisely monitored *in vivo* by ultrasound imaging, and the process of equine follicular recruitment, selection, and dominance is similar to that of other species (40, 47). Results provide evidence for a novel inverse regulation of StAR transcripts in theca interna and granulosa cells of equine preovulatory follicles in response to an ovulatory dose of hCG. Also, the study reports for the first time the cloning of equine StAR from which the complete primary structure of the StAR protein is deduced.

Comparative analyses underscore the highly conserved nature of StAR across species, with the amino acid sequence of the equine protein being more than 86% identical to that of other species (12, 13). However, although the equine protein appears to contain a putative mitochondrial transit peptide within the first 55 amino acids, it does not have the consensus motif for mitochondrial two-step cleavage identified in mouse StAR (8). This divergence from the murine sequence is also observed in human (14), bovine (15), and porcine StAR (21) and argues against a critical role for this region in StAR action. Indeed, mounting evidence in the mouse clearly shows that the steroidogenic action of StAR instead involves C-terminal domains (48, 49). The activity of the protein appears to lie outside of the mitochondria, and mitochondrial import is not required for StAR action (48, 49). Computer analysis of the equine StAR amino acid sequence also revealed several potential phosphorylation sites that could modulate the activity of the protein. Although the functional role of each site remains to be established, results indicate that the potential phosphorylation site located at serine 195, shown in human and mouse to regulate StAR activity (50), is conserved in the equine protein.

Northern blot analyses revealed the presence of one major StAR transcript of about 3.0 kb in follicular extracts and a minor band of about 1.8 kb in a few samples. The finding of multiple equine StAR transcripts is in agreement with the

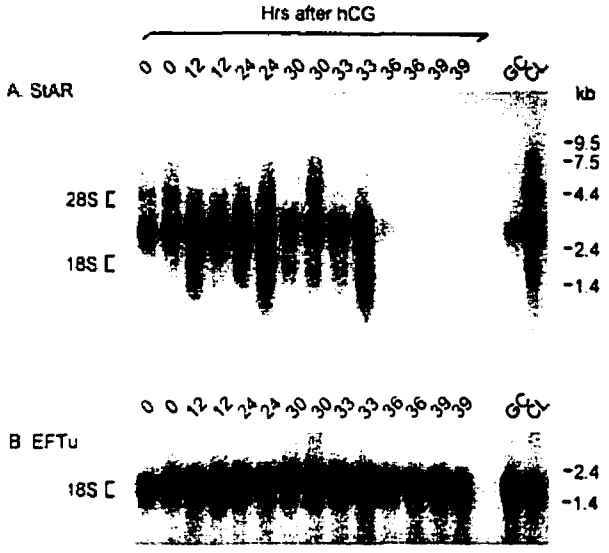

FIG. 6. Regulation of StAR mRNA by hCG in theca interna of equine preovulatory follicles. Isolated preparations of theca interna were obtained from equine preovulatory follicles isolated between 0–39 h after hCG treatment, as described in *Materials and Methods*. In addition, samples of granulosa cells (GC; 39 h) and corpus luteum (CL; day 8 of cycle) were isolated. Samples of total RNA (10 μg/lane; n = 2 follicles/time) were analyzed by Northern blotting using a ^{32}P-labeled equine StAR cDNA probe (A). The same blots were stripped of radioactivity and hybridized with a cDNA encoding the rat EFTu as a control gene for RNA loading (B). *Brackets* on the *left* show migration of 28S and 18S ribosomal bands, and markers on the *right* indicate migration of RNA standards. Filters in A and B were exposed to film at −70 C for 4 and 2 h, respectively.

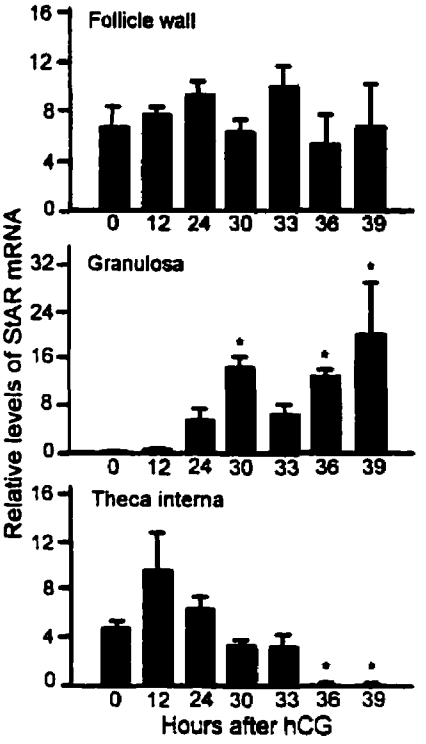

FIG. 7. Relative changes in StAR mRNA levels in equine follicle cells isolated between 0–39 h after hCG treatment. Samples (n = 10 μg) of total RNA extracted from follicle wall (A), granulosa cells (B), and theca interna (C) were analyzed by Northern blotting with the equine StAR cDNA and subsequently with the rat EFTu cDNA as a control gene for RNA loading. After autoradiography (films not shown), the StAR signal intensity was quantified by densitometric analysis and normalized with the control gene EFTu. Results are presented as StAR mRNA levels relative to EFTu (mean ± SEM; n = 4 follicles/time point). *Columns marked with an asterisk* are significantly different ($P < 0.05$) from 0 h post-hCG.

results in other species. Three mRNAs have been observed in mice and rats, including two major bands of 3.4 and 1.6 kb (17, 51). One major transcript of 1.6 kb and two minor mRNAs of 4.4 and 7.5 kb have been reported in human tissues (14, 52), two transcripts of 3.0 ad 1.8 kb were detected in bovine tissues (15, 21, 34, 52), and up to three transcripts have been reported in the pig (21, 34). Only one StAR mRNA of 2.8 kb has been observed in sheep (20). Our cloning results suggest that differences in the lengths of transcripts are attributable to variations in the 3'-UTR, as the short form equine StAR measuring 1.6 kb appeared derived from an internal polyadenylation signal (5'-AATAAA-3') located 22 bp from the end of the clone. However, the functional significance, if any, of multiple StAR transcripts remains unknown.

One key finding of the present study is the reciprocal regulation by gonadotropins of StAR transcripts in theca interna and granulosa cells of equine preovulatory follicles. This result clearly highlights the importance of defining the contribution of each steroidogenic cell type, as Northern blots from whole follicular wall extracts could have erroneously lead to the conclusion that hCG had no effect on StAR expression in equine follicles. The pattern of induction of StAR mRNA in equine granulosa cells compares with that observed in the immature rat model after hCG administration *in vivo* (17, 33) and with the ability of agonists of the protein kinase A pathway to up-regulate StAR expression in granulosa cells *in vitro* (16, 27, 29, 33–36). Also, absence of the transcript in equine granulosa cells isolated before gonadotropin treatment is consistent with results obtained in cattle (53). Interestingly, the increase in steady state levels of StAR mRNA caused by hCG paralleled a significant rise in follicular fluid concentrations of progesterone, suggesting a link between StAR expression in granulosa cells and the onset of follicular luteinization *in vivo*. A more precise understanding of its relative role in the equine follicle should result from further studies on the characterization and gonadotropin regulation of key enzymes involved in equine follicular steroidogenesis. However, a relationship between StAR and steroid hormone production has been clearly established in various systems (11, 14, 23).

In contrast to granulosa cells, high levels of StAR mRNA were observed in theca interna of equine preovulatory follicles isolated before hCG treatment. This observation is not surprising considering the hypertrophied and highly steroidogenic appearance of the theca interna layer in equine preovulatory follicles isolated during early estrus, as characterized under light microscopy (54). Also, elevated levels of StAR transcripts have been reported in theca interna of large follicles in rats (17, 33), cows (53), and humans (29, 30).

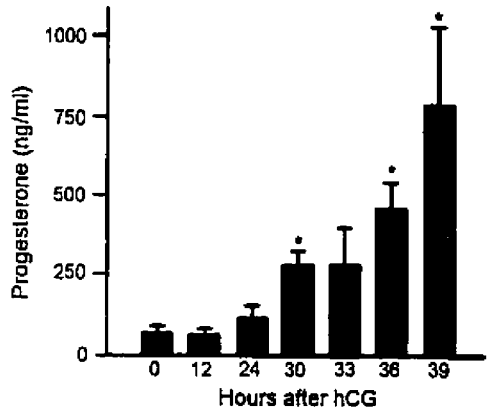

FIG. 8. Follicular fluid concentrations of progesterone in equine preovulatory follicles. Preovulatory follicles were isolated between 0–39 h after hCG treatment, and follicular fluid concentrations of progesterone were determined by specific RIAs. Results are shown as the mean ± SEM (n = 5 follicles/time point, 0–30 and 36 h post-hCG; n = 6 follicles, 33 h post-hCG; n = 3 follicles, 39 h post-hCG). *Columns marked with an asterisk* are significantly different ($P < 0.05$) from 0 h post-hCG.

However, whereas StAR mRNA remained relatively constant until 33 h post-hCG, a dramatic loss in the transcript occurred thereafter in theca interna. This loss is cell type specific, as a concomitant increase in StAR was observed in the neighboring granulosa cell layer. To our knowledge, this is the first time that such a reciprocal regulation of StAR mRNA was simultaneously observed in distinct cellular compartments of ovarian follicles or any other steroidogenic tissue. The loss of StAR transcript in equine theca interna occurred 6–9 h before the expected time of ovulation (37). Although the biological significance of the loss of StAR transcript remains to be precisely established, we believe that it could represent the first biochemical consequence of a putative degenerative process in theca interna of equine follicles just before ovulation (54, 55). Van Niekerk *et al.* (54) reported that, in contrast to other species, the theca interna degenerates at the time of ovulation in mares, and therefore does not contribute to the formation of the corpus luteum. Interestingly, the abrupt disappearance of StAR between 33–36 h post-hCG could suggest a timing for the onset of the degenerative process, thereby providing a paradigm to study its molecular regulation.

In summary, this study describes the primary structure of equine StAR and reports the cloning of two transcripts that differ primarily in the length of the 3′-UTR. The equine protein is composed of 285 amino acids, and its sequence is highly homologous to that of other species. The gonadotropin-dependent and cell-specific regulation of StAR mRNA *in vivo* was studied in a series of preovulatory follicles isolated before and after hCG treatment. The results revealed a unique inverse regulation of StAR mRNA in equine follicular cells, with hCG causing an induction of StAR transcripts in granulosa cells and the disappearance of the message in theca interna. Although these changes are believed to relate to the luteinization of granulosa cells and a putative degeneration of theca interna before ovulation in mares, future studies are needed to better understand the precise role of StAR during equine follicular steroidogenesis.

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