THE UNIVERSITY OF CALGARY

Effects of Cyclin A2 Non-Coding Regions on Reporter Gene Translation During Early Development in *Xenopus laevis*

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCE

CALGARY, ALBERTA

JANUARY, 1997

Abstract

A reporter gene system was employed to analyze the effects of the *Xenopus* cyclin A2 non-coding regions on expression during early development. On their own, the 5' and 3' UTRs (untranslated regions) were able to inhibit reporter translation during late oogenesis and at maturation. At the time of fertilization the individual non-coding regions were unable to inhibit reporter translation until approximately the time of the mid-blastula translation. Transcripts containing the 3' UTR were polyadenylated after fertilization and at the mid-blastula transition. When both non-coding regions flanked a CAT reporter gene, translation was repressed at all stages of development examined. From these data, the 5' and 3' UTRs interact synergistically to prevent translation during early development. Although a poly(A) tail was evident after fertilization, it did not appear to play a role in translation regulation.

Acknowledgments

I thank Dr. Manfred J. Lohka for providing the idea around which this project was built. I would also like to thank Tim Hunt and Mike Powell (ICRF) for providing the cyclin constructs and sequence used in these experiments and I thank Dr. Leon Browder for his guidance and support and for his help in preparing this manuscript. Additionally I would like to acknowledge Sherri Fraser, Mike Carpenter, and my committee members for their helpful suggestions and discussions.

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Abbreviations

I. Units of Measure

bp	base pairs
cm	centimeter
cpm	counts per minute
h	hour
kb	kilobase
kDa	kilodalton
min	minute
nm	nanometer
nt	nucleotide
rpm	revolutions per minute
sec	seconds
S	Svedberg unit (sedimentation coeffecient)
µCi	microcurie
v/v	volume per volume
w/v	weight per volume
x g	gravitational force (where g=9.81 meters/second^2)

II. Reagents

ACA	acetyl co-enzyme A
ATP	adenosine-5'-triphosphate
CTP	cytidine-5'-triphosphate
GTP	guanosine-5'-triphosphate
UTP	uridine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dUTP	2'-deoxyuridine-5'-triphosphate
BSA	bovine serum albumin
$CHCl_3$	chloroform
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
ddH_20	distilled deionized water
EtBr	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
PIPES	1,4-piperazinediethanesulfonic acid
SDS	sodium dodecyl sulfate
TAE	tris acetate/EDTA buffer
TBE	tris borate/EDTA buffer
TEMED	N',N',N',N'-tetramethylethylenediamine
Tris-HCl	tris (hydroxymethyl) aminoethane hydrochloride

III. Enzymes

Rnase	ribonuclease
Dnase	deoxyribonuclease
Sp6 pol	Sp6 RNA polymerase

IV. Other Abbreviations

A260	optical absorbance at 260 nm wavelength
ds	double stranded
ss	single stranded
CaMV	Cauliflower Mosaic Virus
CPE	cytoplasmic polyadenylation element
eIF	eukaryotic initiation factor
EGT	early gastrula transition
IRES	internal ribosome entry site
mRNA	messenger ribonucleic acid
MBT	mid-blastula transition
M.W.	molecular weight
ORF	open reading frame
uORF	upstream open reading frame
RNP	ribonucleoprotein
rpmRNA	ribosomal protein mRNA
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
UV	ultraviolet

INTRODUCTION

1. Overview

Regulation of gene expression can occur at multiple levels: transcription, messenger ribonucleic acid (mRNA) processing and stability, translation, and protein modification and turnover. In most cells, gene expression is regulated predominantly at the level of transcription. However, many biological processes, including proliferation, differentiation and development, are also regulated by modulation of translation rates. Gene regulation at the level of protein synthesis allows cells to respond rapidly to changes in physiological conditions, because activation or repression of mRNAs can occur essentially instantaneously.

During initial development of animal embryos there may be no nuclear gene transcription, and the transfer of genetic information to protein is controlled exclusively at the level of translation (Richter, 1991; Newport and Kirschner, 1982; Gelfand and Smith, 1983). Translational regulation is particularly evident during oocyte maturation and early embryonic development in the frog, *Xenopus laevis*. Frog oocytes contain levels of polyadenylated [poly(A)$^+$] mRNA that far exceed the cell's immediate protein synthesis requirements. In *Xenopus* oocytes as much as 90 per cent of the total poly(A)$^+$ mRNA is non-polysomal, the majority of which is stored as non-translated ribonucleoproteins (RNPs). Translational recruitment of these non-translated pools of masked mRNAs enables rapid gene expression during early embryonic development in the frog and a variety of vertebrate and invertebrate species (Davidson, 1986) and establishes several developmental programs, including the early cell-cycle divisions and germ layer formation (Richter, 1991). In contrast, other mRNAs that are translated in the oocyte are released from polysomes after fertilization.

In *Xenopus*, the first 12 cell cycles of embryogenesis occur in a rapid and synchronous manner (approx. 25 min. per cell cycle) (Newport and Kirschner, 1984). Cell division becomes asynchronous, and the length of the cell-cycle gradually increases after the twelfth cell cleavage (the mid-blastula transition; MBT). The MBT is characterized by an increase in the rate of

zygotic transcription (approximately 200-fold from background), initiation of cell motility and the synthesis of new proteins and activities not present in the early embryo (Newport and Kirschner, 1982). Therefore, critical steps in initial frog embryogenesis depend on the proper temporal and spatial control of maternal messengers. Because all protein synthesis during this early period of development is under translational control, it provides a useful system for the study of the translational regulation of specific messengers.

2. Translation

Translation is an energy-requiring process, in which free amino acids are polymerized in a genetically determined manner to form polypeptides. It requires the presence of messenger RNA, ribosomes, transfer RNA (tRNA), certain cations, amino acids and a number of enzymes and protein factors. In addition, ATP and GTP are required as sources of energy. In eukaryotic cells, the translation of mRNA occurs in four distinct phases: activation of amino acids, initiation, elongation and termination. Each phase is mediated by specific protein factors. The first phase of protein biosynthesis involves the activation of amino acids (using energy from ATP) and does not require the presence of ribosomes. Each amino acid is esterified with a specific tRNA, which requires an unique aminoacyl-tRNA synthetase. These reactions produce the activated amino acids necessary for the synthesis of polypeptides.

The other three phases of protein biosynthesis are dependent upon the presence and activities of ribosomes. Eukaryotic ribosomes are composed of two different subunits (each composed of ribosomal RNAs [rRNAs] and proteins), which associate non-covalently to form a functionally active 80S ribosome: a smaller 40S subunit consisting of an 18S rRNA molecule and 37 proteins and the 60S subunit composed of three rRNAs (5S, 5.8S and 28S) and 47 proteins. The association of the two different subunits forms three functionally distinct domains capable of interacting with tRNA molecules: The peptidyl (P)-site, aminoacyl (A)-site and the exit (E)-site. The P site is responsible for binding initiator methionyl-tRNA (Met-tRNA$_i$) and peptidyl-

tRNAs, the A site binds the incoming aminoacyl-tRNAs and the E site binds the deacylated-tRNAs.

Initiation of protein synthesis involves the interactions of initiation factors, GTP, ATP, Met-tRNA$_i$, mRNA and ribosomal subunits. The binding of Met-tRNA$_i$ and mRNA to ribosomes is promoted by at least 11 initiation factors (Merrick and Hershey, 1996). The interactions of these factors result in an 80S ribosome situated at the initiation codon, which contains Met-tRNA$_i$ bound in the P site of the 60S ribosomal subunit. Once the 80S ribosome is correctly positioned at the initiation codon, the elongation phase of protein synthesis can occur.

The elongation phase of protein synthesis involves translating triplet nucleotide codons into specific amino acids. This decoding is mediated by GTP, elongation factors and aminoacyl-tRNAs and occurs in a sequential manner, as each triplet codon is translated in its order of occurrence on the mRNA. Elongation factor (EF)1α, with bound GTP and aminoacyl-tRNA, directs the binding of the aminoacyl-tRNA in a codon-dependent manner to the mRNA. GTP is then hydrolyzed and EF1α is released, resulting in the correct aminoacyl-tRNA positioned in the A site of the 80S ribosome. Once the correct aminoacyl-tRNA is in the A site, a transpeptidation reaction links it to the peptidyl site residue via a peptide bond. The final step of elongation is the motion of the ribosome relative to the mRNA, which is accompanied by the translocation of the peptidyl-tRNA from the A site to the P site of the 80S ribosome (mediated by EF2 in a GTP-dependent manner). This translocation makes room for a new aminoacyl-tRNA to enter the ribosomal A site, and the cyclic reactions can continue, resulting in a lengthening of the polypeptide chain.

When the 80S ribosome reaches a termination codon (UAA, UGA or UAG), a specific protein factor (release factor; RF) binds to the ribosome A site, in a GTP-dependent manner (RF-GTP), and mediates hydrolysis of the peptidyl-tRNA ester at the P site, hydrolysis of GTP and the release of the completed polypeptide along with RF-GDP (Konecki *et al.*, 1977; Tate and Caskey, 1974). RF is necessary for efficient termination and to allow recycling of ribosomes and mRNA for further translation. RF may also be responsible for controlling the percentage of 40S ribosomal subunits that remain attached to

the mRNA and thus determine the extent of translation reinitiation that occurs on a messenger (Merrick, 1992).

3. Initiation

One of the major points of translational control is the process of initiation, which is the rate limiting step for protein biosynthesis. Initiation occurs either on mRNA already associated with polysomes or with an as yet untranslated mRNA in the form of a messenger ribonucleoprotein particle. Initiation is a complicated process involving mRNA structure, dissociation of 80S ribosomes into subunits, binding of Met-tRNA$_i$ to the 40S ribosomal subunit, binding of mRNA, recognition of the initiation codon and binding of the 60S ribosomal subunit (Merrick and Hershey, 1996) (refer to Figure 1: adapted from Merrick, 1992).

80S ribosomes are in dynamic equilibrium with their subunits. Under physiological conditions the 80S ribosomes are the predominant species and must first dissociate into subunits for initiation to occur (Merrick and Hershey, 1996). Three eukaryotic initiation factors (eIFs) are responsible for shifting the equilibrium to favor ribosome dissociation. The initiation factors eIF1A and eIF3 bind to the 40S subunit and prevent its binding to the 60S ribosomal subunit (Goumans *et al.*, 1980). eIF1A stabilizes Met-tRNA$_i$–40S subunit binding, promotes mRNA-binding and functions in ribosome dissociation and the prevention of 40S dimers (Merrick and Hershey, 1996). eIF3, which contains at least 8 polypeptides, forms an mRNA-protein complex that binds to 40S ribosomal subunits in the absence of other translational components. It interacts with ternary complexes of eIF2, GTP, and Met-tRNA$_i$, is required for mRNA binding and stabilizes Met-tRNA$_i$ binding to 40S ribosomal subunits (Merrick and Hershey, 1996). The initiation factor eIF6 binds exclusively to the 60S ribosomal subunit and prevents reassociation of the 60S ribosomal subunit with the smaller 40S subunit.

During initiation, the translational machinery associates with an mRNA to form a ribosome initiation complex, in which the anti-codon of the initiator Met-tRNA$_i$ interacts with the mRNA initiation codon and establishes the

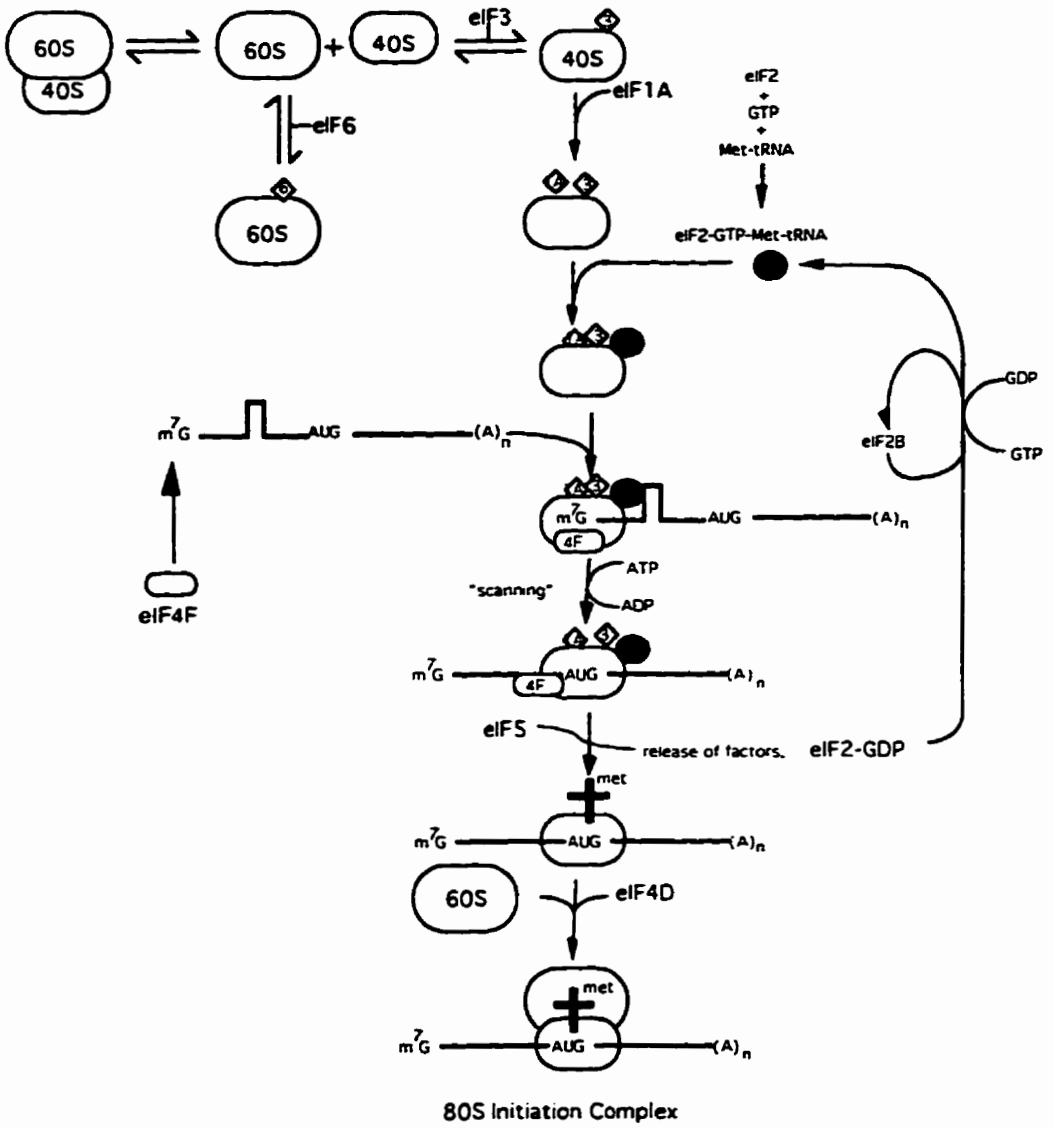


Figure 1: Model of translation initiation showing the formation of the 80S initiation complex (adapted from Merrick and Hershey, 1996)

reading frame of the mRNA (Merrick and Hershey, 1996; Merrick, 1992; Kozak, 1989a). Initially, the ternary complex associates with the 43S ribosomal pre-initiation complex (consisting of the 40S ribosomal subunit, eIF3, and eIF1A). The next step is the binding of mRNA, in association with eIF4 factors, to the 43S ribosome pre-initiation complex. Messenger RNA-binding to the 43S pre-initiation complex is mediated through the recognition of the m^7G cap structure by eIF4E, in association with eIF4A and eIF4G, which make up the cap binding complex, eIF4F. When bound to the mRNA, eIF4F possesses ATP-dependent RNA-helicase activity that melts secondary structure near the 5' terminus (Merrick and Hershey, 1996), which allows the 43S pre-initiation complex to bind to the unfolded mRNA. The initiation factor eIF5 recognizes the 43S-mRNA complex, binds to the 40S ribosomal subunit and promotes the hydrolysis of the GTP carried by eIF2 (Benne and Hershey, 1978). eIF2-GDP is then released from the 40S subunit and recycled through the action of eIF2B.

The last step is the binding of a 60S ribosome subunit to the 40S subunit, which is complexed with Met-$tRNA_i$ and mRNA, to form a translationally active 80S ribosome. Three possible models have been proposed to explain the process of mRNA-binding and initiation codon recognition: (1) pre-initiation complex binding to the 5' terminus of the mRNA, followed by 5'→3' scanning until the initiation codon is recognized; (2) internal initiation due to binding of the ribosome at or upstream of the initiation codon (Merrick and Hershey, 1996); and (3) binding of the pre-initiation complex to the 5' terminal cap followed by non-linear scanning of the mRNA (Futterer *et al.*, 1993; Yuech and Schneider, 1996).

3.1. Scanning Model of Translation Initiation

For the majority of eukaryotic mRNAs, the scanning model of translation initiation is considered by most investigators to be the most plausible method for the recognition of the correct initiation site (Kozak, 1989a). The scanning theory proposes that the primed 43S ribosomal pre-initiation complex, consisting of the 40S subunit in association with initiation factors and Met-$tRNA_i$, binds to the mRNA close to the 5' cap structure and then migrates in a

linear 5'→3' direction. Translational initiation usually begins on the first AUG codon that occurs in the correct context (Kozak, 1989a). After dissociation of the initiation factors, the 60S ribosomal subunit joins the 40S subunit to form the translationally active 80S ribosome, and the elongation phase of translation can proceed (Merrick and Hershey, 1996). After termination, the 80S ribosome is thought to be released from the polysome and then dissociate so that it can participate in another round of protein synthesis.

A variation of the "scanning" model is termination/reinitiation, which predicts that when the 80S ribosome reaches the termination codon, the 60S subunit dissociates and the 40S subunit continues scanning down the messenger until it recognizes the initiation codon of a second open reading frame (Kozak, 1986a). Reinitiation of translation is generally very inefficient, since only a few 40S subunits successfully scan from the termination codon of the first ORF to the initiation codon of the second ORF. In addition, reinitiation of translation is generally believed to occur only if the upstream ORF is relatively short. There is also evidence that the ability to reinitiate at a downstream ORF depends upon the consensus sequence surrounding the upstream termination codon (Grant and Hinnebusch, 1994). The best studied example of reinitiation after termination is the yeast GCN4 mRNA (Hinnebusch, 1994), whose upstream ORFs serve a regulatory function.

3.2. Internal Initiation of Translation

In addition to ribosome binding at the capped 5' terminus of a messenger followed by linear scanning, some transcripts initiate translation at a site within the 5' non-coding region. Members of the picornavirus family have a genome consisting of uncapped mRNA with a large ORF coding for a polyprotein. The long 5' untranslated region (UTR) is able to form a high degree of secondary structure. As well, the 5' UTR contains several AUG codons and small open reading frames, with the upstream AUG codons appearing to be silent, despite the fact that some of the upstream AUG codons occur in a favorable context and should be strong start sites for initiation (Palmenbery, 1989). The translation of picornavirus RNA is unaffected by the formation of a hybrid between picornaviral RNA and their cDNAs up to several

hundred nucleotides at the 5' ends, which according to the scanning model should inhibit scanning by the 40S subunit due to the presence of upstream AUGs and ORFs (Kozak, 1986a; Kozak, 1986b; Shih *et al.*, 1987). As well, cap analogues, which inhibit cap-dependent translation, have no effect on picornavirus mRNA translation (Pelletier *et al.*, 1988). These data suggest a method of translation initiation other than cap-dependent scanning.

Picornavirus mRNA contains a sequence termed an internal ribosome entry site (IRES) (Jang *et al.*, 1989), which is responsible for directing internal initiation of translation. Although the primary sequences of IRESs in picornavirus mRNAs are not well conserved, they do form well-conserved secondary structures (Rivera *et al.*, 1988). Presumably, the majority of the 450 nucleotides in the 5' UTR are required for the formation of secondary and tertiary structure. The only conserved primary sequence motif in picornavirus mRNA is a well-conserved U-rich tract between the IRES and AUG start codon. The actual ribosome entry site is at an AUG codon located at the 3' end of the IRES, approximately 25 nucleotides downstream from a pyrimidine-rich tract (Jackson *et al.*, 1994; Kaminski *et al.*, 1994). Deletion of this U-rich tract results in non-viable virus (Iizuka and Nomoto, 1989) and illustrates the importance of specific sequence elements in the regulation of translation.

The model for the internal initiation of translation requires the initial binding of ribosomes to a potentially unstructured segment located 3' to the IRES and U-rich tract and subsequent scanning of the mRNA from this binding site to the correct AUG initiation codon (Sonenberg and Pelletier, 1989). Insertion of an AUG codon upstream of the poliovirus initiation codon decreases translation efficiency, which implies that some ribosome scanning occurs in the 5' UTR (Kuge *et al.*, 1989). The majority of protein involved in the recognition and utilization of IRES structural domains must be of cellular origin, because the infecting viral RNA is translated before the production of any viral proteins (Ehrenfeld, 1996).

Oh *et al.*(1992) demonstrated that internal ribosome binding is a functional mechanism of translation initiation in *Drosophila* SL2 cells using the *Antennapedia* (*Antp*) homeotic gene of *Drosophilia melanogaster*. The *Antp* mRNAs contain large 5' UTRs with multiple upstream AUG codons, several of which occur in a favorable context for initiation (Cavener, 1987). RNA secondary structures in the long 5' UTR may also prevent scanning and inhibit

translation. Internal ribosome binding as opposed to read-through translation was indicated by several lines of evidence: (1) dicistronic mRNAs lacking AUG codons in the intercistronic spacer blocked translation in contrast to the expected increase in translation that would occur if read-through translation or termination/reinitiation were occurring; (2) translation of the second gene was independent of the first gene as demonstrated by uncapped dicistronic mRNA; (3) stable stem loops inserted upstream of the putative IRES had no effect of translation of the second gene. If termination/reinitiation or read-through translation were occurring, the stem loop would have been an impediment to translation (Oh *et al*, 1992).

Internal ribosome entry has been demonstrated to play a role in translation in normal, uninfected *Drosophila* cells. However, the importance of this finding in translation initiation of vertebrate cells remains to be demonstrated. Because the majority of eukaryotic genes with long 5' UTRs are involved in growth regulation (Kozak, 1991), it is possible that internal translation initiation may be an important regulatory mechanism for mRNAs encoding growth factors, growth factor receptors and oncogenes.

3.3. Cap-Dependent Non-Linear Scanning Model of Initiation

In addition to the cap-dependent or cap-independent linear scanning models of initiation, a poorly-understood mechanism of ribosome shunting or jumping has been described (Futterer *et al.*, 1993; Yuech and Schneider, 1996). This third method requires the interaction of the pre-initiation complex with the 7-methylguanosine cap structure but allows unfavorable secondary structures or sequences in the 5' non-coding region to be bypassed and for initiation to occur normally on a downstream AUG initiation codon.

Examples of this initiation mechanism include the 35S cauliflower mosaic virus (CaMV) RNA (Futterer *et al.*, 1993) and the late adenovirus mRNAs (Schneider, 1995; Yuech and Schneider, 1996). In the case of the CaMV mRNA, a large segment of approximately 600 nucleotides in the 5' UTR, which contains seven small open reading frames (ORFs), is bypassed by the scanning ribosomes. The skipping was inferred based on observations that insertion of a β-glucuronidase (GUS) open reading frame in the middle of

the 35S mRNA 5' UTR reduced reporter gene expression by only 70% (Futterer *et al.*, 1993). The data indicate that ribosomes were able to skip over the entire GUS ORF and initiate at the downstream reporter gene.

Similarly, the insertion of stable stem loop structures in the distal portion of the 5' UTR of the late adenovirus mRNAs had little effect on the frequency of initiation at the authentic site (Schneider, 1995), again indicating an ability for at least some of the ribosomes to skip over regions of the 5' UTR. Recently, the 5' non-coding region, known as the tripartite leader, of late adenovirus mRNAs has been shown to direct both linear and discontinuous scanning when eIF4F is abundant (Yuech and Schneider, 1996). Under conditions of stress, such as late adenovirus infection or heat shock, eIF4F activity is low, and the tripartite leader directs initiation exclusively through the ribosome shunting mechanism (Yuech and Schneider, 1996).

Discontinuous scanning may play an important role in the translation of cell stress mRNAs, which are translated efficiently despite the down-regulation of eIF4F activity (Joshi-Barve *et al.*, 1992). In addition, ribosome shunting may play important regulatory roles in uninfected or normal cells, by minimizing the requirement for the unwinding of long stretches of secondary structure.

4. Initiation Factors

Protein factors initiate and control every step in the process of protein synthesis. Key players in the biosynthesis of proteins are eukaryotic initiation factor (eIF) 4E (the mRNA cap-binding protein) and eIF2. Due to their direct interaction with mRNA, the cap binding proteins are clearly in a position to exert selective control over the translation of different mRNAs (Redpath and Proud, 1994). As well, proteins that interact with these initiation factors and regulate their function may be important for controlling overall cellular translation.

4.1. eIF4 Initiation Factors

The 5' terminal cap structure facilitates mRNA–ribosome binding, a process that requires the participation of three initiation factors (eIF4A, eIF4B and eIF4F) and ATP hydrolysis (Sonenberg, 1988). Secondary structure in the 5' UTR of an mRNA impedes its translation and apparently has to be unwound to permit scanning.(Sonenberg, 1988; Kozak, 1988). Inefficient translation is probably due to interference with the scanning mechanism by which the 40S subunit (and associated factors) contacts the initiation codon. Unwinding of secondary structure is achieved by the ATP-dependent RNA helicase activity of eIF4A, which appears to be more abundant than the other cap binding proteins and can exist both as free eIF4A and as a component of the eIF4F complex (Pause *et al.*, 1994; Rozen *et al.*, 1990).

eIF4E is the least abundant of the initiation factors, is present in limiting amounts and is less abundant than mRNA (Duncan and Hershey, 1987; Ray *et al.*, 1983). In addition, eIF4E is possibly the first initiation factor to contact the mRNA. These observations suggest there may be competition among different mRNAs for eIF4E. Structured transcripts reliant on eIF4E activity to overcome the translational block, imposed by secondary structure, would be translated inefficiently when eIF4E is limiting. Over-expression of eIF4E would relieve this translational block and facilitate translation of structured transcripts, such as those encoding growth-related proteins. This is demonstrated in cell lines over-expressing transfected copies of eIF4E, which display an aberrant morphology, anchorage-independent growth, form foci on a monolayer of cells and cause tumors in nude mice (Lazaris-Karatzas *et al.*, 1990; De Benedetti and Rhoads, 1990). Moreover, over-expression of eIF4E in rodent fibroblast cells results in malignant transformation, which is consistent with the idea that eIF4E is an important transducer of growth signals (De Benedetti *et al.*, 1994; Lazaris-Karatzas *et al.*; 1990; Sonenberg, 1992). In addition, the mRNAs encoding the growth-promoting proteins cyclin D1 (Rosenwald *et al.*, 1993) and ornithine decarboxylase (Shantz and Pegg, 1994) are more efficiently translated in cells that over-express eIF4F. These data suggest that malignant transformation could result from inappropriately

high levels of proto-oncogene expression, whose mRNAs are thought to have a high degree of secondary structure in their 5' UTRs (Kozak, 1991).

eIF4E is found in both phosphorylated and non-phosphorylated forms, consistent with a regulatory role in translation (Koromilas *et al.*, 1992; Rhoads, 1993). eIF4F activity is dependent on phosphorylation of eIF4E on serine-53, which is stimulated by serum, growth factors or phorbol esters (Koromilas *et al.*, 1992). This is demonstrated by the observation that overexpression of wild-type eIF4E, but not of the alanine-53 mutant (which is not phosphorylated), leads to malignant transformation of transfected cells (Koromilas *et al.*, 1992).

4.1.1. Proteins Regulating eIF4E

In addition to initiation factors, cells contain *trans*-acting factors that interact with components of the translational machinery and regulate their functions. Pause *et al.* (1994) have described two low molecular weight human proteins that can complex with eIF4E and inhibit cap-dependent, but not cap-independent, translation. Proteins such as these eIF4E-binding proteins (4E-BPs) appear to affect the ability of eIF4E to contribute to the formation of a functional cap-binding complex (Pause *et al.*, 1994).

After activation, phosphorylation of 4E-BP1 occurs on serine-64, which is phosphorylated *in vitro* by the mitogen-activated protein (MAP) kinases ERK1 (extracellular regulated protein kinase-1) and ERK2 (Lin *et al.*, 1994; Haystead *et al.*, 1994). Consistent with a role for MAP kinase in regulating 4E-BP1 association with eIF4E, phosphorylation of 4E-BP1 by MAP kinase renders the protein incapable of interacting with eIF4E, which is then available to form an active cap-binding complex (Lin *et al.*, 1994). This is also supported by the observation that stimulation of 3T3-L1 adipocytes with EGF or insulin led to an increase in MAP kinase activity. In response to hormones, such as insulin, only cap-dependent translation would be stimulated and mRNAs with extensive secondary structure in their 5' UTR would be translationally upregulated (Sonenberg, 1996). This is exemplified by the translation of messengers in response to insulin, which stimulates specifically cap-dependent translation. In 3T3-L1 cells, ornithine decarboxylase

translation increases approximately 30 fold after insulin stimulation, despite the presence of high amounts of secondary structure in its 5' UTR, whereas general translation is increased only 2-fold (Manzella *et al.*, 1991; Gallie and Traugh, 1994).

The interaction of eIF4E with the 5' terminal cap structure is not prevented by the binding of either 4E-BP1 or 4E-BP2 to eIF4E (Lin *et al.*, 1994, Pause *et al.*, 1994). However, it has been shown by Haghighat *et al.* (1995) that the 4E-BPs and eIF4G cannot bind simultaneously to eIF4E. It was subsequently demonstrated that the prevention of simultaneous binding was due to a common motif for eIF4E-binding (Mader *et al.*, 1995). In addition, the cap-binding complex eIF4F binds better than eIF4E to the mRNA cap structure (Lee *et al.*, 1985). Therefore, the prevention of eIF4F assembly would interfere with efficient cap-binding and result in decreased translation rates.

In response to modulation of eIF4E function, heterogeneous requirements or abilities to compete for eIF4F by different mRNAs would not merely change the global rate of protein synthesis, but also alter specific patterns of protein expression. Regulation may not be restricted to the eIF4E subunit of the eIF4F complex. Cells infected with poliovirus are unable to translate capped mRNAs due to inactivation of the initiation factor, eIF4F (Wyckoff, 1993) as a result of eIF4G cleavage. It is also likely that the other components may also be subjected to regulation or be targeted as a means of bypassing normal translational control.

4.2. eIF2 Initiation Factors

The activities of initiation factors eIF2 and eIF2B affect the reinitiation of translation and the recycling of ribosomes for protein synthesis (Vazquez *et al.*, 1994). eIF2 regulation is likely to play a role in the global regulation of translation rates, but there is also evidence that its regulation can result in mRNA-specific modulation of translation. eIF2, which is composed of three non-identical subunits, mediates the binding of Met-tRNA$_i$ to the ribosome in a GTP-dependent manner; when bound to GDP, eIF2 is unable to bind Met-tRNA$_i$. eIF2 is released from the ribosome bound to GDP as an inactive binary complex. In order to bind another Met-tRNA$_i$, this GDP must be exchanged for

GTP. Since the GDP-GTP exchange rate of eIF2 is very low, another initiation factor, eIF2B, is required for the replacement of bound GDP and eIF2 recycling (Redpath and Proud, 1994) (refer to Figure 1: adapted from Merrick and Hershey, 1996).

The phosphorylation of eIF2B correlates positively with increased translation rates, whereas the phosphorylation of eIF2 results in the inhibition of translation and suppression of cell growth. Specifically, it is phosphorylation of the α-subunit of eIF2, on serine-51, that inhibits GDP-GTP nucleotide exchange on eIF2 (Hinnebusch, 1996). eIF2-GDP containing phosphorylated eIF2α has a higher affinity for eIF2B than does non-phosphorylated eIF2-GDP. Therefore, the formation of ternary complexes is decreased because eIF2B is no longer able to facilitate the GDP-GTP exchange reaction on phosphorylated eIF2. Because eIF2 is present at considerably higher levels than is eIF2B, the phosphorylation of eIF2α is sufficient to inactivate the majority of eIF2B present in the cell (Clemens, 1996; Merrick and Hershey, 1996).

5. mRNA Structures Affecting Translation

Several features of an mRNA's structure might determine its ability to compete for a discriminatory factor and hence influence the efficiency of initiation complex formation (Pelletier and Sonenberg, 1985). Interacting with protein factors, they can also directly enhance or repress translation initiation. These structural elements include: the 5' terminal cap and its accessibility, the initiation codon (usually AUG), the context surrounding the initiation codon, the length of the 5' UTR, the amount and stability of secondary structure in the 5' UTR, the absence of upstream AUG codons or open-reading frames (Merrick and Hershey, 1996), as well as the presence of specific elements in the 3' UTR (e.g., cytoplasmic polyadenylation elements) (Richter, 1991; Richter, 1993; Jackson, 1993; Sheets *et al.*, 1994)).

In addition to regulation by components of the translational machinery, regulation of translation may also occur through other specific protein and mRNA interactions. Messenger RNA molecules are capable of forming a variety of structures, including helices, loops, bulges and pseudoknots, which

may play important roles, along with specific sequences in the 5' and 3' UTRs, in the recognition and binding of proteins (Draper, 1989). In some RNAs these structures regulate translational initiation (e.g., ferritin; Hartford and Klausner, 1990). Sequences in the 3' UTR can also direct mRNA to specific regions of the cell from which repressors are excluded, thereby activating their translation (Curtis *et al.*, 1995).

5.1. m^7G Cap Structure and Accessibility

At the extreme 5' end of all cytoplasmic mRNAs is a modified guanosine nucleotide (7-methylguanosine), termed the 5' cap, which is linked to the next nucleotide by a 5'-5' triphosphate group [m^7(5')Gppp(5')N]. The m^7G cap structure stimulates the translation of mRNAs in cell lysates, *Xenopus* oocytes and in cells transfected with mRNAs. The scanning process, which aligns the ribosome with the initiation codon, is thought to involve the binding (directly or indirectly) of various proteins to the cap structure. The cap-binding proteins are likely to be the first initiation factors to interact with the mRNA, and therefore, subsequent binding of the ribosome is presumably dependent on the interaction of these cap-binding proteins with the mRNA (Redpath and Proud, 1994). The accessibility of the m^7G cap structure to eIF4F appears to be especially important for efficient protein synthesis (Lawson *et al.*, 1988).

5.2. Initiation Codons and Open Reading Frames

Generally, in eukaryotic cells the AUG codon nearest the 5' terminus is the unique site of translation initiation, provided that the AUG codon occurs in a favorable context (Kozak, 1995). The consensus sequence gccGCCACC**AUG**G is considered to be the strongest signal for initiation codon recognition in mammalian cells (Kozak, 1986a; Kozak, 1987; Kozak, 1989a; Merrick and Hershey, 1996). The most important residues are the purine at -3 (the A of AUG is +1) and the guanosine at +4. Two mechanisms account for most exceptions to the initiation of translation on the first AUG codon. Reinitiation at a downstream initiation codon may be possible when the 5' proximal AUG codon is followed shortly by a termination codon (Kozak,

1987). A second mechanism that allows access to downstream AUG codons is "leaky" scanning. "Leaky" scanning (Kozak, 1989a) involves the passing over of initiation codons that weakly match the consensus sequence. If a purine is absent at -3 or guanosine is not present in position +4, some 40S subunits will bypass the first AUG codon and initiate at a downstream site (Kozak, 1987; Kozak, 1995). In addition, AUG codons less than about 10 nucleotides from the 5' terminus are passed over in favor of other AUG codons, perhaps due to a requirement for sufficient length to allow both m^7G-cap and AUG interactions on the mRNA (Kozak, 1989b).

Upstream AUG codons decrease the frequency of initiation at the downstream AUG initiation codons (Merrick and Hershey, 1996). Similarly, small upstream open reading frames (uORFs) in the 5' UTR decrease translation of the downstream major ORF. In some cases these uORFs play a role in the regulation of translation as exemplified by the yeast GCN4 mRNA. The unique induction of GCN4 translation under starvation conditions is mediated by four short uORFs in the leader of GCN4 mRNA. These uORFs act as *cis*-regulatory elements to prevent ribosomes from initiating translation at the GCN4 start site when nutrients are abundant. However under conditions of nutrient deprivation, eIF2 is phosphorylated, and the *cis*-acting uORFs are much less effective in repressing GCN4 translation (Hinnebusch, 1994).

5.3. Translational Control by the 5' UTR

Selective regulation specific to certain mRNAs or subsets of mRNAs may arise by virtue of the interaction of specific or general factors with either structural features of mRNAs (especially secondary structure in the 5' terminal region) or specific sequences of the mRNA. These mRNAs must compete for a limiting component of the initiation machinery, which acts as an mRNA discriminatory factor due to its different affinities for specific messengers (Pelletier and Sonenberg, 1985). Messenger RNA-binding proteins may function as *trans*-acting factors by either facilitating initiation factor binding to the mRNA or by preventing their interaction (Redpath and Proud, 1994).

5.3.1. Secondary Structure in the 5' UTR

Eukaryotic 5' UTRs range in length from a few nucleotides up to 1000 nucleotides, with the majority consisting of 50 - 70 nucleotides, and establish the intrinsic rate of translation of an mRNA (Merrick and Hershey, 1996). Inefficient translation of messengers may be a result of leaders that are very short or as a result of secondary structure due to complementary nucleotides in their 5' UTRs (Merrick and Hershey, 1996; Kozak, 1986b; Kozak, 1989b; Oliveira *et al.*, 1993; Fu *et al.*, 1991). Secondary structure near the 5' terminal cap has the greatest effect on mRNA-ribosome binding, but stem loop structures anywhere in the 5' UTR can inhibit ribosome scanning (Kozak, 1989b). This secondary structure must be unwound by the concerted actions of the eIF4 initiation factors to permit scanning of the 40S ribosomal subunit toward the initiation codon (Jaramillo *et al.*, 1990; Rhoads, 1993). Under normal conditions, eIF4F is present in limiting amounts, and there will be positive discrimination in favor of the more efficiently translated mRNAs. Although most eukaryotic transcripts lack extensive secondary structure in their 5' UTRs, transcripts that encode growth related proteins, such as ornithine decarboxylase, oncoproteins, growth factors, and growth factor receptors typically have long, guanosine/cytosine (G/C)-rich 5' UTRs with the potential to form stable secondary structure (Kozak, 1989b; Manzella and Blackshear, 1990). In normal somatic cells this stable secondary structure results in poor translation because of the impediment to translation initiation. The importance of the 5' UTR in post-transcriptional regulation is demonstrated by the human transforming growth factor-β1 mRNA, which contains a stable stem loop structure in the 5' UTR that inhibits its translation (Kim *et al.*, 1992).

The translation of ferritin mRNA is a well–studied example of post-transcriptional regulation due to stable secondary structure in the 5' UTR. Ferritin mRNA translation is repressed by the binding of a specific protein (the iron regulatory factor; IRF) to a stem-loop motif (iron responsive element; IRE) in the 5' UTR of the messenger (Hartford and Klausner, 1990; Melefors and Hentze, 1992). The IRF is a 98 kilodalton (kd) iron–sulfur protein with high affinity for the IRE under conditions of iron starvation and low affinity when the iron–sulfur center is complete. For efficient regulation of translation by iron,

the IRE must be within 40 nucleotides of the 5' terminal cap, even though *in vitro* binding of IRF is not influenced by the position of the IRE (Hartford and Klausner, 1990; Melefors and Hentze, 1992).

The presence of stable secondary structure in the 5' UTR is an important regulatory element for determining translation efficiency. However, precise secondary structures are difficult to define due to possible long-range RNA-RNA interactions and RNA-protein binding that may influence mRNA structure (Merrick and Hershey, 1996).

5.3.2. Specific Primary Sequence Elements in the 5' UTR

Although secondary structure has been demonstrated to be an important regulatory element in many cases, it is not always responsible for inhibition of translation. In some cases the actual sequence codes for a regulatory element that binds specific proteins and regulates translation initiation. Furthermore, other elements in the 5' UTRs are known to have regulatory functions, such as the 7-methylguanosine cap (discussed previously).

The translation of vertebrate ribosomal protein mRNAs (rpmRNAs) is regulated in a growth-dependent manner, illustrated by their selective shift from polysomes in growing cells into messenger ribonucleoprotein (mRNP) particles (subpolysomal fraction) in quiescent cells (Meyuhas *et al.*, 1996). The 5' UTR of rpmRNAs functions as a *cis*-acting regulatory element, altering the translational efficiency of these mRNAs in response to the accumulation of ribosomal RNA (rRNA) (Meyuhas *et al.*, 1996). The appearance of newly-synthesized rpmRNAs during *Xenopus* embryogenesis occurs at gastrulation and precedes the onset of ribosomal protein synthesis, which occurs in tailbud embryos (Baum and Wormington, 1985). rpmRNAs have the following structural features: short 5' UTRs (approx. 40 nucleotides) with limited ability to form secondary structure of significant stability, a lack of upstream AUG codons and a cytosine residue at the cap site, followed by 4-13 pyrimidines (termed the 5' terminal oligopyrimidine tract; 5' TOP) (Meyuhas *et al.*, 1996). Initial evidence that translational regulation resides in the 5' UTR comes from experiments in which fertilized *Xenopus* eggs were injected with chimeric

mRNAs. These experiments demonstrated that the 35 nucleotides of the *Xenopus* rpS19 mRNA 5' UTR were sufficient to confer translational repression in a development-dependent pattern (Mariottini and Amaldi, 1990).

Developmental or growth stimuli may lead to variable extents of recruitment of rpmRNAs onto polysomes (Meyuhas *et al.*, 1987), suggesting differences in the affinity of the individual rpmRNAs for *trans*-acting factors. In addition, individual *Xenopus* embryos exhibit different developmental patterns of polysomal recruitment of the same species of rpmRNA (Bagni *et al.*, 1992), perhaps reflecting diversity in the amount or activity of *trans*-acting factors in these individuals. The translational control of 5' TOP-containing mRNAs could be carried out by interactions between specific and general translation factors (Meyuhas *et al.*, 1987). The translation of these mRNAs would be completely repressed by the attachment of a specific protein to the 5' TOP. Translation could initiate upon displacement of the repressor by a general component of the translational apparatus (Lodish, 1974).

5.4. Translational Control by 3' Elements

In addition to translational control mediated by the 5' UTR, the 3' UTR may be completely or partially responsible for regulating the translation of specific messengers. This control can be exerted through specific sequence elements in the 3' UTR, polyadenylation or through the length of the 3' non-coding region itself. Besides acting on their own to regulate translation, elements in the 3' UTR may act cooperatively with the 5' UTR. As well, the interactions of specific proteins with elements in the 3' UTR can regulate translation.

5.4.1. Sequence Elements in the 3' UTR

During spermatogenesis in mice, protamine mRNAs are stored polyadenylated (approximately 150 adenosine residues) in the cytoplasm before they are translated (Braun, 1991). After 7 days in the cytoplasm there is shortening of the poly(A) tail, and translation initiates (Braun, 1991). Because deadenylation seems to occur slightly later than translational activation, it is

probably not a primary cause of activation. Further experiments characterized a 60 nucleotide motif at the distal 3' end of the mRNA that is necessary and sufficient for the appropriate temporal regulation of translation *in vivo* (Braun, 1991). Proteins that specifically bind this 3' UTR motif can be found in developing spermatids during the stages when these mRNAs are masked, but not in cells in which protamines are being expressed (Braun, 1991). An 18 kd protein binds specifically to the 3' UTR motif of protamine mRNA and inhibits translation in an rabbit reticulocyte (RRL) translation assay of mRNAs containing the 3' UTR motif of protamine 2 mRNA (Braun, 1991). These observations provide insights into how protein–mRNA interactions at the 3' end of an mRNA can regulate translation.

5.4.2. Polyadenylation

It is likely that the effects of the 5' terminal cap and the poly(A)$^+$ tail are not totally independent and that interactions between the 5' and 3' non-coding regions of the mRNA can stimulate translation. Because changes in polyadenosine [poly(A)] tail length closely parallel changes in the translation rates of specific messengers during development, cytoplasmic polyadenylation has been implicated as a regulator of translation (Richter, 1995). Once transcribed in the nucleus, most mRNAs are polyadenylated and then exported to the cytoplasm, where they are subject to both removal and lengthening of their poly(A) tails by cytoplasmic factors. The mRNAs synthesized during oogenesis can be grouped into two broad classes. One class is polyadenylated mRNAs that are efficiently translated in oocytes. Two examples are *Xenopus* actin and ribosomal protein mRNAs, which retain fairly long poly(A) tails and are efficiently translated during oogenesis. However, shortly after nuclear envelope breakdown at maturation, their poly(A) tails are shortened or removed, and their translation ceases (Jackson, 1993). In addition, other mRNAs are deadenylated after fertilization and are removed from polysomes. These messengers are stored in the cytoplasm of developing oocytes as masked, untranslated mRNAs with short poly(A) tails (30-50 A residues) and undergo polyadenylation and translational activation either at maturation or after fertilization (Jackson, 1993). These events are

dependent on signals that are located within the untranslated regions of the messengers

Polysome recruitment and subsequent translational activation as a result of polyadenylation occur in a messenger-specific manner in response to different developmental stimuli (Wickens, 1990; Wormington, 1993; Paris and Richter, 1990; Sheets *et al.*, 1994). Changes in poly(A) tail length are presumed to regulate translation through the interactions of specific protein factors and sequence elements in the 3' UTR. To determine whether poly(A) elongation was actually causal for translation or merely correlative, McGrew *et al.* (1989) first characterized the *cis*-acting sequences necessary for polyadenylation and then determined whether they were also required for translation. Using *in vitro*-synthesized mRNAs injected into *Xenopus* oocytes, they demonstrated that two 3' UTR sequences were necessary for cytoplasmic polyadenylation: the A_2UA_3 hexanucleotide, which functions in nuclear pre-mRNA cleavage and polyadenylation, and a U-rich sequence with the general structure of UUUUUAU, called the cytoplasmic polyadenylation element (CPE; Fox *et al.*, 1989; Paris and Richter ,1990; Simon *et al.*, 1992; Sheets *et al.*, 1994). In addition, an mRNA that contains the wild type *cis*-acting elements but whose 3' terminus is blocked by cordecypin (3'-dA) is neither polyadenylated nor recruited for translation (McGrew *et al.*, 1989). The precise spacing of a CPE relative to the A_2UA_3 hexanucleotide sequence can regulate the timing of polyadenylation (Simon *et al.*, 1992). In the absence of specific 3' UTR sequences that promote cytoplasmic polyadenylation, mRNAs are deadenylated and translationally repressed during *Xenopus* oocyte maturation (Fox and Wickens, 1990; Varnum and Wormington, 1990), and a similar case can be made for the regulation of certain transcripts after fertilization.

5.4.3. Length of the 3' Non-Coding Region

Tanguay and Gallie (1996) examined the effect of the length of the 3' non-coding region on the regulation of poly(A)$^-$ and poly(A)$^+$ chimeric mRNA expression in transiently-transfected Chinese hamster ovary (CHO) cells. Both the translational efficiency and the stability of poly(A)$^-$ mRNA were

increased as the length of the 3' UTR increased (Tanguay and Gallie, 1996). Their observations suggested that the length of the 3' UTR, in addition to the sequence, is an important mRNA property that can influence translation.

In addition, very short 3' UTRs, consisting of only 4 bases, reduced the *in vitro* translation from poly(A)$^-$ mRNAs (Tanguay and Gallie, 1996). Based on these observations, it was concluded that increasing the length of the 3' UTR increases the translational efficiency of poly(A)$^-$ mRNAs (Tanguay and Gallie, 1996). It is probable that the increase in translational efficiency is a result of an increase in the local concentration of translational machinery in the vicinity of an mRNA due to increased 3' UTR length. However, in poly(A)$^+$ mRNAs the rate of translation both *in vivo* and *in vitro* remained largely unaffected by increasing 3' non-coding region length.

Tanguay and Gallie (1996) proposed a model to explain how increasing the length of the 3' UTR can effect initiation. Following termination, 40S subunits may remain associated with the mRNA and continue to travel across the 3' UTR. The continued association of a 40S subunit with the 3' UTR would raise the local concentration of ribosomes on the mRNA and increase the likelihood that the 40S subunit would be re-recruited for translation. Increasing the length of the 3' UTR would, therefore, increase the time over which the 40S subunit remains associated with a given mRNA and consequently increases its chance for re-recruitment. The increase in translation conferred by a long 3' UTR would be a passive mechanism, in contrast to the effects of protein factors and structural elements located in the 3' UTR (CPEs).

6. Oligopyrimidine Tracts

Another important regulatory motif that has been examined in recent years is polypyrimidine (pPy) tracts. These homopyrimidine- and pyrimidine-rich tracts may be located in the functional regions of 5' or 3' UTRs of mRNAs and may play roles in splicing, initiation, cytoplasmic degradation and polyadenylation.

Sequences containing 8 - 14 pyrimidine residues have been found in the 5' UTR of all vertebrate ribosomal protein mRNAs (Kaspar *et al.*, 1993) and

confer translational regulation (Mariottini and Amaldi, 1990; Levy *et al.*, 1991). Removal or mutation of the polypyrimidine tract abolished the translational regulation (Kaspar *et al.*, 1992; Levy *et al.*, 1991). Other mRNAs containing the pyrimidine-rich tracts in their 5' non-coding regions have been described, although in most cases a functional role has not been demonstrated (Pinsky *et al.*, 1985; Chitpatima *et al.*, 1988; Makirdes *et al.*, 1988).

Polypyrimidine tracts have also been demonstrated to play a functional role in the 3' UTR of messenger RNAs. Polyuridine and AU-rich elements frequently occur in the 3' non-coding regions of eukaryotic mRNAs. The presence of uridine residues in these tracts appears to be very important for function and mutations are less tolerated than mutations in the adenosine residues (Morris *et al.*, 1993). The 3' UTR of the GM-CSF mRNA, which is uridine rich and contains the sequence AUUUA, is sufficient to destabilize β-globin mRNA when chimeric messengers are used for micro-injections (Shaw and Kamen, 1986). The AU-rich element (ARE) is generally composed of multiple copies of the AUUUA motif (Shaw and Kamen, 1986) and is responsible for the degradation of many cytogenic and oncogenic mRNA (Shaw and Kamen, 1986; Caput *et al.*, 1986) In addition, the polyadenylation signals of mRNAs are uridine-rich and can control both the timing and extent of polyadenylation, as well as translational regulation.

Clearly, polypyrimidine tracts are important regulatory motifs and must be considered when examining elements responsible for the translational regulation of any messenger mRNA.

7. Interactions Between the 5' and 3' Non-Coding Regions

In vivo studies suggest that efficient translation requires the 5' terminal cap and the poly(A) tail to function interdependently (Gallie, 1991). In uncapped messengers, the translational efficiency of poly(A)$^+$ mRNA is not substantially greater than that of poly(A)$^-$ mRNA translation. Only when the messenger is capped does the addition of a poly(A) tail substantially stimulate translational efficiency. Because the poly(A) tail requires the 5' terminal cap structure in order to serve as a regulator of translation, it may be that interactions between the poly(A) tail (mediated by poly(A) binding protein;

PAB) and the 60S ribosomal subunit are important in controlling 80S initiation complex formation (Sachs and Davis, 1989; Gallie, 1991). In addition, the poly(A) tail–PAB protein complex may be involved in earlier events in translational initiation, such as cap recognition.

In vitro, eIF4F and PAB protein bind to the cap and poly(A) tail, respectively (Sachs *et al.*, 1987; Sonenberg, 1988). Certain discrepancies between *in vitro* and *in vivo* protein synthesis data can be explained in terms of interactions between the poly(A)-PAB and cap binding proteins. These discrepancies include the fact that translation was an order of magnitude less cap-dependent *in vitro* than *in vivo*; the poly(A) tail had no effect *in vitro* and the 5' terminal cap and poly(A) tail had no effect on messenger stability *in vitro* (Gallie, 1991). This is also supported by the observations of Laskey *et al.*, (1977), who inferred from their studies that *Xenopus* oocytes can only translate about 2 nanograms (ng) of mRNA, irrespective of whether the RNA is endogenous or injected. The oocyte itself contains at least 50 ng of poly(A)$^+$ mRNA, and although oocytes contain an abundance of ribosomes, aminoacylated-transfer RNAs (tRNAs) and mRNAs, fewer than 5% of each of these is involved actively in translation (Richter, 1993). Therefore, some component(s) of the translational machinery must be rate limiting. The *in vitro* lysates may fail to reflect an *in vivo* environment in a number of ways: Because embryos or cells have a limited capacity for protein synthesis, it is possible that certain factors are missing or damaged when using *in vitro* systems. The demonstration that eIF4E preferentially associates with intermediate filaments (Zumbe *et al.*, 1982) and the observation that some polysomes are cytoskeleton-associated (Lenk *et al.*, 1987; Howe and Hershey, 1984) supports the hypothesis that the missing factor may be a single protein or part of a framework, such as the cytoskeleton. Association of mRNA with the cytoskeleton may be a necessary prerequisite for the synergistic effects between the 5' terminal cap and the poly(A) tail to occur, mediated by the activities of other, as yet, uncharacterized factors.

8. mRNA Stability

Cells can control the translation of some transcripts by regulating the stability of the mRNA. Messengers that are rapidly degraded will not be available to the translational machinery and will therefore be down-regulated as opposed to stable transcripts, which are available to the synthetic machinery for a longer time.

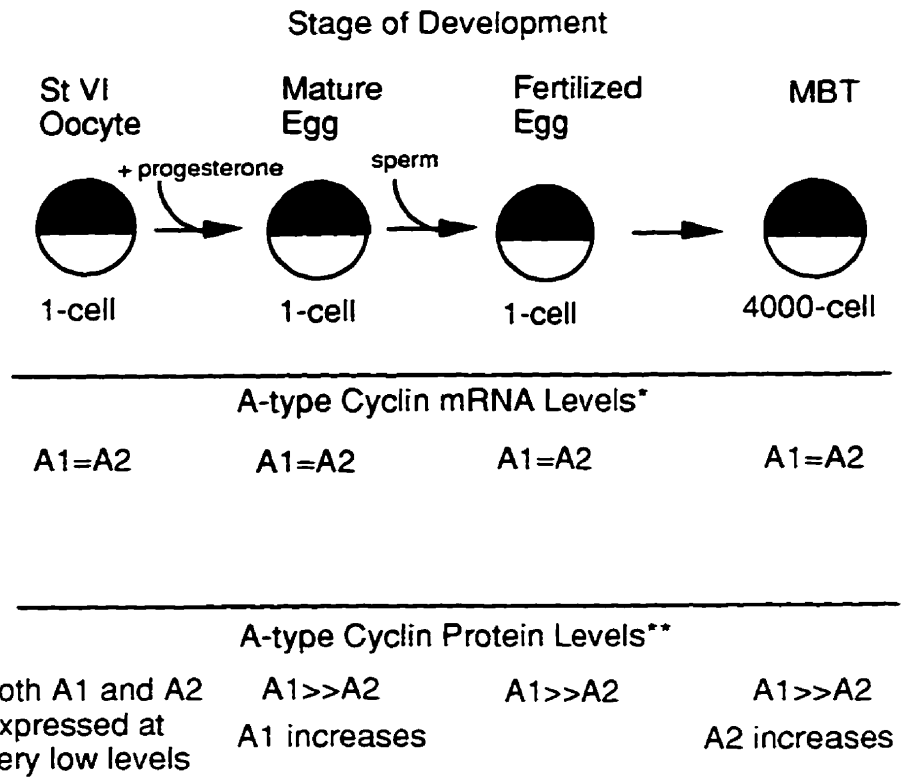
A number of different factors play a role in regulating mRNA stability, including polyadenylation, translation and the interactions of specific proteins. Adenosine residues at the 3' end of a message can protect the molecule from exonuclease activity at this end of the molecule (Nudil et al., 1976). Some mRNAs can be stabilized by adding inhibitors of protein synthesis, which suggests either an association of nucleases with ribosomes or lability of components of the degradation machinery (Atwater et al., 1990).

9. Translational Control of Cyclin mRNAs

The cyclins are a family of proteins encoded by maternal mRNAs in *Xenopus* eggs and early embryos. Cyclin polypeptides accumulate during interphase and are destroyed during mitosis at about the time of entry into anaphase (Swenson *et al.*, 1986). Cyclins are essential activating subunits for a family of structurally-related protein kinases, the cyclin dependent kinases (CDKs; Pines and Hunter, 1994). Cyclin–CDK complexes are involved in the regulation of many of the important transitions of the cell cycle, such as decisions to enter mitosis and to begin DNA replication (Pines and Hunter, 1994). The cyclins can be divided into two subclasses: the G1 or START cyclins and the mitotic cyclins (Sherr, 1993). In mammalian cells, the G1 cyclins include cyclins D and E, and the mitotic cyclins are A, B1 and B2 (Sherr, 1993). The roles of the individual cyclin–CDK complexes in the cell cycle are not well defined, with the exception of the cyclin B–$p34^{cdc2}$ complex. Most cyclins have similar substrate specificity. Thus, the specificity of these complexes *in vivo* probably involves temporal control of the synthesis of cyclin and CDK components (Pines and Hunter, 1994).

Recent experiments have indicated that the addition and removal of poly(A) from both A-type and B-type cyclin transcripts is extensively regulated, due to specific sequences in the 3' UTRs of these mRNAs (Sheets *et al.*, 1994). These effects were demonstrated using chimeric mRNAs, in which a luciferase-coding region was joined to the 3' UTRs of both cyclin A1 and B1. These chimeric messengers received poly(A) tails of the same length and at the same time as the endogenous mRNAs, indicating that signals in the 3' UTR were sufficient to regulate both temporal and quantitative control of poly(A) addition (Sheets *et al.*, 1994). In addition to regulating the addition of poly(A), the 3' UTR also regulated the translation of these messengers, determining both when and how much translation occurred. The results of Sheets *et al.*, (1994) suggest that elements within the 3' UTRs of cyclin mRNAs control when and if activation occurs, as well as the extent of activation.

The expression pattern of the A-type cyclins suggests that they are developmentally regulated (Howe *et al.*, 1995). This is confirmed by examining the levels of the A-type cyclin mRNAs and proteins during development (Figure 2). It has been observed that the maternally-stored pools of mRNAs encoding both A1 and A2 cyclin proteins were stable until the onset of gastrulation, at which time they were rapidly degraded (Howe *et al.*, 1995). Both cyclin A1 and cyclin A2 messengers were present in nearly equivalent amounts in stage 6 oocytes (Howe *et al.*, 1995). After fertilization, cyclin A1 mRNA levels remained constant until stage 9.5 and became undetectable by stage 11. In contrast, cyclin A2 mRNA remained stable after stage 11 when cyclin A1 messengers were not observed (Howe *et al.*, 1995). In unfertilized eggs and during the early cleavage stages, the levels of cyclin A2 protein were very low. Cyclin A2 protein levels increased rapidly after the MBT, reaching a constant level by the neurula stage. Unlike cyclin A2, cyclin A1 mRNA and protein were present in embryos until the beginning of gastrulation at which time they were rapidly degraded (Howe *et al.*, 1995). The maternal store of both cyclin A1 and A2 messengers was stable before the early gastrula transition at which time they were degraded rapidly (Howe *et al.*, 1995).



* both A1 and A2 mRNAs are stable. A1 mRNA levels start to decrease around the time of the EGT.
** A1 protein is expressed after maturation and begins to taper off by the MBT, disappearing sometime after the EGT. A2 protein is not made significantly until after the MBT.

Figure 2: Ontogenic profile showing the relative levels of expression of cyclin A1 and A2 for the examined stages of development

10. Objective

Research in Dr. Leon Browder's laboratory is directed towards examining the translational regulation of specific messengers during development using *Xenopus laevis* as the model system. Translational regulation can be examined using a simple experimental system of injecting chimeric messengers containing the chloramphenicol acetyl transferase (CAT) reporter gene into *Xenopus* oocytes, unfertilized eggs, fertilized eggs and embryos. This system allows us to quantify differences in the translational expression of chimeric molecules conferred by the non-translated regions of the native molecules. These differences in translational activity can be quantitated throughout the early developmental stages of *Xenopus laevis*, providing insight into how specific translational control can determine and regulate gene expression.

Based on the results of other investigators, it appears that maternal mRNAs regulating key events in the embryo are often repressed during oogenesis, becoming activated either during meiotic maturation or shortly after fertilization. Other maternal mRNAs remain repressed throughout early development. It is not yet known how many different biochemical mechanisms are responsible for this complex control, although a number of distinct mechanisms have already been described. An unresolved problem in developmental biology is how mRNAs are retained in an inactive state and become translationally competent at either oocyte maturation or fertilization. Homologous proteins such as different members of the cyclin family (e.g., cyclin A and B), which show different levels of translational activity during development (Nurse, 1990; Draetta, 1990), provide an excellent opportunity to examine this question. The specific elements regulating the translation of transcripts are usually located within the 5' or 3' flanking regions of the mRNAs. It has already been demonstrated by Sheets *et al.* (1994) that the 3' UTR is responsible for the translational regulation of the cyclin A1 messenger. It remains to be seen whether the translation of cyclin A2 is regulated by a similar mechanism.

The aim of this project was to investigate the translational regulation of *Xenopus* A-type cyclins during early development Using chimeric

messengers, with a CAT reporter gene, I have examined the roles of the 5' and 3' UTRs of cyclin A2 on the translation and stability of chimeric messengers. The questions examined in these experiments include: determining whether either the 3' UTR or the 5' UTR functions as a regulator of mRNA translation and/or stability; examining whether the 5' and 3' UTRs function synergistically to regulate translation and/or mRNA stability and determining if the polyadenylation of the chimeric messengers is controlled in part by the 3' UTR. However, these experiments do not explore the possibility that the coding sequence may affect the function of the 3' and 5' non-coding regions (see Discussion).

MATERIALS AND METHODS

1. Materials

1.1. Frogs

Sexually mature male and female *Xenopus laevis* frogs (Nasco, Inc.) were kept in tanks with continuously flowing dechlorinated water and were fed Frog Brittle (Nasco, Inc.). The frogs were handled in accordance with the principles contained in the "Care of Experimental Animals" manual published by the Canadian Council of Animal Care.

1.2. Chemicals, Enzymes and Other Materials

All chemicals were purchased from BDH Inc., except for the following: Agarose and Dithiothreitol (DTT) were from Gibco/BRL; Bacto-agar was purchased from Difco; Yeast extract was purchased from Becton Dickinson; Formamide, Sodium Iodide, Hydrochloric Acid, Iso-Amyl Alcohol and Formaldehyde were from Fisher Scientific; Ampicillin was purchased from Boehringer Mannheim; HEPES, Ficoll type-400, Glucose, Penicillin G and Streptomycin were purchased from Sigma Chemical company.

Radiolabeled iso-blue [α-^{33}P]UTP was purchased from Amersham; radiolabeled [α-^{35}S]dATP, [α-^{35}S]ATP and [^{14}C]-chloramphenicol were purchased from either NEN, Amersham, or ICN.

Enzymes and reagents were purchased from the following sources: Restriction enzymes and DNA- and RNA-modifying enzymes were purchased from Fisher/Promega, except for Geneclean™ and RNaid™ Kits from Bio 101 Inc.; Proteinase K, Calf Intestinal Alkaline Phosphatase, Lysozyme, λ-DNA and tRNA from Boehringer Mannheim; RNAguard, RNase A, Taq DNA polymerase, dNTPs and ^{T7}Sequencing™ Kits from Pharmacia Biotech; T7 RNA polymerase and Trizol from Gibco/BRL.

Silica gel PE Sil G plates were purchased from Whatman; Polaroid Instant film type 57 was from Treck Hall; Kodak XAR5 X-ray film was from

Eastman Kodak Co.; Kimax type-51 capillary tubes were purchased from Kimble; Micro-injection apparatus was from Hamilton; Agarose gel apparatus was from Pharmacia; Mini Polyacrylamide Gel Apparatus was from BioRad, Thermal cycler for PCR was from Perkin-Elmer, BAS 1000 Phosphoimager, 20x40cm Imaging Plates (Type BAS-IIIs) and MacBAS v2.x software were from Fuji.

2. Methods

2.1. Plasmid DNA Manipulations

2.1.1. Growth Conditions

Transformed bacterial strains, were cultured overnight at 37°C in Luria-Bertani (LB) broth (10 gm Bacto-tryptone, 5 gm Bacto-yeast extract, 10 gm NaCl, d.d. H_2O to 1000 ml, pH to 7.5 and sterilized by autoclaving). To make media for plates, 14.4 gm of agar was added per liter of medium before autoclaving. Ampicillin was added at a concentration of 50 µg/ml to select for the plasmids of interest.

2.1.2. Plasmid Purification

Plasmid DNA purification was performed essentially as described by Birnboim and Doly (1979). Plasmid DNA carrying an ampicillin resistance gene as a selectable marker was prepared from stable transformants of *Escherichia coli* (*E. coli*) XL-1 blue or DH5-α cells (XL-1 blue cells were used for plasmid constructs that contained an A_{73} tract, in order to prevent loss of adenosine residues). 50 ml of an overnight bacterial culture were pelleted by centrifugation at 5000xg for 10 min and resuspended in 3 ml of GTE buffer (50 mM glucose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA; pH 8.0). The bacterial suspension was lysed in 3 ml of lysis buffer (0.2 N NaOH, 1% SDS) by inverting the tube gently to mix. 3 ml of a 3 M potassium/5 M acetate neutralization solution (60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml of d.d. H_2O) were added, mixed by inversion and then centrifuged at 20,000xg for 15 min. The supernatant was collected, transferred to a clean 15 ml Falcon tube, and 30 µl of an RNase A stock solution (10 mg/ml RNase A in d.d. H_2O) were added and incubated at 37°C for 1 hour. The supernatant was extracted with an equal volume of phenol:chloroform three times and was precipitated with one volume isopropyl alcohol at -20°C for 15 minutes. The plasmid DNA was pelleted by

centrifugation at 20,000xg for 15 min and washed one time with 70% ethanol, dried and dissolved in 400 µl d.d. H_2O.

To obtain high quality plasmid DNA for sequencing or for the polymerase chain reaction, a polyethylene glycol (PEG) precipitation step was added to the above protocol (Sambrook *et al.*, 1989). The plasmid DNA was precipitated by the addition of 125 µl of 4 M NaCl and 525 µl of autoclaved 13% PEG_{8000} to the resuspended plasmid DNA and incubating on ice for 20 min. The precipitated DNA was pelleted by centrifugation at 20,000xg for 15 min at 4°C. After washing once with 70% ethanol and drying, the plasmid DNA was resuspended in 100 µl d.d. H_2O and stored at -20°C.

2.1.3. Plasmid construction

Reporter plasmids were based on a pGEM-3 plasmid containing the gene for chloramphenicol acetyltransferase (790 base pairs) inserted between the *Hin* dIII and *Bam*HI sites of the multiple cloning site. The 790 nucleotides of the CAT reporter included a small 5' UTR (approximately 50 nucleotides) and a small 3' UTR of approximately 100 nucleotides (Figure 3). The use of Sp6 RNA polymerase yielded sense RNA from *in vitro* transcription reactions.

Plasmid pGEM-CATA2 contains the 200 nucleotides of the *Xenopus* cyclin A2 3' UTR inserted downstream of the CAT reporter gene between the *Bam*HI and *Xba*I restriction sites. The 3' UTR was isolated by PCR amplification using primers engineered to have the appropriate restriction sites for insertion into pGEM-CAT, transcription with Sp6 RNA polymerase yields a transcript of 990 nucleotides (Figure 3A). Plasmid, pGEM-A2CAT, contains the 5' UTR of *Xenopus* cyclin A2 cloned by PCR with primers engineered to contain *Hin*dIII sites and inserted into the *Hin*dIII site upstream of the CAT reporter translation start site and results in a full length transcript of 1050 nucleotides when transcribed with Sp6 RNA polymerase (Figure 3B). The 260 nucleotides of the cyclin A2 5' UTR were inserted in the sense orientation and confirmed by sequencing and restriction enzyme digests. Plasmid pGEM-A2CATA2 contains the 3' UTR of *Xenopus* cyclin A2 inserted downstream of the CAT reporter gene between the *Bam*HI and *Xba*I restriction enzyme sites and the 5' UTR of *Xenopus* cyclin A2 inserted into the *Hin*dIII site

upstream of the CAT reporter gene (Figure 3C). *In vitro* transcription with Sp6 RNA polymerase yielded a transcript of 1250 base pairs.

2.1.4. Quantitation of DNA and RNA

DNA and RNA quantitations were determined with the use of a PYE Unicam SP1750 ultraviolet spectrophotometer. Samples were diluted in sterile d.d. H_2O and placed into quartz cuvettes, and absorbance values at 260 nm and 280 nm were obtained. One A_{260} unit was considered to be equivalent to 50 µg of double-stranded (ds) DNA or 37 µg of single-stranded (ss) RNA. An $A_{260/280}$ ratio indicated levels of purity with a value of 1.8 being considered essentially free of contaminating protein in a dsDNA preparation. An $A_{260/280}$ ratio of 2.0 was used to determine if RNA solutions were free from contaminating DNA or phenol.

2.2. Restriction Enzyme Digestion

Restriction enzyme digestion was performed according to the manufacturer's conditions with the assumption that 1 U of enzyme could digest 1 µg of DNA in 1 hour under optimal conditions. The digested DNA products were mixed with one-sixth volume of 6X DNA loading buffer (50% glycerol, 1 mM EDTA; pH 8.0, 0.25% bromophenol blue) and electrophoresed through a 1% agarose gel (0.3 gm of agarose in 30 ml of 1X TAE and then mixed with 2 µl of 10 mg/ml of ethidium bromide) in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA; pH 8.0) at 100 mA at room temperature. Lambda DNA digested with *Hin*dIII served as a molecular size marker. The DNA bands were visualized under UV light and photographed using Polaroid Type 57 Film. The DNA sample was then cleaned by Geneclean™ and stored in d.d. H_2O at -20°C.

If necessary, the DNA fragments were further cleaned by running the entire restriction digestion on an agarose gel. The appropriate fragment was then excised from the gel, weighed and minced in 3 volumes NaI (w/v) solution. The gel was melted by heating the gel-NaI mixture at 55°C. The

resulting DNA-NaI solution was then cleaned using the Geneclean™ Kit. The fragment was eluted in d.d. H_2O and stored at -20°C until needed.

2.3. PCR, DNA Cloning and Bacterial Transformation

2.3.1. Polymerase Chain Reaction (PCR)

Plasmid DNA for PCR amplification was prepared as stated previously and diluted to a concentration of 0.2 ng/µl. Appropriate primers, which would amplify the region of interest, were synthesized by the Morris Moloney Laboratory (University of Calgary) and were diluted to a concentration 20 µM before use in the reactions. A typical PCR reaction contained: 10 µl 10X Buffer (containing Mg^{2+}, resulting in a final concentration of 1.5 mM), 10 µl 10X dNTP mix (10 mM each dNTP), 5 µl each primer, 5 µl template DNA (1 ng or less), 64 µl d.d. H_2O and 1 µl Taq polymerase. The reaction was overlayed with 100 µl mineral oil before amplification. PCR amplification was performed for 25 cycles as follows: one cycle at 95°C for 5 min; 24 cycles at 95°C for 1 min, 50°C for 1 min, 72°C for 1 min; with a final extension at 72°C for 10 min. The entire reaction was then resolved on a 1% agarose gel, and the PCR product was excised from the gel and cleaned by Geneclean™. The purified PCR product was stored at -20°C until needed.

2.3.2. Cloning

Typical sub-cloning reactions were performed as described by Sambrook *et al.* (1989). DNA vectors and fragments to be cloned were purified from restriction digests directly or from agarose gels by Geneclean™.

Once the appropriate purified fragments were obtained, both fragments and vector were quantitated spectrophotometrically and checked by electrophoresis for quality. For use in blunt-end ligations or ligations involving a single restriction enzyme cut site, linearized vector DNA was treated with calf intestinal phosphatase (1 U/5 µg DNA) according to the manufacturers instructions. After dephosphorylation, the vector DNA was purified by

Geneclean™ and re-quantitated before use in ligations. For "sticky-end" ligations, a final reaction volume (typically 10 µl) contained: insert and vector DNA (3:1 ratio, approximately 100 ng of vector DNA), 1 µl of 10x ligase mix (10x buffer is 200 mM Tris-HCl, 50 mM $MgCl_2$, 50 mM DTT, 5 mM ATP) and 1 unit T4 DNA ligase. The ligation reaction was carried out at room temperature for 1 hour or at 4°C overnight.

2.3.3. Preparation of Competent Bacteria

E. coli DH5-α or XL1-blue cells (Stratagene) were grown in 5 ml of sterile LB broth overnight. 1 ml of the bacterial suspension was inoculated in a 50 ml sterile LB broth and grown to an optical density (A_{600}) of 0.5 to 1.0. The bacteria were pelleted in a Sorval RC-5B centrifuge at 6000xg using a SS-34 fixed angle rotor for 10 min at 4°C, resuspended in 20 ml of sterile 50 mM $CaCl_2$ on ice for 15 min and centrifuged again as above. The pellet was then resuspended in 2 ml of 50 mM $CaCl_2$, and 500 µl of sterile glycerol were added to 2 ml of competent bacteria. The bacteria were aliquoted at 100 µl/tube and stored at -80°C.

2.3.4. Transformation of Competent Bacteria

Competent bacteria (100 µl) were mixed with 10 µl of ligated DNA and left on ice for 30 min. The cells were heat-shocked at 42°C for 90 sec and transferred to ice for an additional 2 min. The cells were then mixed with 100 µl LB broth and left at 37°C for 30 min. The transformed bacteria were plated onto an ampicillin-containing (50 µg/ml) LB-agar plate and incubated overnight at 37°C.

2.3.5. Screening for Constructed Plasmids

After overnight incubation of transformed bacteria, 5 to 10 individual colonies were selected and cultured separately in 5 ml of LB broth containing 50 mg/ml of ampicillin at 37°C overnight. 1.5 ml of each culture were

transferred to a 1.5 ml microfuge tube and pelleted in a micro-centrifuge at 16,000xg for 5 min. The pellets were resuspended in 200 µl of resuspension buffer (50 mM glucose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA; pH 8.0), and 200 µl of lysis buffer (0.2 N NaOH, 1% SDS) were added and the reactions mixed by inversion. The reactions were neutralized by the addition of 200 µl neutralization solution (60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml of d.d. H_2O) and again mixed by inversion (Birnboim and Doly, 1979). The bacterial slurry was micro-centrifuged for 15 min at 16,000xg to pellet the cellular debris. The supernatant was transferred to a clean 1.5 ml microfuge tube, and 10 µl of an RNase A solution (10 mg/ml stock) were added and the plasmid preparation incubated at 37°C for 1 hour. The plasmid preparation was extracted once with phenol:chloroform (1:1 v/v), the aqueous layer was transferred to a clean 1.5 ml microfuge tube and 1 volume of isopropyl alcohol was added and incubated at -80°C for 15 min. The DNA was pelleted by microcentrifugation, washed once with 70% ethanol and air dried. The dried DNA was resuspended in 20 µl d.d. H_2O and analyzed by restriction enzyme digestion.

2.4. DNA Sequencing

2.4.1. Preparation of Sequencing Template

Plasmid DNA was purified and cleaned from bacterial culture as described previously. Double-stranded plasmid DNA for sequencing was prepared as described by Pharmacia Biotech. Briefly, 2 µg of plasmid DNA were denatured in 0.2 M NaOH for 10 min at room temperature and neutralized in 1.4 M ammonium acetate, pH 5.4. The DNA was precipitated in 2.5 volumes of ethanol before use. The DNA was then sequenced using radiolabeled ^{35}S-dATP for visualization. Sequencing was performed by the Sanger dideoxy chain termination method (Sanger, 1977) using a T7 polymerase based kit (^{T7}Sequencing™). The appropriate primers were obtained from the University Core DNA Services (University of Calgary) and were diluted to a working concentration of 5 pmol/µl.

2.4.2. Gel Preparation and Electrophoresis

Denaturing polyacrylamide sequencing gels (44 cm x 20 cm) were prepared as described in the BioRad literature. Typically, 6% polyacrylamide gels containing 7 M urea were used. Stock solutions of 40% acrylamide (38:2 acrylamide/bis-acrylamide) were used in preparing gels. Before electrophoresis, gels were pre-run until the appropriate temperature was reached (approximately 50°C). Electrophoresis buffer was 1X TBE (900 mM Tris-HCl, 900 mM boric acid, 20 mM EDTA; pH 8.0). Samples were denatured in loading buffer (80% formamide, 1M EDTA; pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol), heated to 90°C, loaded onto the gel and electrophoresed at 50 W for 1 - 3 h. Gel temperature was maintained over the running period. After electrophoresis, the gel was dried and exposed to Kodak XAR5 film overnight at -80°C.

2.5. *In vitro* Synthesis of mRNA

2.5.1. DNA Template Preparation

Plasmids to be used for the production of mRNA were digested with the appropriate restriction enzyme, following the manufacturer's directions, to completion. The linearized plasmid DNA was cleaned from the digestion reaction by Geneclean™, diluted to 0.2 µg/µl in d.d. H_2O and stored at -20°C until used for transcription. If restriction enzymes that generated 3' protruding ends were used for plasmid DNA linearization, then the linear DNA was treated with Klenow DNA polymerase before transcription. The Klenow fragment of DNA polymerase, at a concentration of 5 U/µg DNA, was added to the linear DNA solution and incubated at 22°C for 15 min. After Klenow treatment, the DNA was stored at -20°C.

2.5.2. *In vitro* Transcription of Translatable mRNA

Translatable, capped mRNA was synthesized according to standard transcription protocols (Melton *et al.*, 1984; Melton, 1987; Yisraeli and Melton, 1989). To prepare mRNA *in vitro*, the following reagents were added to an autoclaved 1.5 ml microfuge tube in the order recommended by Promega Biotech: 10 µl of 5X transcription buffer (200 mM Tris-base, pH 7.5, 50 mM $MgCl_2$, 20 mM spermidine, 50 mM NaCl), 5 µl of 100 mM DTT, 2.5 µl of RNAguard (35 U/µl), 2.5 µl each of 10 mM ATP, CTP, and UTP, 2.25 µl of 10 mM m^7GpppR (Ambion Inc.), 2.5 µl of 2 mM GTP, 2.5 µl of linearized template DNA (0.5 µg), 10 units of T7 or Sp6 RNA polymerase and DEPC-d.d. H_2O to a volume of 50 µl. The reaction was incubated at 37°C for 2 hours, after which the DNA template was digested by 3 units of DNase for 15 min at 37°C. The mRNA was cleaned using an RNaid™ kit (Bio101). A 50 µl transcription was typically adsorbed to 20 µl RNA matrix and eluted in 30 - 50 µl sterile water. The amount of RNA was determined spectrophotometrically, and the quality was determined by running an aliquot through an ethidium bromide-containing 1.0% agarose-TAE gel. The purified mRNA was stored at -80°C until used for micro-injections.

2.5.3. *In vitro* Synthesis of Radiolabeled, Translatable mRNA

Plasmid DNA was linearized with the appropriate restriction endonuclease for run-off transcription. The transcription reaction for the synthesis of radiolabeled mRNA was carried out essentially as described by Melton *et al.*, (1984). A transcription reaction (typically 20 µl) contained: 4 µl 5x transcription buffer (200 mM Tris-base, pH 7.5, 50 mM $MgCl_2$, 20 mM spermidine, 50 mM NaCl), 2 µl of 100 mM DTT, 1 µl of RNA guard (35 U/µl), 1 µl of 10 mM ATP, 1 µl of 10 mM CTP, 0.5 µl of 10 mM UTP, 1 µl of 2 mM GTP, 1 µl of 10 mM m^7GpppR (Ambion Inc.), 1.5 µl of [α-^{33}P]UTP (15 µCi at 10 µCi/µl), 1 µl of linearized template DNA (0.2 µg), 10 units of T7 or Sp6 RNA polymerase and DEPC-H_2O to a volume of 20 µl. The transcription reactions were incubated at 37°C for 2 hours. Contaminating DNA was removed by the

addition of 1 µl of RNase-free DNase (40 U/µl) and incubating at 37°C for 15 min. Radiolabeled mRNA was purified by RNaid™ kit and typically eluted in 15 µl DEPC-H_2O. 1 µl of purified RNA was counted in a liquid scintillation counter to determine specific activity of the capped mRNA, and another 1 µl was resolved on a 4% acrylamide/7 M urea gel to check for termination products. Before use in micro-injections, the RNA was quantitated at 260 nm and diluted to the proper concentration.

2.6. Handling of Gametes and Embryos

2.6.1. Preparation of Oocytes

A fully mature female was anesthetized in 0.25% Tricaine, and ovarian fragments were removed from the body cavity and placed in Modified Barth's-HEPES Solution (MBS-H: 10 mM HEPES-KOH, 0.88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca[NO_3]_2$, 0.41 mM $CaCl_2$; pH 7.6) (Kay and Peng, 1991). The body wall was sutured using Ethicon-4-0 plain gut, and the skin was sutured with Ethicon-3-0 braided silk. The frog recovered in shallow tap water until she was able to swim. For storage of oocytes for up to 3 days, the MBS-H was supplemented with 10 µg/ml each penicillin-G and streptomycin sulfate. Stage VI oocytes were removed manually from the ovarian tissue using watchmaker forceps (Dumont No. 5) and transferred to a fresh dish of MBS-H until used. Stage VI oocytes are full-grown oocytes (1.0-1.2 mm diameter), with a white band around the equator (Dumont, 1972).

For experiments examining translational regulation during oocyte maturation, the oocytes, still surrounded by the follicle cells, were incubated in 10 µg/ml progesterone in MBS-H, (Smith, 1989) until germinal vesicle breakdown (GVBD; usually 6-7 hours after the addition of progesterone) after microinjection of the reporter constructs. Only matured oocytes, as evidenced by the presence of a punctate white spot in the animal hemisphere, were selected and used for analysis.

2.6.2. Preparation of Sperm Suspensions

Testes were removed from a Tricaine-anesthetized male. They were placed in 4.5 ml 110% Marc's Modified Ringer's (MMR) (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA; pH 8.0, 5 mM HEPES; pH 7.8, 10 µg/ml penicillin G, 10 µg/ml streptomycin sulfate) supplemented with 0.5 ml Fetal Calf Serum and could be stored for several days at 4°C (Newport and Kirschner, 1982). For *in vitro* fertilization, a testis was macerated in a few drops of 100% MBS-H buffer.

2.6.3. Preparation of Fertilized Eggs

Mature female frogs that had not ovulated for at least two months were stimulated to lay eggs by the injection of 800 I.U. human chorionic gonadotropin (HCG) subcutaneously into their dorsal lymph sac about 12 hours before the eggs were required (Heikkila *et al.*, 1985). The unfertilized eggs were collected at room temperature in 34% MBS-H buffer. Fertilized eggs were prepared for micro-injection by the addition of macerated testis in 100% MBS-H to unfertilized eggs and shaking at room temp for 5 min, then allowing them to sit at room temp for 15 min. If the majority showed evidence of fertilization, the batch was dejellied with 2% cysteine-HCl in Steinberg's solution (60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca[NO_3]_2$, 0.83 mM $MgSO_4$, 10 mM HEPES; pH 7.4) neutralized to pH 7.8 with 10 M KOH. The embryos were washed 5 times with approximately 100 ml Steinberg's solution and used immediately. The first cleavage occurred within the first 1.5 hours after fertilization.

2.6.4. Preparation of Coenocytic Embryos

Dejellied, fertilized eggs were placed in the interface between a 50% and a 5%-Ficoll type-400 solution (in MMR solution) before the first cleavage and centrifuged at 500xg for 10 min. (Newport and Kirschner, 1982). Under these conditions, the centrifugation blocked cytokinesis but not karyokinesis. After centrifugation, the eggs were rinsed with 5%-Ficoll/MMR solution

thoroughly and kept in 5% Ficoll/MMR for micro-injection. The untreated eggs from the same fertilization were kept in Steinberg's solution and used as a control to determine comparable developmental stages.

2.7. Micro-injections of *Xenopus* Oocytes, Eggs and Embryos

2.7.1. Needle Preparation

Needles used for micro-injections were made with glass capillary tubes (1.5 - 1.8 x 100 mm, Kimax-51). The tubes were siliconized with Sigmacote, dried either overnight at room temperature or for 1 hour at 65°C, rinsed with chloroform, dried and autoclaved. They were then pulled to form needles using a Narashige needle-puller (magnet setting 6; 17 amps).

2.7.2. Micro-injections of Synthetic mRNAs

A MicroLab P pipettor was adapted for use in microinjection according to Hitchcock and Friedman (1980) and adjusted to deliver 50 nanolitres per cell. In all experiments, molar equivalents of RNA were injected to ensure that the amount of CAT coding capacity was equal when injecting RNA with different-sized upstream and downstream elements. After injection, the cells were transferred to medium (MBS-H for oocytes, MMR for coenocytes and matured oocytes, and Steinberg's solution for embryos) containing 4-5% Ficoll Type 400. Progesterone-matured oocytes were incubated in MMR, pre- and post-injection, that lacked Ficoll Type 400.

2.8. Sample Preparation and Assays

2.8.1. CAT Assays, Thin Layer Chromatography, and Quantitation

The samples were incubated at room temperature for 2 hours and then washed with 0.25 M Tris-HCl, pH 7.8 (CAT Assay buffer) in 1.5 ml tubes. At

this point the samples could be frozen at -80°C. The buffer was removed, and each sample was homogenized in 100 µl CAT assay buffer. The samples were then centrifuged at 16,000xg in a microfuge for 5 min. The supernatant was transferred to a new tube. The samples could be frozen at -80°C at this point.

An appropriate amount of each sample was diluted to 120 µl with CAT assay buffer. Care was taken to ensure that all CAT assays were within the linear range for the reaction (between approximately 5 per cent and 70 per cent conversion to the acetylated form). To each sample 40 µl of reaction mix were added (0.67 ml ^{14}C-chloramphenicol, 70 µg acetyl co-enzyme A in CAT assay buffer), and the reaction was incubated at 37°C for 30 min. The reaction was stopped by the addition of 1 ml of ethyl acetate. After vigorous mixing, the samples were microfuged at 16,000xg. for 5 min, and 900 µl of the ethyl acetate were transferred to a new tube. The solvent was evaporated by drying in a Savant SpeedVac attached to a water aspirator.

Each dried sample was dissolved in 10 µl ethyl acetate and spotted on TLC plates. The reactants were resolved in about 50 min by ascending chromatography in 95% chloroform:5% methanol. The plates were dried and exposed to Kodak XAR5 film or exposed to a Fuji Imaging Plate (type Bas-IIIs) for 3 hours or overnight and then read in a BAS-1000 phosphoimager system.

To determine the amount of chloramphenicol that was acetylated (and hence the amount of enzyme present in the sample), each lane on the TLC plate was either cut out, and the lower major spot down to the origin was counted separately from the upper two spots up to the solvent front or the image was analyzed on the phosphoimager using Fuji MacBAS v2.x software.

2.8.2. Protein Assays

The protein concentrations of oocytes and embryos were determined using Bradford's protein assay method (Bradford, 1979). Typically, 1 oocyte or embryo, homogenized in a 0.25 M Tris buffer, pH 7.8, was analyzed for protein concentration by comparison of the A_{595} reading to a standard curve. The standard curve was generated using BSA, diluted to concentrations ranging

from 0 µg - 20 µg and analyzed at 595 nm in a PYE Unicam SP1750 ultraviolet spectrophotometer.

2.8.3. Recovery of Radiolabeled mRNA

After micro-injection of radiolabeled transcripts, total mRNA was isolated essentially as described by Kay and Peng (1991). Briefly, oocytes or embryos were homogenized in 50 µl RNA extraction buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA; pH 8.0, 0.5% SDS) containing 400 µg/ml proteinase K, using sterile tissue homegenisers. After homogenization, another 200 µl of RNA extraction buffer were added to each sample, and the samples were incubated at 37°C for 1 hour. The samples were extracted twice with an equal volume of phenol:chloroform and once with an equal volume of chloroform. The RNA in the aqueous phase was then precipitated by the addition of 1/10th volume 3 M sodium acetate and 2 volumes ice cold 95% ethanol and incubating at -20°C overnight. The precipitated RNA was pelleted by centrifuging at 16,000xg for 10 min, washed once with 70% ethanol and allowed to air dry. The pellet was resuspended in 100 µl DEPC-H_2O, and the mRNA was then precipitated by the addition of 1 volume 8 M LiCl and incubating at -20°C overnight. The RNA was again pelleted by centrifugation at 16,000xg for 10 min. The pellet was washed once with 70% ethanol and allowed to air dry. The pellet was resuspended in 2 µl diethyl pyrocarbonate (DEPC)-treated water followed by addition of 2 µl gel dye solution ('Stop solution' from ^{T7}Sequencing™ kit), loaded onto a 4% (Tris-borate-EDTA-urea) polyacrylamide gel and run at 45 W until the bromophenol blue dye front had just reached the bottom of the gel. The gel was dried and exposed to Kodak XAR5 film overnight at -80°C. For quantitation of recovered mRNA, the dried gel was exposed to a Fuji Imaging Plate (type Bas-IIIs) for 5 hours or overnight and then read in a BAS-1000 phosphoimager system. Data were analyzed using the BAS 1000 software from Fuji.

2.8.4. Determination of Polyadenylation

2.8.4.1. Oligo-d(T) Cellulose

To determine whether the chimeric messengers were polyadenylated after microinjection into oocytes, eggs or embryos, an oligo-d(T) cellulose batch purification method was employed following the guidelines of Sambrook *et al.* (1989). Approximately 0.03 gm of oligo-d(T) cellulose were resuspended in 1 ml of 0.1 N NaOH, washed 3 times with 1 ml of DEPC-H_2O and 5 times with 1m of 1X loading buffer (20 mM Tris, pH 7.6, 0.5 mM LiCl, 1 mM EDTA; pH 8.0, 0.1% Sarkosyl) in preparation for use. Total RNA, containing the ^{33}P-labeled chimeric messenger for analysis, was isolated from 20 oocytes, eggs or embryos as described previously. The isolated RNA was resuspended in 20 µl DEPC-H_2O, and an equal volume of 2X loading buffer (40 mM Tris, pH 7.6, 1 mM LiCl, 2 mM EDTA; pH 8.0, 0.2 % Sarkosyl) was added to the sample. The mRNA was heated to 65°C for 5 min and added to 1 ml of an 1X loading buffer/oligo-d(T) cellulose slurry in a 1.5 ml microfuge tube. The samples were rotated at 4°C for 30 min to allow binding of the polyadenylated mRNA to the matrix. The matrix was pelleted at 1500xg for 4 min at 4°C and resuspended in 1 ml of 1X loading buffer, rotated for 5 min and again pelleted at 1500xg to wash. The wash steps were repeated 5X, and the matrix was resuspended in 500 µl elution buffer (10 mM Tris, pH 7.6, 1 mM EDTA, 0.05% SDS)) and rotated for 5 min at 4°C. The matrix was pelleted, and the eluant was removed to a clean 1.5 ml microfuge tube, and the elution was repeated another four times. mRNA was precipitated from the eluant by the addition of 0.1 volumes 3 M sodium acetate and 2 volumes 95% ethanol and incubating at -20°C for 15 min. The precipitated mRNA was pelleted at 16,000xg for 15 min at 4°C, dried and resuspended in 2 µl DEPC-H_2O and 2 µl loading dye. For quantitation and visualization of recovered mRNA, the dried gel was exposed to a Fuji Imaging Plate (type Bas-IIIs) overnight and then read in a BAS-1000 phosphoimager system. Data were analyzed using the BAS 1000 software from Fuji.

2.8.4.2. Oligonucleotide/RNase H Digestion Assays

Reporter transcripts that indicated the presence of a poly(A) tail, as determined by analysis with oligo-d(T) cellulose, were further characterized by the use of an oligonucleotide/RNase H treatment as described by Brewer and Ross, (1988). Briefly, total RNA was recovered from early embryos as described previously in section 2.8.3. Approximately 5 ng of micro-injected RNA were recovered for analysis. The air-dried mRNA was resuspended in 20 µl 1 mM EDTA and heated at 80°C for 10 min. The RNA was removed, cooled on ice, and 0.5 µg of oligonucleotide (complementary to nucleotides 600 - 621 of the 3' coding region of CAT) were added and the reaction incubated at room temperature for 10 min. 1 µl of a 4 M KCl solution was added to each reaction, and again the reactions were incubated at room temperature for 10 min. Finally, 20 µl of TM buffer (40 mM Tris-HCl; pH 8.0, 56 mM $MgCl_2$) and 0.5 µl of Rnase H (0.5 Units) were added to the reactions and incubated at 37°C for 25 min. As a control, 0.2 µg of poly$(T)_{15}$ (Boehringer Mannheim) was added to a duplicate set of samples when the oligonucleotide primer was added. Digestion of samples lacking poly$(T)_{15}$ with RNase H released a 376 nucleotide fragment from 0 hour samples. After 2.5 hours post-injection a smear of increasing size from 376 nucleotides was evident. Samples containing poly$(T)_{15}$, which removed the poly(A) tail from the 3' UTR fragment, resulted in loss of a visible smear (a discrete band was seen). This control served to illustrate that the smear itself was actually a polyadenosine tail. The digested RNA was recovered by phenol/chloroform extraction and precipitation with 0.1 volumes 3 M sodium acetate/2 volumes 95% ethanol and incubating at -80°C for 20 min. The precipitated mRNA was pelleted at 16,000xg for 15 min at 4°C, dried and resuspended in 2 µl DEPC-H_2O and 2 µl loading dye. The samples were then resolved on a 4% acrylamide gel and exposed to Kodak XAR5 film overnight at -80°C. For quantitation and visualization of RNase H-digested mRNA, the dried gel was exposed to a Fuji Imaging Plate (type Bas-IIIs) overnight and then read in a BAS-1000 phosphoimager system. Data were analyzed using the BAS 1000 software from Fuji.

RESULTS

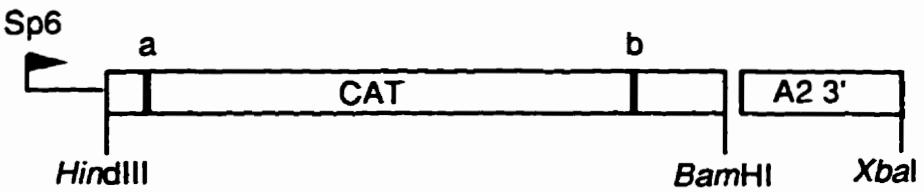
Micro-injection of synthetic mRNA into *Xenopus* oocytes and early embryos was used to investigate the effects of the 5' and 3' non-coding elements on the translation of mRNA. Constructs containing a CAT reporter gene in combination with the 5' or 3' UTR of *Xenopus* cyclin A2 sequences were used for the *in vitro* transcription of translatable mRNA (Figure 3). Approximately 0.5 nanograms of messenger RNA [corrected for the number of transcripts relative to a CAT(A)$^{-}$ transcript] were micro-injected into late stage VI oocytes or early embryos, and translation was allowed to occur for 2 hours (6 hours or until GVBD for progesterone-matured oocytes) before samples were collected for analysis of CAT activity, recovery of injected mRNA or determination of the status of polyadenylation.

1. Effects of the *Xenopus* Cyclin A2 3' Non-Coding Region on Reporter Translation

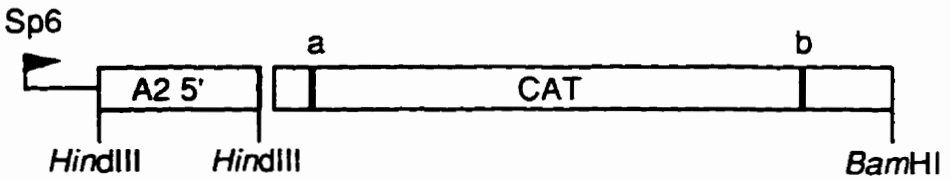
Because the translation of *Xenopus* cyclin A1 has previously been demonstrated to depend upon sequences located within the 3' non-coding region of the mRNA (Sheets *et al.*, 1994), I examined whether a similar mechanism exists for the translational regulation of cyclin A2 mRNA during oogenesis and early development.

The 3' UTR of cyclin A2 mRNA from nucleotide 1502 to 1687 was cloned through PCR, and *Bam*HI and *Xba*I restriction enzyme sites were added to facilitate ligation into a CAT reporter construct (Figure 3A and Figure 4). These 200 nucleotides include a putative cytoplasmic polyadenylation element (CPE) shown in bold-face at position 1600 to 1607 and a uridine-rich element (underlined) at position 1650 to 1666. Neither the significance nor the function of these sequence elements was tested in these experiments. The normal consensus sequence for nuclear polyadenylation (A_2UA_3), which is usually located downstream of the CPE, cannot be identified in this sequence. Although the A_2UA_3 consensus sequence is not evident, there are sequences present that may function in this role (Figure 4),

A. pGEM-CATA2



B. pGEM-A2CAT



C. pGEM-A2CATA2

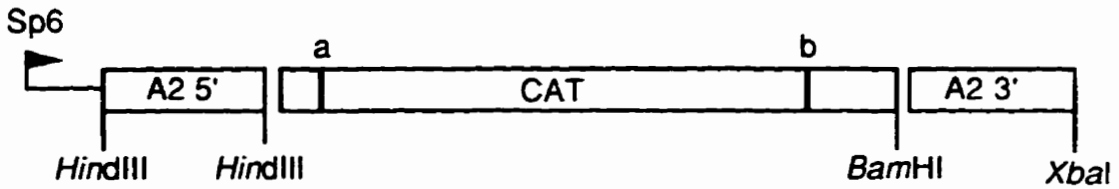


Figure 3: Schematic drawings of linearized reporter constructs used for testing the effects of the *Xenopus* cyclin A2 5' and 3' non-coding regions on the translation of CAT mRNA. All transcripts were synthesized *in vitro* and micro-injected into oocytes or early embryos for testing. A, pGEM-CATA2 linearized with *Xba*I yielding a reporter construct of 990 nucleotides when transcribed with Sp6 RNA polymerase *in vitro*. B, pGEM-A2CAT linearized with *Xba*I yielding a reporter construct of 1050 nucleotides when transcribed with Sp6 RNA polymerase *in vitro*. C, pGEM-A2CATA2 linearized with *Xba*I yielding a reporter construct of 1250 nucleotides when transcribed with Sp6 RNA polymerase *in vitro*. (a) denotes the position of the AUG start codon of CAT; (b) indicates the position of the CAT stop codon, which is approximately 100 nucleotides upstream of the BamHI site.

*gaucc*AUAAA CUUGAAGAAU GGACUCUAAA AUGUAUAUUG

AUAAAAGCAC AUGAUGUACA GUUAA**UUUUU UAU**CAUAUAG

GCUUUGGUUG UAUAUCUCAA ACUUCAACAU GACGUUUGGA

GCGACAGAAU UAUGAAGCAA AGUAAUUUUU UUUUUAUUUU

AAAAGGUUUU AAAUUUUGUA AAAACGGAA*ucuag*

Figure 4: Sequence of the *Xenopus* cyclin A2 3' non-coding region from nucleotide 1502 to 1687. The *Bam*HI and *Xba*I sites used for directional cloning are indicated in lowercase. The putative nuclear polyadenylation element and CPE are in bold face, and the polyuridine sequence is underlined.

but the role of these sequences was not examined in this project. Perhaps the lack of the normal consensus A_2UA_3 sequence has functional implications for the translation of the pGEM-CATA2 or pGEM-A2CATA2 reporter constructs during oogenesis and development. As well, there are a number of AU-rich elements within the 3' UTR that may play functional roles in the translation of the transcript.

Stage-specific modulation of reporter gene expression was observed for the micro-injected, pGEM-CATA2 reporter construct (Figure 5). The cyclin A2 3' UTR inhibited CAT activity in stage VI oocytes and in oocytes matured in 10 µg/ml progesterone by approximately 60 per cent when 0.5 nanograms of mRNA were injected. At the time of fertilization repression was alleviated, and the reporter gene was expressed at levels 50 per cent higher than the level of a pGEM-CAT(A)$^-$ control. Using coenocytic embryos, staged at the mid-blastula transition by comparison to control embryos, it was demonstrated that at approximately the time of the MBT (stage 8), repression was restored, and pGEM-CATA2 expression was repressed approximately 25 per cent. A Kruskel-Wallis test for non-parametric statistics (Systat 5.2.1 for the Macintosh) was employed for analysis to account for the normalization of the data relative to CAT(A)$^-$. Fluctuations in reporter expression during late oogenesis and early development were highly significant relative to control transcripts lacking the 3' non-coding region (see Table 4; Appendix A).

The differences in the levels of reporter translation during late oogenesis and early development appear to be due to differences in the translatability of the pGEM-CATA2 transcripts during early development and not a result of mRNA instability (Figure 6 and Table 1). At all stages of development, greater than 69 per cent of the micro-injected, radiolabeled mRNA could be recovered from the oocytes or early embryos after 2 or 6 hours post-injection when 0.5 nanograms of mRNA were injected. Correcting the relative levels of expression for the per cent of micro-injected mRNA recovered did not significantly affect the results.

Because the 3' non-coding region contains the elements responsible for regulating polyadenylation (which has been demonstrated to control both the timing and extent of translation for specific mRNAs), the status of polyadenylation of reporter constructs containing the 3' UTR of cyclin A2 was

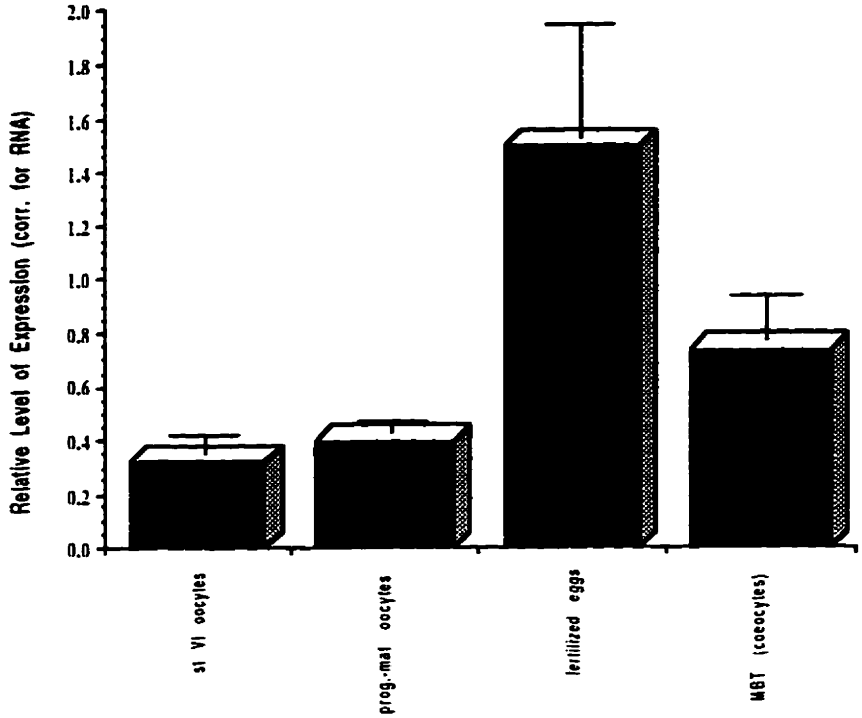


Figure 5: Effects of the cyclin A2 3' UTR on reporter gene expression during late oogenesis and early development of *Xenopus*. After micro-injection, translation was allowed to proceed for 2 hours (6 hours or until GVBD for progesterone-matured oocytes). The relative level of CAT activity was determined by comparison to a pGEM-CAT(A)$^-$ control, which was set at 1.0. All values were corrected for the percentage of micro-injected RNA recovered after incubation. Standard deviation (error bars) was calculated from relative levels of CAT expression.

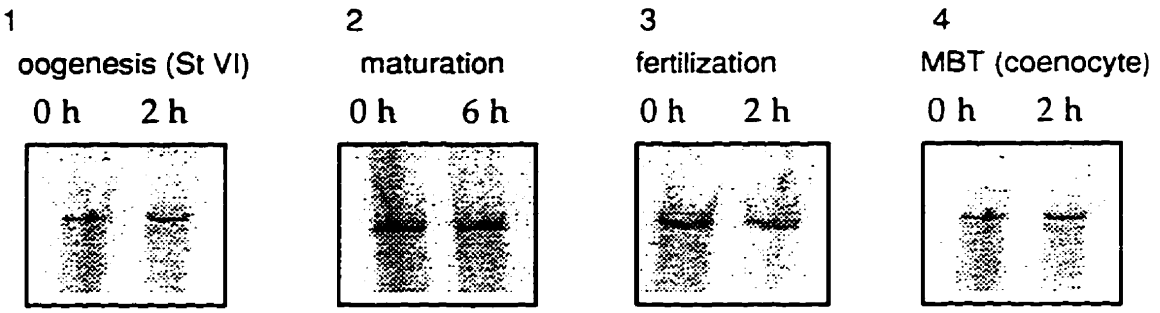


Figure 6: Recovery of micro-injected, radiolabeled pGEM-CATA2 mRNA from late oogenesis and early developmental stages of *Xenopus laevis*. Results are quantitated in Table 1. 1) oogenesis (Stage VI oocytes); 2) progesterone-matured stage VI oocytes; 3) fertilized eggs; 4) coenocytic embryos staged at the MBT.

Table 1: Effects of the *Xenopus* Cyclin A2 3' UTR on CAT reporter gene expression during early development (2 hour translation).

Stage of Development	Relative Level of Expression*	% RNA Recovered	Relative Level of Expression Corrected for RNA	Status of Polyadenylation
stage VI oocytes	0.251 ± 0.036	77.62 ± 4.93	0.323 ± 0.064	---
maturation	0.307 ± 0.102	78.12 ± 5.7	0.393 ± 0.045	---
fertilization	1.036 ± 0.356	68.9 ± 4.6	1.50 ± 0.413	polyadenylated
MBT (coenocyte)	0.559 ± 0.092	75.36 ± 7.4	0.741 ± 0.172	polyadenylated

* Expression relative to CAT(A)$^-$ reporter after 2 hours, where expression of CAT(A)$^-$ reporter equals 1.0. Standard deviation (± values) was calculated from relative levels of CAT expression or from computer analysis of mRNA recoveries. Dashed line indicates that polyadenylation did not occur.

examined by passing the recovered RNA over an oligo-d(T) cellulose matrix. Micro-injected pGEM-CATA2(A)- mRNA was recovered from both fertilized eggs and from coenocytic embryos staged at the mid-blastula transition but not from stage VI oocytes or progesterone-matured oocytes (Figure 7) as a result of interactions between the oligo-d(T) and polyadenosine residues. To examine further the possibility that the 3' terminus of these transcripts was polyadenylated after micro-injection, the samples were analyzed by an oligonucleotide/RNase H assay digestion. Digestion of the samples with RNase H released a 376 nucleotide fragment consisting of the 196 nucleotides of the cyclin A2 3' UTR and 200 nucleotides from the 3' coding sequence of CAT. The presence of a smear instead of a discrete band for the 376 nucleotide 3' fragment illustrated in figure 8 (lower band) indicated that transcripts containing the 3' UTR of cyclin A2 were polyadenylated to different extents after fertilization when micro-injected into fertilized eggs or into coenocytic embryos staged at the MBT (incubated for 2.5 hours at room temperature). Additionally, the addition of a $poly(T)_{15}$ oligonucleotide to the digestion reaction resulted in the loss of a smear and the return of a discrete band at approximately 376 nucleotides, which demonstrates that the smear is a result of a variable number of adenosine residues. These effects were quantitated using the phosphoimager software to demonstrate that there was an increase in radioactivity in the region above the band after fertilization. Analysis of the 376 nucleotide fragment at the 0 hour time point indicated that 77 per cent of the radiolabeled RNA was located at approximately this size. After 2.5 hours post-injection, in both fertilized eggs and coenocytic embryos, approximately 74 per cent of the radiolabeled RNA was located above the 376 nucleotide fragment (data not shown) and only 27 per cent was located at approximately 376 nucleotides. This quantifiable increase is a result of polyadenylation, because the addition of $poly(T)_{15}$ to the 2.5 hour sample decreased the percentage of RNA above 376 nucleotides by 30 - 40 per cent (data not shown).

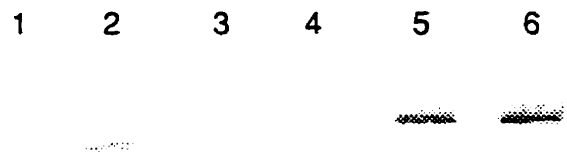


Figure 7: Recovery of micro-injected, radiolabeled pGEM-CATA2 mRNA by oligo-d(T) cellulose. 1) pGEM-CAT(A)$^-$ negative control, 2) pGEM-CAT$(A)_{73}$ positive control, 3) stage VI oocytes, 4) progesterone-matured stage VI oocytes, 5) fertilized eggs, 6) MBT (coenocytic embryos).

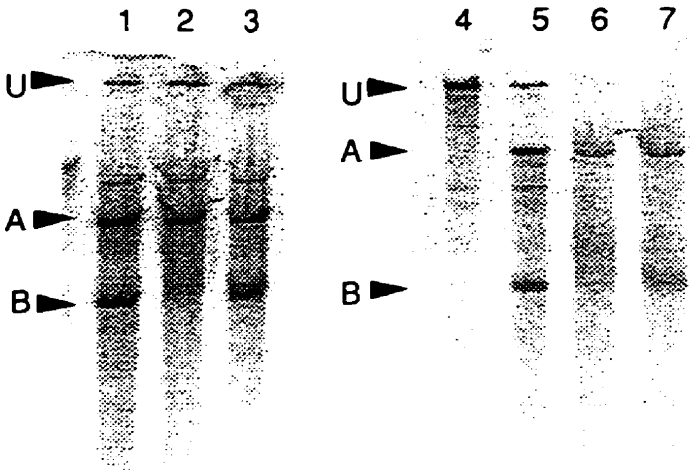


Figure 8: RNase H/oligonucleotide digestions of micro-injected, radio-labeled pGEM-CATA2 mRNA in *Xenopus* early embryos: 1) 0 hr from fertilized eggs; 2) 2.5 hr from fertilized eggs; 3) 2.5 hr + poly$(T)_{15}$ from fertilized eggs; 4) undigested RNA, 5) 0 hr from coenocytic embryos; 6) 2.5 hr from coenocytic embryos; 7) 2.5 hr + poly$(T)_{15}$ from coenocytic embryos. The upper band (A) corresponds to the remainder of the CAT coding sequence after digestion, and the lower band (B) indicates the 376 nucleotide fragment of interest. The presence of undigested RNA is indicated (U).

2. Effects of the *Xenopus* Cyclin A2 5' Non-Coding Region on Reporter Translation

Cyclin A2 protein is not normally expressed in significant amounts before the early gastrula transition (EGT) during *Xenopus laevis* development (Howe *et al.*, 1995), in contrast to the case of cyclin A1. The results indicate that CAT transcripts containing the 3' UTR from cyclin A2 do not reflect the behavior of native cyclin A2 transcripts during late oogenesis and early development (Howe *et al.*, 1995). Based on these observations, I examined the effects of the *Xenopus* cyclin A2 5' non-coding region on the translation of a CAT reporter during late oogenesis and early development.

The sequence of the 260 nucleotides of the *Xenopus* cyclin A2 5' UTR from nucleotide 1 to 260 is shown in Figure 9A. The sequence was cloned utilizing PCR, and *Hin*dIII linkers were added at either end (lowercase) to facilitate cloning it upstream of the CAT reporter gene translation start site (Figure 3B). A single upstream AUG codon is found at positions 23-25 (underlined). The upstream AUG codon does not occur in a favorable context, lacking a guanosine residue at position 4 (Kozak, 1986a; Kozak, 1989a; Kozak, 1995) and therefore would probably not have a significant effect on repressing translation. The actual translation start site for the native cyclin A2 molecule was not included in the 260 bp fragment. The 260 nucleotides have the potential to form secondary structure with a predicted stability of ΔG=-73 kcal/mol determined using the Fold algorithm of Zuker and Stiegler (1981) in the GCG sequence analysis software package (Figure 9B). The predicted stability of -73 kcal/mol is sufficient to inhibit translation, however, it remains to be demonstrated whether this predicted structure is real and significant.

Similar to the case for the 3' UTR alone, the 5' UTR of cyclin A2 inhibited CAT reporter gene translation during late oogenesis and at the MBT (Figure 10). In stage VI oocytes and in progesterone-matured oocytes, the 5' UTR negatively affected the translation of the CAT reporter gene. Micro-injection of 0.5 nanograms of mRNA, corrected for the number of RNA transcripts relative to CAT(A)$^-$, resulted in a 55 per cent and 70 per cent inhibition, respectively. Fertilization resulted in a loss of translational

A)

*agcuu*AUUCC GGAAAAAGCA GCUGCUGAUG UAGUGAAGGG

AUUGCUAUAG AGGCGCAUU GAGAAGCGGU GGGAGUGGGA

GGCUUUAGAU CAAUAUACCG GCAUACAAGA GGGAAGGGGU

CUAAGGCCG AGCUGCAGAC GGUAACCCAC AGUCUUUUAC

UUACACCACU AGGGAGGGCA GGGGACGUGC AACCGCGCAC

UCUCCUACAG GCGCCAGUCC UUCCCCGCUU UCUUCUUCCC

UCCUGGUCGG UAUCCGCACU*acgt*

B)

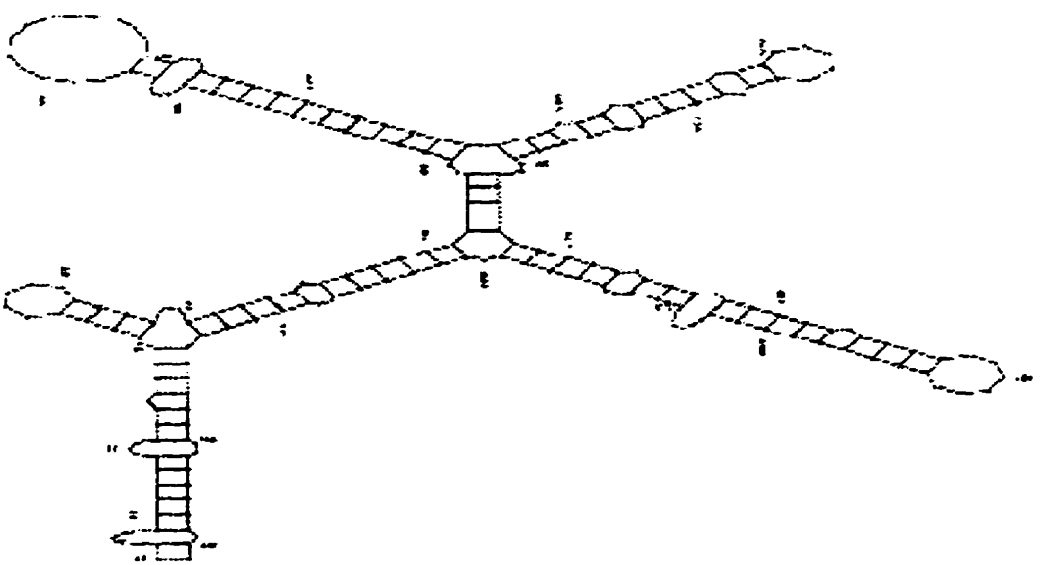


Figure 9: Sequence and putative secondary structure of the *Xenopus* cyclin A2 5' non-coding region. A. Sequence of the 5' UTR indicating the presence of an upstream AUG codon (underlined); B. Secondary structure predicted using the Fold algorithm of Zuker and Stiegler (1981) in the GCG sequence analysis software package. The stability of this structure is ΔG=-73 kcal/mol.

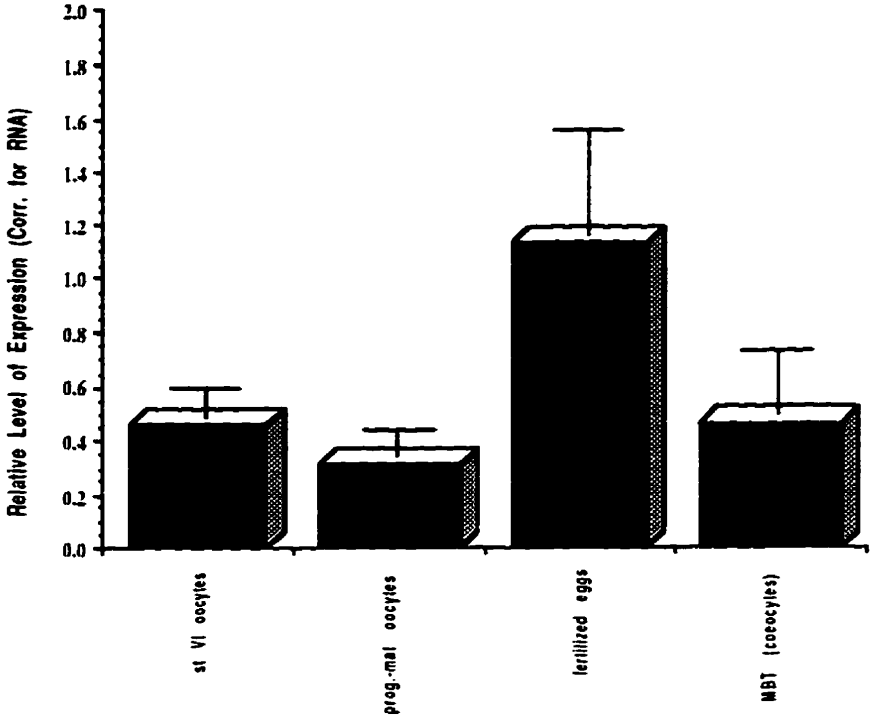


Figure 10: Effects of the cyclin A2 5' UTR on reporter gene expression during late oogenesis and early development of *Xenopus*. After micro-injection, translation was allowed to proceed for 2 hours (6 hours or until GVBD for progesterone-matured oocytes). The relative level of CAT activity was determined by comparison to a CAT(A)$^-$ control, which was set at 1.0. All values were corrected for the percentage of micro-injected RNA recovered after incubation. Standard deviation (error bars) was calculated from relative levels of CAT expression.

repression, and the pGEM-A2CAT reporter transcript was translated at levels equal to a CAT reporter gene lacking the 5' non-coding sequence. Later in development, around the time of the mid-blastula transition, translational repression was again evident with reporter expression repressed 44 per cent (examined using coenocytic embryos micro-injected with 0.5 nanograms of capped pGEM-A2CAT mRNA). These results were analyzed using Kruskal-Wallis non-parametric statistics, which assigned a high degree of significance to the changes in CAT reporter gene expression during the four developmental stages analyzed (see Table 4; Appendix A). The statistics indicated that the expression was repressed relative to the controls in stage VI oocytes and in progesterone-matured oocytes. At fertilization, statistical analysis shows a significant increase in translation relative to the levels of translation observed in both stage VI oocytes and progesterone-matured oocytes. Subsequently, at the MBT, the translational repression was significant relative either pGEM-CAT(A)- controls.

The majority (greater than 68 %) of the mRNA micro-injected into oocytes or early embryos could be recovered after 2 hours of translation (6 hours for progesterone-matured oocytes) demonstrating that the translational repression was not due to mRNA stability changes during development (Figure 11 and Table 2).

As expected, micro-injected transcripts containing only the 5' UTR of *Xenopus* cyclin A2 could not be recovered from either oocytes or early embryos by oligo-d(T) cellulose, indicating that they were not polyadenylated during any stage of development tested (Figure 12). Because there was no evidence for polyadenylation, an oligonucleotide/RNase H digestion assay was not employed to analyze the 3' UTR for the extent of polyadenylation.

3. Combinatorial effects of the *Xenopus* Cyclin A2 5' and 3' Non-Coding Regions on Reporter Translation

My observations on the translation of CAT reporter genes, containing either the 3' or 5' non-coding sequences of cyclin A2 alone, demonstrated that neither element by itself could account for the pattern of translation of native cyclin A2 during *Xenopus* late oogenesis and early development. To determine if the 5' and 3' UTR's cooperated to influence translation, a reporter

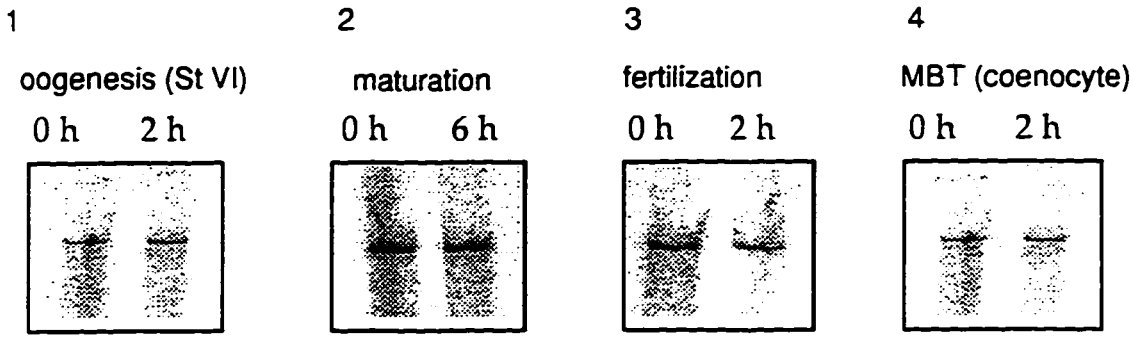


Figure 11: Recovery of micro-injected, radiolabeled pGEM-A2CAT mRNA from late oogenesis and early developmental stages of *Xenopus laevis*. Results are quantitated in Table 2. 1) oogenesis (Stage VI oocytes); 2) progesterone-matured stage VI oocytes; 3) fertilized eggs; 4) coenocytic embryos staged at the MBT.

Table 2: Effects of the *Xenopus* Cyclin A2 5' UTR on CAT reporter gene expression during early development (2 hour translation).

Stage of Development	Relative Level of Expression*	% of RNA Recovered	Relative Level of Expression corrected for RNA	Status of Polyadenylation
stage VI oocytes	0.375 ± 0.058	82.34 ± 12.7	0.455 ± 0.105	---
maturation	0.287 ± 0.082	84.12 ± 5.7	0.311 ± 0.098	---
fertilization	0.803 ± 0.312	70.6 ± 3.2	1.13 ± 0.388	---
MBT (coenocyte)	0.331 ± 0.183	71.63 ± 3.81	0.462 ± 0.247	---

* Expression relative to CAT(A)$^-$ reporter after 2 hours, where expression of CAT(A)$^-$ reporter equals 1. Standard deviation (± values) was calculated from relative levels of CAT expression. Dashed line indicates polyadenylation did not occur.

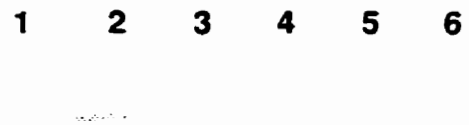


Figure 12: Recovery of micro-injected pGEM-A2CAT mRNA by oligo-d(T) cellulose. 1) pGEM-CAT(A)˙ negative control, 2) pGEM-CAT$(A)_{73}$ positive control, 3) stage VI oocytes, 4) progesterone-matured stage VI oocytes, 5) fertilized eggs, 6) MBT (coenocytic embryos).

construct containing the CAT reporter gene and both the 5' and 3' UTRs of cyclin A2 was made (pGEM-A2CATA2) (Figure 3C).

Transcripts containing both the 5' and 3' non-coding elements were translationally repressed compared to the controls at all stages of late oogenesis and early development tested (Figure 13). In stage VI oocytes and progesterone-matured oocytes, the translation of approximately 0.5 nanograms of micro-injected mRNA was repressed approximately 80 per cent relative to a CAT(A)$^-$ control. Although there was a 25 per cent increase in pGEM-A2CATA2 reporter translation between stage VI oocytes and fertilized eggs, the level of translation of the pGEM-A2CATA2 reporter construct in fertilized egg was still repressed by 65 per cent relative to the control lacking both the 3' and 5' UTR. It appears that both the 5' and 3' UTR function synergistically to repress translation during early *Xenopus* development. The effect does not appear to be strictly additive of the inhibition of the two non-coding elements because when either the 5' or 3' UTR alone was present in fertilized eggs, there was no repression of reporter translation, using equivalent numbers of mRNA transcripts. This would seem to suggest a synergistic effect between the 3' and 5' end of the molecule that prevents activation of translation at the time of fertilization.

As illustrated in figure 14 and table 3, the inhibitory effect of the 5' and 3' UTRs on pGEM-A2CATA2 reporter translation was not due to differences in mRNA stability during late oogenesis and early development. Greater than 66% of the micro-injected radiolabeled RNA could be recovered from either oocytes or early embryos after 2 hours (6 hours for progesterone-matured oocytes).

As was the case with the 3' UTR alone, transcripts containing both the 3' and 5' UTR of cyclin A2 were polyadenylated after fertilization and in coenocytic embryos staged at the MBT, as determined by oligo-d(T) cellulose analysis of the recovered RNA (Figure 15). The polyadenylation of the injected RNA was confirmed by RNase H/oligonucleotide digestion of the recovered RNA (Figure 16). Samples from 0 hour timepoints released a 376 nucleotide fragment (consisting of 196 nucleotides from the 3' UTR of cyclin A2 and 200 nucleotides from the 3' coding sequence of CAT; lower band) when digested with Rnase H. Digestion of RNA from either fertilized eggs or coenocytic embryos (after 2.5 hours post-injection) indicated a smear of

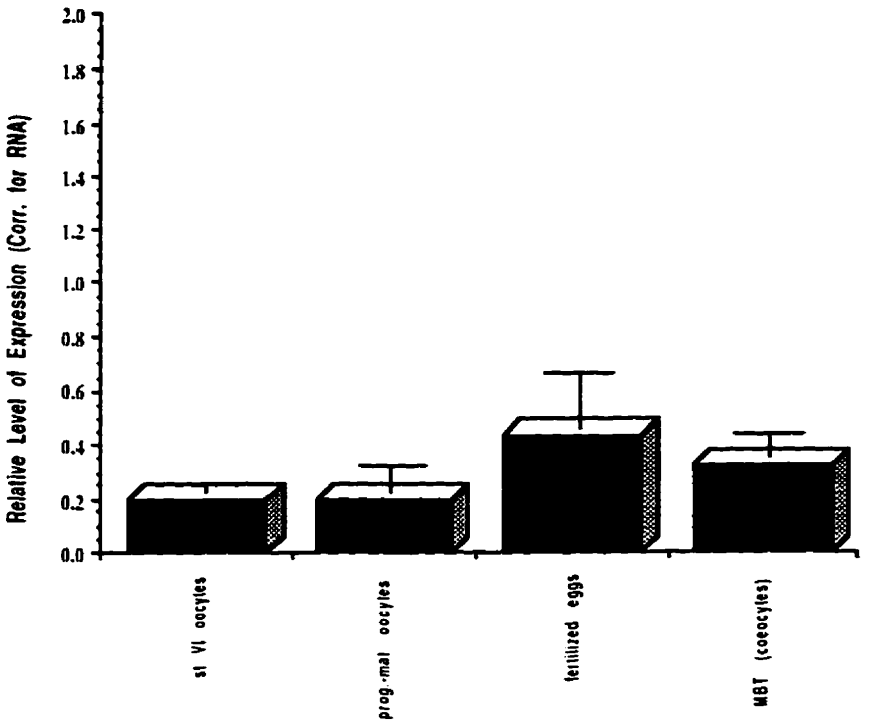


Figure 13: Effects of the cyclin A2 5' and 3' UTRs on reporter gene expression during late oogenesis and early development of *Xenopus*. After micro-injection, translation was allowed to proceed for 2 hours (6 hours or until GVBD for progesterone-matured oocytes). The relative level of CAT activity was determined by comparison to a pGEM-CAT control, which was set at 1.0. All values were corrected for the percentage of micro-injected RNA recovered after incubation. Standard deviation (error bars) was calculated from relative levels of CAT expression.

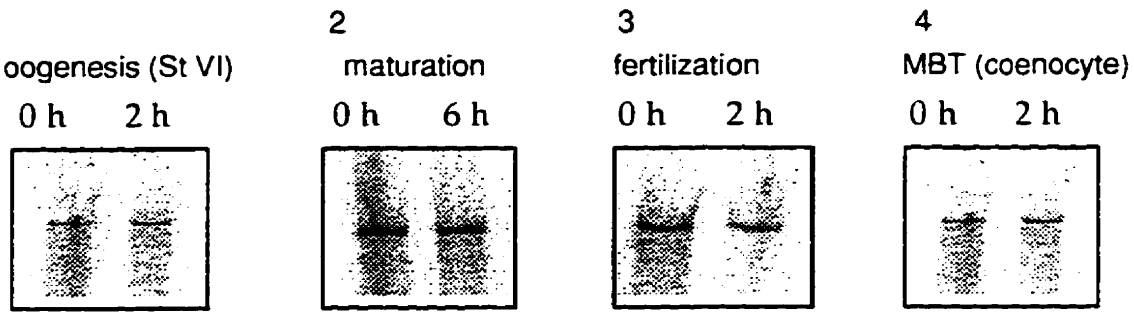


Figure 14: Recovery of micro-injected, radiolabeled pGEM-A2CATA2 mRNA from late oogenesis and early developmental stages of *Xenopus laevis*. Results are quantitated in Table 3. 1) oogenesis (Stage VI oocytes); 2) progesterone-matured stage VI oocytes; 3) fertilized eggs; 4) coenocytic embryos staged at the MBT.

Table 3: Effects of the *Xenopus* cyclin A2 3' and 5' UTRs on CAT reporter gene expression during early development (2 hour translation).

Stage of Development	Relative Level of Expression*	% of RNA Recovered	Relative level of Expression corrected for RNA	Status of Polyadenylation
stage VI oocytes	0.133 ± 0.020	66.65 ± 5.1	0.199 ± 0.032	---
maturation	0.167 ± 0.092	83.26 ± 5.7	0.201 ± 0.097	---
fertilization	0.314 ± 0.124	72.9 ± 4.87	0.431 ± 0.210	polyadenylated
MBT (coenocyte)	0.251 ± 0.135	79.1 ± 4.95	0.317 ± 0.097	polyadenylated

* Expression relative to CAT(A)$^-$ reporter after 2 hours, where expression of CAT(A)$^-$ reporter equals 1. Standard deviation (± values) was calculated from relative levels of CAT expression. Dashed line indicates polyadenylation did not occur.

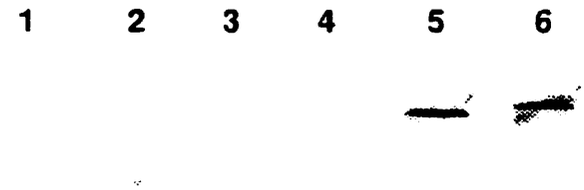


Figure 15: Recovery of micro-injected pGEM-A2CATA2 mRNA by oligo-d(T) cellulose. 1) pGEM-CAT(A)$^-$ negative control, 2) pGEM-CAT$(A)_{73}$ positive control, 3) stage VI oocytes, 4) progesterone-matured stage VI oocytes, 5) fertilized eggs, 6) MBT (coenocytic embryos).

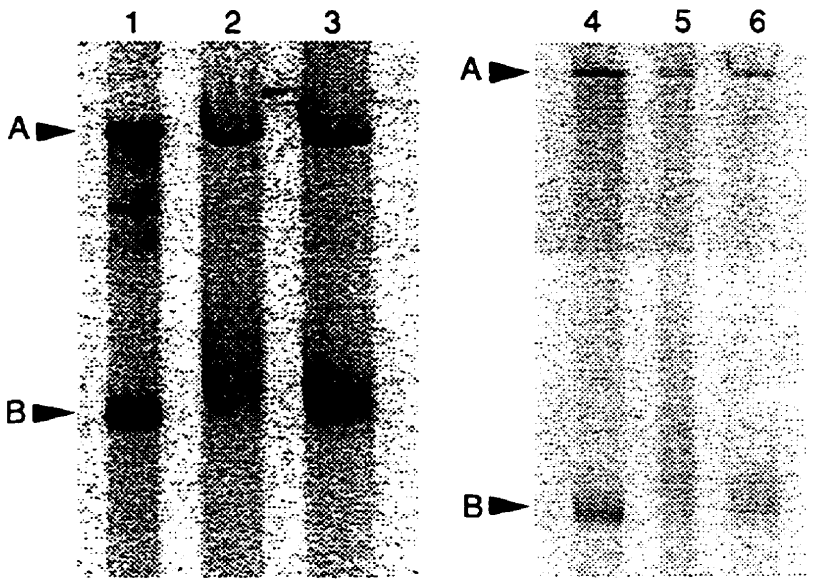


Figure 16: RNase H/oligonucleotide digestions of micro-injected pGEM-A2CATA2 mRNA in *Xenopus* early embryos: 1) 0 hr from fertilized eggs; 2) 2.5 hr from fertilized eggs; 3) 2.5 hr + poly$(T)_{15}$ from fertilized eggs; 4) 0 hr from coenocytic embryos; 5) 2.5 hr. from coenocytic embryos; 6) 2.5 hr + poly$(T)_{15}$ from coenocytic embryos. The upper band (A) corresponds to the remainder of the CAT coding sequence after digestion, and the lower band (B) indicates the 376 nucleotide fragment of interest.

increasing size from 376 nucleotides. The sequence elements contained within the *Xenopus* cyclin A2 3' non-coding region enable the reporter transcripts to be polyadenylated during post-maturation stages (fertilization, MBT) but do not allow polyadenylation to occur before fertilization. Additionally, the addition of a poly$(T)_{15}$ oligonucleotide to the digestion reaction resulted in a loss of a smear and the return of a discrete band, which demonstrates that the smear was a result of a variable number of adenosine residues. These effects were quantitated using the software to demonstrate that there was a quantifiable increase of the region above the band and the band itself following fertilization. Analysis of the 376 nucleotide fragment at the zero hour time point indicated that 81 per cent of the radiolabeled RNA was located at approximately 376 nucleotides in size. After 2.5 hours post-injection, in both fertilized eggs and coenocytic embryos, approximately 65 per cent of the radiolabeled RNA was located above 376 nucleotides (data not shown). This quantifiable increase is a result of polyadenylation because the addition of poly$(T)_{15}$ to the digestion (2.5 hour sample) decreased the percentage of radiolabeled mRNA above 376 nucleotides by approximately 36 per cent (data not shown).

DISCUSSION

Gene expression can be regulated at a multitude of points: from the time of gene transcription to the actual activation of the protein. Translation is an interesting method of regulation, because the mRNA is available to the protein synthesis apparatus in the cytoplasm but it is kept in an untranslated state. Messenger RNA transcripts can be retained in this untranslated state by mechanisms that are either intrinsic to the mRNA molecule itself or due to its interaction with *trans*-acting factors.

The differential translation of cyclin A1 and A2 messengers found in the *Xenopus* oocyte and early embryo suggested that these maternal transcripts are translationally regulated. Cyclin A1 is the predominant A-type cyclin protein synthesized throughout early development until approximately the time of the early gastrula transition (EGT; Howe *et al.*, 1995). Cyclin A2 is not expressed significantly until the time of the EGT, which suggests that the messenger itself contains elements responsible for its translational regulation.

The roles of the 5' and 3' non-coding regions of the *Xenopus* cyclin A2 mRNA, in translational regulation, were examined using a reporter gene expression system. The effects of the 5' and 3' non-coding regions on CAT reporter gene translation were examined at four early developmental stages of *Xenopus*: stage VI oocytes, progesterone-matured stage VI oocytes, fertilized eggs and coenocytic embryos staged at the MBT. These stages provided a broad overview of early *Xenopus* development from the time of late oogenesis to maturation and up until the onset of zygotic transcription.

1. Effects of the 3' UTR

The *Xenopus* cyclin A2 3' UTR, consisting of 200 nucleotides of the 3' non-coding region, was inserted downstream of the CAT reporter gene termination codon (pGEM-CAT A2). In stage VI oocytes and in progesterone-matured stage VI oocytes, the 3' UTR alone was sufficient to inhibit translation by at least 2-fold relative to messengers lacking the 3' UTR (Figure 5). After fertilization, the 3' non-coding region itself was unable to repress CAT reporter gene translation, which suggests possible activation of *trans*-acting factors or

alleviation of repressors that are either directly or indirectly involved in the translational regulation of cyclin A2 . Later in development, at approximately the time of the MBT (analyzed using coenocytic embryos staged at the MBT) the translational inhibition was again evident, possibly due to a down-regulation of *trans*-acting factors at the onset of zygotic gene transcription.

Many different possibiliities exist to explain the observed stage-specific regulation of reporter translation. Activation or down regulation of specific or general components of the translational machinery directly or *via trans*-acting factors could account for the observed pattern of expression. Another possibility is the saturation of limiting factors. The activity of these factors may be low, either due to low levels of the factors or low activity levels, during oogenesis and maturation, which would prevent effecient translation. After fertilization, increased activity or levels of these transcripts would prevent saturation and allow for increased levels of translation.

One likely candidate for regulating translation in a stage-specific manner is polyadenylation. Many examples have been characterized where the polyadenylation of the message is wholly or partly responsible for regulating translation. There are two functionally distinct polyadenylation activities in the cell: The first lacks specificity and occurs while the transcript is still in the nucleus; the second requires specific sequences, is cell-type specific and occurs in the cytoplasm (Richter, 1996). Generally the sequences that regulate the polyadenylation of mRNAs are located within the 3' non-coding region of the message. Cytoplasmic polyadenylation generally functions to control translation and activation of non-translated mRNA pools (Richter, 1996). Experimental evidence suggests that there are two different sets of signals for polyadenylation. The first is active during oocyte maturation, and the second controls polyadenylation during embryogenesis (Paris *et al.*, 1988; Paris and Philippe, 1990).

In contrast to the developmental expression of cyclin A1, which is regulated entirely by the 5' UTR and polyadenylation (Sheets *et al.*, 1994), polyadenylation of cyclin A2 does not appear to be sufficient for translational regulation during early *Xenopus* development. Messengers containing the 3' UTR of cyclin A2 were apparently polyadenylated in a stage-specific manner, as indicated by mRNA recovered by oligo-d(T) cellulose and confirmed through the use of an RNase H/oligonucleotide assay (Figure 8). In spite of

the translational repression of the native transcript throughout oogenesis and early development the 3' UTR of cyclin A2 was able to promote polyadenylation and moderate stimulation of translation after fertilization.

Polyadenylated RNA was recovered from both fertilized eggs and from coenocytic embryos staged at the MBT. The inability to recover polyadenylated messengers from either stage VI oocytes or from progesterone-matured oocytes indicated that the sequence elements contained with in the 3' UTR are responsible for controlling post-fertilization polyadenylation but not maturation-specific polyadenylation (Paris *et al.*, 1988; Paris and Philippe, 1990). This result appears to agree with an analysis of the 3' UTR sequence, which shows the presence of a CPE at position 1571 to 1579, that does not have an A_2UA_3 hexanucleotide consensus sequence (the nuclear polyadenylation element) located in close proximity. Experiments have demonstrated that the lack of either sequence element prevents maturation-specific translation (Paris and Richter, 1990; Paris and Philippe, 1990; McGrew *et al.*, 1989). In addition, work by Fox *et al* (1989) discovered that point mutations in the A_2UA_3 hexanucleotide would prevent maturation-specific polyadenylation. Although the consensus sequence for the nuclear polyadenylation signal (A_2UA_3) is not evident in the sequence for the *Xenopus* cyclin A2 gene, a detailed analysis of sequences in the 3' UTR was not performed, and it is not clear whether any of the other AU-rich sequence elements may function in this regard. In addition, there have also been published examples of transcripts whose polyadenylation is not regulated by their 3' UTR and lack either a A_2UA_3 hexanucleotide sequence or a CPE. For example, Standart and Dale (1993), discovered that the polyadenylation of ribonucleotide reductase mRNA in fertilized egg extracts of *Spisula* required neither a CPE or A_2UA_3 for polyadenylation to occur. In *Drosophilia* embryos, at least three mRNAs (*bicoid*, *Toll*, and *torso*) undergo cytoplasmic polyadenylation but have not yet had the sequences responsible defined (Salle *et al.*, 1994), although the CPE and A_2UA_3 alone are probably not sufficient.

In addition, not all polyadenylation is regulated by *cis*-sequences located in the 3' UTR of an mRNA. The *Drosophilia* Mst87F transcript is synthesized in primary spermatocytes, stored in an translationally inactive form for 3 days during spermiogenesis and then undergoes cytoplasmic

polyadenylation and translational activation (Schafer *et al.*, 1990). Schafer *et al.*, localized the regulatory region to the 5' UTR at a position +28 relative to the transcription start site. This regulatory region is composed of the consensus sequence ACAUCAAAAUUU (Schafer *et al.*, 1990).

The lack of polyadenylation at maturation may have important implications in gene control, because it would prevent the activation of cyclin A2 at an inappropriate time. Although polyadenylation occurs after fertilization, it is not clear if this event has an significant effect on translation rates. The 3' UTR can exert translational control in a number of ways: the presence of specific sequences that can interact with specific proteins (Braun, 1991), regulation of polyadenylation (Richter, 1993) or through regulating mRNA stability (Atwater et al., 1990).

The length of the 3' UTR has also been implicated as being an important structural element in translational regulation (Tanguay and Gallie, 1996). This effect of length is independent of actual sequences located within the 3' UTR and is more pronounced in transcripts that are not polyadenylated, which has been postulated to indicate that the 3' UTR may be serving as a reservoir for ribosomes (Tanguay and Gallie, 1996). However, in the case of the 3' UTR of cyclin A2, the actual length of the poly(A) tail could not be determined from this experimental data, because side-by-side comparisons of poly(A)$^+$ and poly(A)$^-$ transcripts were not examined. However, in stage VI and progesterone-matured oocytes (stages in which polyadenylation did not occur) the 200 nucleotides of the cyclin A2 3' UTR did not increase reporter gene translation relative to a CAT(A)$^-$ control. In addition, the presence of a poly(A) tail at fertilization results in increased pGEM-CATA2 translation, but at the MBT a decrease in translation was observed therefore, one can conclude that the actual length of the 3' UTR is not as important in this case as are the sequences contained within this region. One *caveat* when postulating effects of the 3' UTR length on translation is that it does not take into account the presence of approximately 100 nucleotides derived from the CAT reporter gene. There is no evidence that these nucleotides affect reporter translation, but proof of this hypothesis requires the complete removal of these nucleotides before insertion of the cyclin A2 3' UTR (Tanguay and Gallie, 1996).

Another important regulatory motif is an oligopyrimidine tract in the 3' non-coding regions. The 3' UTR of cyclin A2 contains long stretches of oligopyrimidines within its 3' UTR; however, the importance of these region was not examined in these experiments. Because no identifiable A_2UA_3 hexanucleotide was found, it is possible that these oligonucleotide stretches are functioning in the control of polyadenylation. Additionally, these elements may also been involved in mRNA stability or in the binding of *trans*-factors.

2. Effects of the 5' UTR

Many oncogenes and growth-related genes are translationally regulated completely or in part by secondary or primary structures within their 5' UTRs (Merrick and Hershey, 1996; Kozak, 1986b; Kozak, 1989b; Oliveira *et al.*, 1993; Fu *et al.*, 1991). The presence of secondary structure due to complementary nucleotide pairing results in inefficient translation under normal conditions. A number of constructs were made to examine the role of the cyclin A2 5' UTR on reporter translation during development. The Fold algorithm of Zueker and Steigler (1981), in the GCG sequence analysis software package (Figure 9), predicts a stable secondary structure of ΔG=-73 kcal/mol, which would be expected to inhibit translation under most conditions. During most of early development this is clearly the case. However, at the time of fertilization, this translational impediment is lost. The problem with predicting secondary structure is that different programs will predict different amounts of secondary structure (Draper, 1991), with corresponding differences in the predicted free energies. Possible methods to predict secondary structure include X-ray crystallography, which is difficult because RNA is hard to make into pure crystals, or 2-dimensional nuclear magnetic resonance (NMR). Predicting only the secondary structure for a particular region of mRNA fails to account for other interactions that may occur with different parts of the molecule. In addition, RNAs are flexible molecules and may adopt multiple conformations (Draper, 1991). Therefore, predicting a single structure is probably not the complete picture. Heating the micro-injected RNA to 65°C before micro-injection, which should melt secondary structure and allow more efficient translation, had no effect on CAT reporter

gene expression (data not shown). This would seem to indicate that secondary structure is not involved in inhibiting translation, although it is possible that as the RNA cooled in the needle or in the oocyte or embryo after micro-injection, the secondary structure reformed. Reversing the orientation of the 5' UTR was also attempted, but again this had no effect on the translation of the reporter gene (data not shown), demonstrating that the inhibitory effect observed is not orientation dependent. However, this does not address whether the inhibitory effect is a result of primary sequence or secondary structure in this region.

3. Effects of the 5' and 3' UTR

When either the 3' or 5' UTR of cyclin A2 flanked a CAT reporter gene an increase in CAT activity was observed after fertilization. Thus, neither the 5' UTR nor the 3' UTR alone appeared to be responsible for the translational regulation of cyclin A2. This result is in contrast to the translational regulation of cyclin A1, whose 3' UTR appears to be necessary and sufficient for translation (Sheets *et al.*, 1994). However, when both the 5' and 3' UTR flanked a CAT reporter gene an increased level of CAT activity was not observed at the time of fertilization (Figure 13). This result would support a synergistic model of translation regulation, where both the 5' and 3' non-coding regions of the transcript play a role. Evidence that the ends of the molecule interact synergistically has been accumulating for the past few years (Kuge and Richter, 1995; Gallie, 1991). Building on the work of Sachs and Davis, (1989), which demonstrated genetically and biochemically that the poly(A) binding (PAB) protein may interact with the 60S ribosomal subunit to regulate translational efficiency, Gallie (1991) described a synergistic effect between the poly(A) tail and the 5' methylguanosine cap. Both the translational efficiency and message stability were increased by either the cap or poly(A) tail, although the synergistic effect was a function of translation (Gallie, 1991). The results of my experiments also suggest a cooperation between the two ends of the molecule. The two ends of the molecule would likely be in close proximity due to folding or protein-RNA interactions. The close proximity of the two ends of the molecule could facilitate or repress

translation in a number of ways: the interactions of the 3' UTR with the 5' UTR (possibly mediated by *trans*-acting factor) could facilitate or prevent initiation from occuring; folding of the RNA molecule that brings the two ends together could also prevent necessary factors from binding.

However, these experiments do not preclude the possibilty that the 3' and 5' non-coding regions function in *trans* to regulate translation. It is possible that interaction between two different messengers could serve a regulatory role, eliminating the need for interactions between the 5' and 3' UTRs of an individual transcript. This model is intriguing in that it is possible that unrelated messengers could function to regulate the translation of specific messengers and eliminate the need for protein interactions. Complementary interactions between mRNA molecules could prevent the association of initiation factors with an mRNA and prevent effecient translation.

4. Other Considerations

Recent evidence in our laboratory (Browder, personal communication) has suggested a larger role for the coding sequence itself in the translation process than previously suspected. Specifically, when the Luc reporter gene was substituted for the CAT reporter gene, either the 5' or 3' UTR alone functioned as stimulatory elements, in contrast to the case of the CAT reporter, where translation was equal to the level of the reporter alone. Approximately a 20-fold increase was seen for transcripts containing the 3' UTR of cyclin A2 and a 2-fold increase for transcripts containing the 5' UTR alone. These results are not overly surprising, as the data suggest interactions between the 5' and 3' UTR, in addition to published reporters of synergistic effects (Gallie, 1990). It seems likely that in addition to the non-coding regions, the coding region itself would be important, because it would play a large role in the overall folding of the molecule as well as containing other possible regulatory sequences.

One consideration when looking at mRNA translation is the stability of the messengers. In some cases transcripts are rapidly degraded to prevent translation from occurring. At other times, mRNA transcripts may be stabilized; those transcripts that are available to the translational machinery for a longer

time will result in more protein being synthesized. In the case of cyclin A2 expression, messenger stability does not appear to be an important determinant of translational efficiency. The messengers are not rapidly degraded as a means of translational control during early *Xenopus* development as determined by northern blot analysis (Howe *et al.*, 1995), which is supported by this work with reporter constructs. Greater than 65 per cent of the micro-injected mRNA could be recovered for all the transcripts tested during the four developmental stages examined. Additionally, the stability of the transcripts did not seem to play a role in the level of expression. Although, the transcripts were stable as determined by mRNA recoveries, the level of expression at most stages (other than fertilization) was below that of the CAT(A)$^-$ reporter alone. Therefore, it is some other property of the transcripts, probably due to specific *cis*-sequences or *trans*-acting factor, that is responsible for the levels of translation observed.

Because the levels of expression of transcripts containing the 5' or 3' non-coding regions varied depending on the reporter gene used, it is unclear if any relevance can be assigned to these data when trying to determine the expression of the native molecule. In order to correlate the translation of reporters with intrinsic properties of a transcripts non-coding regions, it would be necessary to run comparisons with the native molecule, through northern blot analysis and mutagenesis studies to determine conclusively what effect the UTRs are having on the regulation of translation of the native molecule.

The effects of the 5' and 3' non-coding regions of *Xenopus* cyclin A2 demonstrated stage-specific CAT reporter gene expression during early development. Individually, either the 5' or 3' UTR alone can inhibit the efficient translation of a CAT reporter gene during early development except for early post-fertilization development. The presence of both non-coding regions is necessary for preventing increased CAT activity of the reporter during all stages of development tested, including fertilization. Neither the 3' UTR nor the 5' UTR has any effect on messenger stability, although only transcripts containing the 3' UTR are polyadenylated. From these experiments, polyadenylation occurs post-fertilization, and the levels of CAT activity at both oogenesis and maturation are consistent with a lack of polyadenylation. However, after fertilization there is not an increase in translation that correlates with polyadenylation of the messenger (pGEM-A2CATA2; Table 3). This is

different from the cyclin A1 example, where polyadenylation has been demonstrated to control translation (Sheets *et al.*, 1994).

5. Future Directions

The effects of the cyclin A2 non-coding regions on reporter expression were examined during early *Xenopus* development until the MBT. However, the native cyclin A2 is not expressed significantly until the EGT. Therefore, it was not surprising that inhibition was observed in these experiments. Although these experiments demonstrate that both the 3' and 5' UTRs of cyclin A2 contain the information required for translational regulation during early *Xenopus* development, there remain unanswered questions. These questions include the isolation and charaterization of the individual sequence elements located within the non-coding regions that are responsible for regulation. Perhaps the most important question involves the differences in behavior observed when reporter genes are substituted. Is this effect due to size alone (Luc transcripts are three times larger than CAT transcripts and approximately two times larger than the native cyclin A2 transcript) or due to differences in secondary structures and folding. Possibly the coding regions themselves contain signals that function to regulate translation.

The interaction of proteins with the non-coding regions should be examined to gain a clear understanding of how the translational regulation occurs. Either specific *trans*-acting factors or the general translation machinery could be responsible for the observed effects on CAT activity, and it would be important in the future to attempt to characterize any possible protein-mRNA interactions. The possibility of *trans* effects of the messengers themselves is an intriguing hypothesis. Perhaps the 5' and 3' UTR's could be transcribed individually and used to try and inhibit translation in micro-injection assays in order to explore this possibility.

The role of polyadenylation was not examined closely in these experiments. Future experiments could be performed to not only further define the role of polyadenylation, but also to characterize the extent of polyadenylation that occurs and to analyze the *cis*-sequences responsible.

Additionally, the role of cyclin A2 in early development might be explored. If cyclin A1 is the predominant A-type cyclin in early development, why are cyclin A2 messengers present in the cell? This is intriguing when one considers that cyclin A2 is not made significantly until after the start of zygotic transcription. Perhaps low levels of cyclin A2 protein are necessary for proper development.

As with any scientific project, there will always remain unanswered questions and unexplored areas of interest. The best one can achieve is to lay a solid foundation upon which future experiments can be based.

APPENDIX A

Table 4: Statistical values for non-parametric analysis of data using the Kruskel-Wallis Test.

Figure	Micro-injected RNA [compared to CAT(A)-]*	Stage	p-value	sample size (n)
5	pGEM-CATA2(A)-	Stage VI	**0.001**	22
5	pGEM-CATA2(A)-	Maturation	<u>0.049</u>	11
5	pGEM-CATA2(A)-	Early Embryo	0.983	26
5	pGEM-CATA2(A)-	Late Embryo	<u>0.045</u>	18
10	pGEM-A2CAT(A)-	Stage VI	**0.001**	22
10	pGEM-A2CAT(A)-	Maturation	**0.001**	11
10	pGEM-A2CAT(A)-	Early Embryo	0.543	26
10	pGEM-A2CAT(A)-	Late Embryo	**0.0001**	16
13	pGEM-A2CATA2(A)-	Stage VI	**0.001**	21
13	pGEM-A2CATA2(A)-	Maturation	**0.001**	11
13	pGEM-A2CATA2(A)-	Early Embryo	**0.001**	23
13	pGEM-A2CATA2(A)-	Late Embryo	**0.001**	18

*All species of mRNA are compared to the level of pGEM-CAT(A)- translation for each stage of development; p-value is the probability that the data could be due to chance ($p \leq 0.05$, the value is statistically significant and is indicated by underlining; $p \leq 0.001$, the value is highly significant and is indicated by bold-faced type); Sample size (n) refers to the number of times a particular experiment was performed.

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