C/EBPβ (NF-M) IS ESSENTIAL FOR THE GROWTH ARREST-SPECIFIC TRANSCRIPTIONAL INDUCTION OF P20K

SELENA KIM

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of

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ABSTRACT

p20K is a gene whose expression is transcriptionally induced in the growth arrested G_0 phase of the cell cycle (quiescence). Since the presence of p20K is well-correlated with growth arrest, it can be used as an indicator of the growth arrested state. The purpose of this study was to generate a better understanding of how p20K is transcriptionally regulated. As a marker of growth arrest, an elucidation of p20K's regulation would contribute to an understanding of the mechanisms regulating the growth arrest state as a whole.

The promoter of p20K contains a 48-base pair segment which is essential and sufficient for quiescence-driven expression. This promoter unit was named the Quiescence-Responsive Unit (QRU). The QRU contains two consensus binding sites for the CCAAT/Enhancer Binding Protein (C/EBP) family of transcription factors. C/EBP factors were therefore tested as candidate regulators of the QRU. Ectopic C/EBP expression strongly activated the QRU, and its quiescence-responsiveness was abrogated by mutating either of the two C/EBP binding sites. Studies with a dominant-negative version of C/EBPβ supported the contention that C/EBP factors are necessary for QRU activity. In an Electrophoretic Mobility Shift Assay, a quiescence-specific complex named C1 could specifically bind the first C/EBP site of the QRU. Complex C1 could be supershifted by incubation with an antibody specific for C/EBPβ, confirming its involvement. In summary, this study proves that C/EBPβ is essential for quiescence-specific induction of p20K, a growth arrest-specific gene.

ACKNOWLEDGEMENTS

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I would like to thank fellow labmates Natalie Rodrigues, Mark Gagliardi, and Claudia Campos for friendship, coffee, humour and tolerance. My other friends among the students and faculty of our department also made my time at York a lot of fun. A special thank you to Dr. Andrew White and his lab for not kicking me off their computer. I am also grateful to Daniel Laskey for helping me with my illustrations.

Finally, I would like to thank my family for their encouragement. This work would not have been possible without their support.

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LIST OF ABBREVIATIONS

AD	activation domain
bp	base pairs
bZip	basic and leucine zipper domain
camKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic AMP
CKI	cdk inhibitor
Cdk	cyclin dependent kinase
cDNA	complimentary DNA
C/EBP	CCAAT/Enhancer Binding Protein
CEF	chicken embryo fibroblasts
CHOP	C/EBP Homologous Protein
cMGF	chicken myelomonocyte growth factor
CR	conserved region
CRE	cAMP response element
Dex	dexamethasone
DNA	deoxyribonucleic acids
EGF	Epidermal Growth Factor
G_1	gap 1
G_2	gap 2

Gas	growth arrest-specific
Gadd	growth arrest and DNA damage-induced
G-CSF	Granulocyte-Colony Stimulating Factor
IL	Interleukin
kb	kilo base pairs
kDa	kilodaltons
LAP	Liver Activating Protein
LIP	Liver Inhibitory Protein
LPS	lipopolysaccharide
LZ	leucine zipper
MAPK	Mitogen-Activated Protein Kinase
Mix	methylisobutylxanthine
mRNA	messenger RNA
NFM	Nuclear Factor Myeloid
PCNA	Proliferating Cell Nuclear Antigen
pH	-log $[H^+]$
PKA	Protein Kinase A
PKC	Protein Kinase C
$PPAR_\gamma$	Peroxisome-Proliferator Activating Receptor Gamma
pRB	Retinoblastoma protein
PyMT	Polyoma Middle T
QRU	Quiescence-Responsive Unit

RNA	ribonucleic acids
RSV	Rous Sarcoma Virus
S	synthesis
SRE	Serum Response Element
TCF	Ternary Complex Factor
TD	transcriptional activation domain
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor Alpha
YY1	Yin-Yang 1

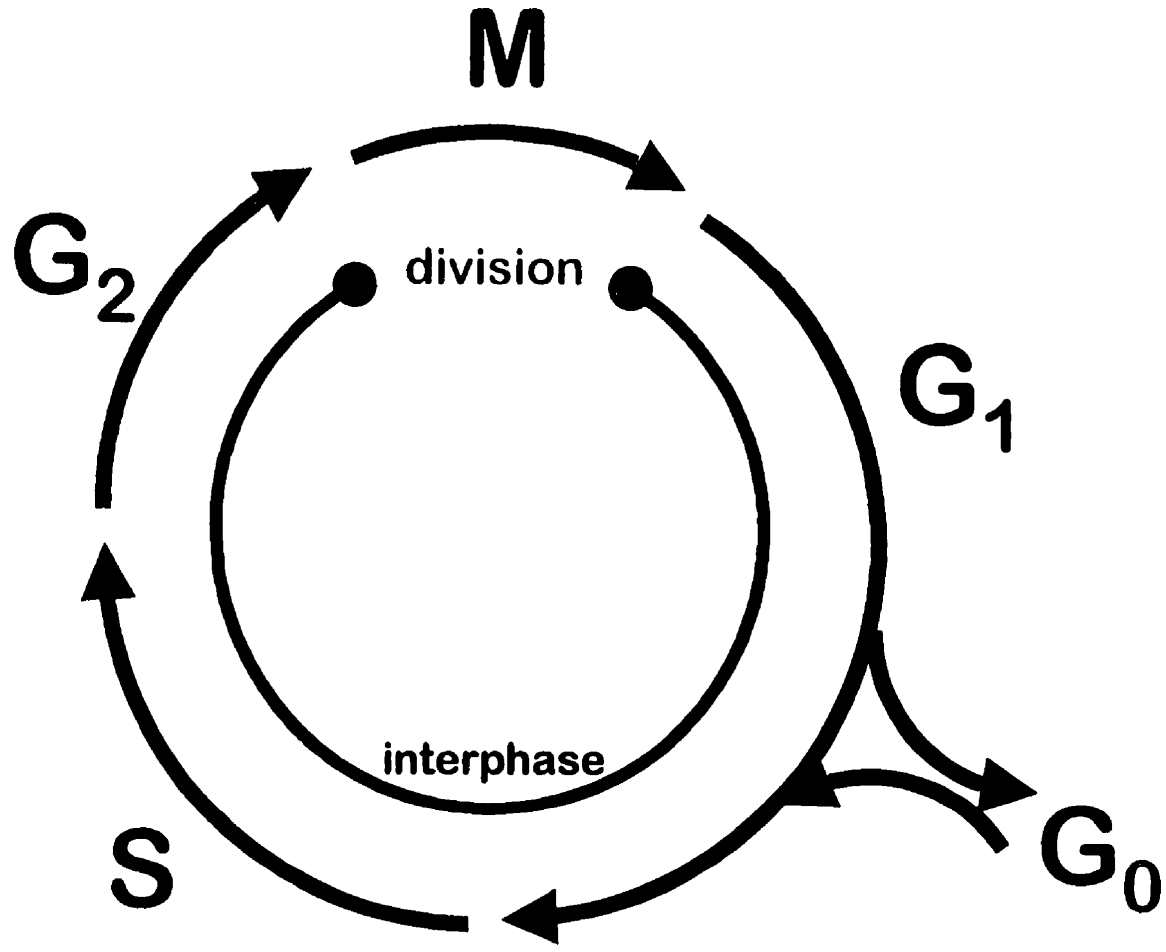
INTRODUCTION

I) The growth arrested-state

The most conspicuous events of the cell cycle, DNA synthesis and mitosis, are each preceded by distinct and directed programs of activity - G_1 and G_2, respectively. They are not mere preparatory phases; an assessment of environmental circumstances occurs within, followed by a decision of whether or not to proceed to the next step of the cell cycle. Progression to S phase from G_1 is decided by an evaluation of the levels of one or several positively or negatively acting factors. A lack of nutrients, mitogenic growth factors, or space may inhibit progression. As well, depending on the cell type, the presence of negatively acting factors may cause a similar arrest. For example, Transforming Growth Factor β (TGFβ) is an inhibitory growth factor in many cell types (Moses *et al.*, 1990). Nitric oxide has been found to be a DNA synthesis inhibitor in PC12 neuronal cells; activation of nitric oxide synthase (mediated in this case by Nerve Growth Factor) will arrest cells in G_0 (Peunova and Enikolopov, 1995). As well, genotoxic insults will result in growth arrest, presumably in order to allow time for DNA repair (Zhan *et al.*, 1994) . In any of these cases, a nonproliferating state called G_0 or quiescence is entered. This state can last as long as its cause persists, but the cells retain their potential to re-enter the cell cycle should favourable conditions return (Pardee, 1989).

Figure 1: A Typical Eukaryotic Cell Cycle.

This figure shows the various stages involved in a cell cycle. Mitosis (M) is the stage in which cell division occurs, producing two daughter cells. Interphase refers to the remainder of the cell cycle and can be divided into three stages: Gap1 (G_1), Synthesis (S), and Gap2 (G_2). If conditions are unfavourable for proliferation, cells exit G_1 and growth arrest in the quiescent state, G_0. These cells retain the ability to cycle and if favourable conditions return, they can re-enter G_1 and proceed to S phase.


M
G2
division
G1
interphase
S
G0

At this point a distinction should be made between quiescence (a reversible growth arrest state) and senescence, which is irreversible growth arrest. They are very distinct states, although the expression patterns of some genes overlap both senescense and quiescence (eg., Wang, 1985). All normal cells in culture can proliferate for a limited number of population doublings, after which they senesce. Senescence is quite different from quiescence in that senescent cells are stably and irreversibly arrested with a G_1 DNA content. Although metabolically active, many of their cellular functions are altered and they can never replicate, even when stimulated with growth factors. Senescent cells also acquire a resistance to apoptosis. The percentage of senescent cells present in any given organism is directly correlated with age; this resistance to apoptosis may explain this observation (reviewed by Cristofalo and Pignolo, 1996; Campisi, 1996).

Cells arrested in G_0 are fundamentally different to cycling cells in G_1. They were initially distinguishable by their delay in re-entering the cell cycle upon mitogenic stimulation. In Swiss 3T3 cells, a lag time of eight hours was documented (Zetterberg and Larson, 1983). They also differ biochemically; G_0 cells tend to be smaller, and general levels of macromolecular synthesis and activity decrease (Pardee, 1989, Hall and Lane, 1994, Epifanova and Brooks, 1994). Yet, there are certainly unique processes actively occurring in quiescent cells. It has been known for many years that if a resting cell is fused with a proliferative cell, S phase entry of the heterodikaryon will be suppressed (Polunovsky *et al.*, 1983). G_0 cells release secreted inhibitors such

as TGFβ (Moses *et al.*, 1990), contactinhibin (Wieser *et al.*, 1990), and chalones, which are small proteins or glycoproteins with antiproliferative activity (Laerum *et al.*, 1984, Reichardt *et al.*, 1987) Many proteins exist in modified or alternative forms during quiescence. For example, in contact-inhibited glioma cells, Protein Kinase C family members α and δ are expressed at markedly higher levels (Moreton *et al.*, 1995). The state of the tumor suppressor pRb may also be used as a marker of growth arrest. pRb always exists in the unphosphorylated state in circumstances of growth suppression, but it is phosphorylated following mitogenic stimulation (Chen *et al.*, 1989). In its unphosphorylated state, it is though to sequester the E2F transcription factor, which may modulate the transcription of some growth arrest-regulated genes (Weintraub *et al.*, 1992).

Underlying cell cycle progression is the sequential assembly and activation of cyclin-dependent kinases (cdks). This process is controlled by external signals such as growth factors. Each cdk complex consists of a catalytic subunit (the cdk itself) as well as a cyclin, the regulatory subunit. Different cyclins are required for passage through different cell cycle phases. Cdks positively mediate cell cycle progression; cell cycle arrest is mediated by cdk-inhibitors (CKIs) (reviewed by Peter and Herskowitz, 1994).

CKIs bind cdk complexes, inhibiting cell cycle progression. p27 is a particularly relevant CKI; its activity is well-correlated with quiescence (reviewed by Sherr, 1994). Contact-inhibition and TGFβ treatment can both increase levels of

active p27 (Polyak *et al.*, 1994), and quiescence can be abrogated by expressing p27 antisense in fibroblasts (Rivard *et al.*, 1997). The evidence shows that p27 is central to G_0 growth arrest.

2) Growth arrest-specific (gas) gene expression

Lumpkin *et al.* showed in 1986 that mRNA injection from a quiescent cell into a cycling cell will halt its progression. This hinted that growth arrest-specific transcription was occurring. Growth arrest-specific genes have been sought relatively recently. After a wave of research studying immediate-early gene expression upon the commencement of mitogenesis, it was realized that the appropriate maintenance and regulation of quiescence could be equally important. The majority of cells comprising the adult vertebrate organism are growth-arrested or quiescent. In many cancers, the ability to regulate the entry into G_0 is lost or modified; cycling may continue under conditions which would normally be unfavourable for proliferation (Pardee, 1989). In fact, much of the work done in an attempt to elucidate growth arrest-pathways was performed in order to understand the effects of the tumor suppressor gene p53. p53 is a transcription factor which is mutated in more than 50% of all cancers. p53 was found to have a growth inhibitory effect and therefore was a potential regulator of growth state-specific genes. Progress has been made in understanding p53's role in growth arrest, but there remains much more to be done in order to understand growth arrest as a whole; both p53-dependent and independent pathways of growth arrest and the regulation of genes specific to these states are still very poorly characterized. It is

improbable that these pathways will be found to be entirely distinct; gene expression patterns in response to growth arrest induced by agents such as wild-type p53, growth-inhibitory cytokines, genotoxic insults, or the other previously discussed growth arrest signals are likely to overlap (reviewed by Liebermann *et al.*, 1995). Therefore, work done towards studying the regulation of growth arrest-specific gene expression is apt to have broad implications in the understanding of cell homeostasis.

The criteria for a growth arrest-regulated gene are that it must be upregulated in growth arrested cells and downregulated upon induction of proliferation. The majority of growth arrest-specific genes have been discovered by hybridization subtraction of cDNAs induced in the growth arrested state from those produced in actively dividing cells. This was the method of discovery of the gas (growth arrest-specific) series of genes, the gadd (growth arrest and DNA damage) genes (Schneider *et al.*, 1988, and Fornace *et al.*, 1989) and the quiescins (Coppock *et al.*, 1993). A few growth arrest-regulated genes were initially discovered at the protein level. p20K was found by analysis of two-dimensional gel electrophoresis of proteins synthesized by normal quiescent vs. growing transformed chicken heart mesenchymal cells (Bédard *et al.*, 1987b). Statin, the first growth arrest-regulated gene discovered, was isolated by making a monoclonal antibody from mice injected with the cytoskeletal extract of an aged human fibroblast culture. It was found to stain the nuclei of nonproliferating fibroblasts (Wang, 1985).

a) Functions of gas genes

In terms of function, it remains to be determined for a significant portion of gas genes discovered to date. Many have been correlated with the induction of growth arrest or the inhibition of DNA synthesis, but the mechanisms by which they work have not yet been elucidated. With regards to the genes whose functions have been more thoroughly investigated, many of these functions make sense in consideration of the supposed needs of the growth-arrested cell. Some gas genes are transcription factors. One of the better-characterized transcription factors encoded by a gas gene is C/EBP Homologous Protein 10 (CHOP10/gadd153), a divergent member of the CCAAT/Enhancer Binding Protein (C/EBP) transcription factor family. CHOP is homologous to the typical C/EBP members in that it possesses the hallmark domains for DNA binding and heterodimerization, but it has differing amino acids in its DNA binding domain which are not shared with any other C/EBP family members (Ron and Habener, 1992). CHOP is believed to have a pivotal role in altering programs of gene expression in response to stress. Indeed, it can undergo inducible phosphorylation by p38 MAP Kinase, a stress-responsive kinase (Wang and Ron, 1996).

CHOP is presently believed to function in dual roles: it can inhibit some transcription factors from activating their usual targets, and it may also be involved in directly activating novel target sequences (Ubeda *et al.*, 1996). Its inhibitory effect has been seen with the C/EBP(CCAAT Enhancer Binding Protein) family and also the ATF/CREB family of transcription factors. CHOP can heterodimerize with members

of the C/EBP family, producing an altered DNA sequence binding specificity. When CHOP heterodimerizes witth ATF3, it forms an inactive heterodimer (Chen *et al.*, 1996).

Several gas genes are believed to have structural functions. It may be that alteration of cell structure would be useful in maintaining structural integrity and therefore cell survival in conditions inadequate for proliferation. Cells are also known to undergo morphological changes upon exit from growth arrest, and an accumulation of certain structural gas gene products may facilitate such structural alterations. Components of the microfilament system or extracellular matrix may also have a role in inducing or maintaining quiescence by participating in signal transduction pathways (Brancolini and Schneider, 1994).

gas1 is a putative integral plasma membrane protein; sequence analysis has revealed two transmembrane domains. Although further details of its function in this regard are unknown, it seems to have an antiproliferative function. When ectopically expressed, it can inhibit DNA synthesis and the transition to S phase (Del Sal *et al.*, 1992). These effects of gas1 are known to require expression of the wild-type p53 transcription factor, but not p53's trans-activation function (Del Sal *et al.*, 1995). This defines a pathway for p53-induced growth arrest which is distinct from the other well-characterized p53-dependent G_1 arrest pathway, in which p53 transcriptionally induces the expression of p21 (El-Deiry *et al.*, 1993). This gas1-mediated pathway was found to require a proline-rich region of p53 which is a potential binding site for

SH3 domains. This proline motif may therefore be a docking site important for transmitting an antiproliferative signal (Ruaro *et al.*, 1997).

Many gas genes appear to favour proliferation and survival. Their putative role in growth arrest may be to maintain proliferative potential should favourable conditions return for cell cycle re-entry.

A growth arrest-specific gene was discovered in NIH 3T3 fibroblasts to encode Platelet-derived growth factor α-Receptor (PDGFαR) (Lih *et al.*, 1996). PDGFα is known to be a growth factor important in the mitogenic response (Pledger *et al.*, 1977) and in early stage embryogenesis following growth arrest (Schatteman *et al.*, 1992). Therefore, an accumulation of the receptor for PDGFα may enhance the responsiveness of the cell to PDGFα, therefore facilitating competence for cell cycle re-entry.

The functions of several gas genes are unknown, an example being p20K. p20K is a 20 kiloDalton protein cloned from Chicken Embryo Fibroblasts (CEF), whose expression is induced by quiescence. It is upregulated by serum-deprivation, contact-inhibition, and inhibition of DNA synthesis (Mao *et al.*, 1993). Repressors include transformation by Rous Sarcoma Virus (RSV), and hormone stimulation by epidermal growth factor or insulin (Bédard *et al.*, 1989). p20K mRNA is found in lung, spleen (Bédard *et al.*, 1989), and in chondrocytes (Cancedda *et al.*, 1990). p20K is secreted, but a significant portion remains cell-associated (Bédard *et al.*,

1987b). Recent work suggests that p20K may act as a survival factor in density-arrested CEF (Slonimsky and Bédard, unpublished observations).

p20K is a member of the lipocalin superfamily of proteins. This is a large, diverse group; members tend to share approximately 20-30% homology. The members are typically small extracellular proteins which bind and transport hydrophobic molecules such as retinoids and fatty acids. Other better characterized members of this family include retinoid-binding protein, α-1-microglobulin, and purpurin, all of which are known to bind retinoids. Besides being important in vision, retinoids are also known to be important in the regulation of cell growth, development, and survival (reviewed by Flower, 1994).

b) Transcriptional regulation of gas genes

Transcriptional regulation of gas genes has not been extensively studied in the majority of cases, although it is relatively well-characterized for certain genes. Discovering the mechanisms which regulate individual gas genes is of importance, since these factors are likely to be crucial for the maintenance of the growth arrest state.

Dissection of the gadd153 promoter has shown that an 800-bp fragment is sufficient for DNA damage-induced promoter activation in a dose-dependent manner. Within this 800-bp region, consensus binding sites for the C/EBP, SP-1, and AP-1

transcription factors are observed, suggesting that these factors may be involved in gadd153's expression. Interestingly, prostaglandin-A2, an agent which induces strong growth arrest, has no effect on the intact gadd153 promoter, even though it increases mRNA levels of gadd153 in Chinese Hamster Ovary and HeLa cells. Therefore, the transcriptional regulation seen with DNA damaging agents appears separate from growth arrest-induced regulation, which may occur transcriptionally or post-transcriptionally. (Luethy *et al.*, 1990).

gas1 is the only gas series gene regulated at the transcriptional level. A 665-bp promoter fragment is required for maximal quiescence-specific induction, but a 300-bp fragment contained within can also drive regulated expression, although at reduced levels. In gelshift assays, growth state-specific nucleoprotein complexes were seen within this 300-bp region. Interestingly, footprinting analysis with this region showed identical protection patterns in nuclear extracts made from growing versus quiescent cells. A size difference could be seen on gelshift, however. This implies that the protein(s) of interest have a similar or identical DNA binding preference. They may differ in size, possible heterodimerization partner, or post-translational modification. The identity of these proteins remains unknown. A computer search of this promoter region revealed no consensus binding sites. Multimerization and insertion of this sequence into a reporter gene revealed no growth state-specific activity (deMartin *et al.*, 1993).

The gadd45 gene houses putative OCT-1, CCAAT, AP-1 and p53 transcription factor binding sites. The p53 site is likely to be activated at least in conditions of DNA damage, since gadd45's activation in this situation is p53-dependent (Zhan *et al.*, 1993). Conversely, gadd45's activation by serum starvation is p53-independent, since it can still be seen in p53-deficient cells. The isolated gadd45 promoter is not as well induced by DNA damage and growth arrest as the intact gene; sequences outside of the promoter are probably important as well (Hollander *et al.*, 1993). Candidate transcription factors have been found that may be central in regulating gadd45's expression. When C/EBPα is ectopically expressed along with a cotransfected reporter plasmid containing a 1.5 kb promoter fragment for gadd45, significant trans-activation is observed. This activation is negated by c-myc co-expression. It is speculated that perhaps C/EBPα mediates gadd45's growth arrest-specific expression, and c-myc comes into play in times of proliferation (Constance *et al.*, 1996, Marhin *et al.*, 1997).

p20K is known to be controlled at least in part at the transcriptional level; the promoter is induced by serum-starvation in transient expression assays. Promoter dissection has revealed that a 48-bp promoter fragment spanning residues -217 to -169 is essential and sufficient for quiescence-specific activation. This region has been named the Quiescence-Responsive-Unit (QRU) (Mao *et al.*, 1993).

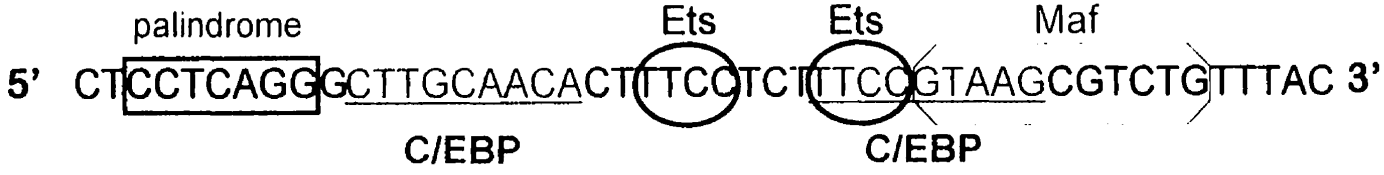
Within the p20K promoter outside of the QRU, there are several consensus binding sites: a pRb control element, a myc consensus sequence, and a TGFβ control element. Within the QRU good consensus sites for the ets and C/EBP families of transcription factors are found. A segment of the QRU also shows homology to a promoter segment in the Retina-specific differentiation gene QR1. This element, called the A box, is essential for the growth arrest-specific transcription of QR1. The A box is believed to be a maf transcription factor consensus binding site (Pouponnot *et al.*, 1995), therefore maf transcription factors may also be considered candidates for regulation of the QRU (fig. 2).

3) Candidate regulators of p20K's QRU

a) maf: Maf transcription factors belong to the "bZip" superfamily of transcription factors, which all harbour basic DNA binding domains adjacent to highly conserved leucine zipper dimerization domains. Other family members include the immediate-early genes jun and fos, the CREB/ATF family, and the C/EBP family (Kataoka *et al.*, 1994a). The first member of the maf family discovered was v-maf, which was cloned from the chicken musculoaponeurotic fibrosarcoma virus AS42. Its cellular homologue, c-maf, is conserved across species (Nishizawa *et al.*, 1989). Several other members have been discovered, and the maf family can now be divided into two subgroups.

Figure 2: The Quiescence-Responsive Unit (QRU) of the p20K Gene.

The 48-base pair QRU is displayed with consensus binding sites for various transcription factors indicated. Perfect consensus sequences are seen for C/EBP and ets factors, but the maf element only has limited homology to the maf consensus. A palindromic sequence is marked at the 5' end. The significance of this palindrome is unknown in that it does not match a consensus binding site for any known transcription factors.


palindrome
Ets
Ets
Maf
5' CTCCTCAGGGCTTGCAACACTTTCCTCTTTCCGTAAGCGTCTGTTTAC 3'
C/EBP
C/EBP

seem unaffected by mitogens (Kataoka *et al.*, 1994a). They do seem to correlate with the expression of differentiation-specific genes, however (Pouponnot *et al.*, 1995).

b) ets: Interestingly, mafB can interact with the ets-1 transcription factor in an inhibitory manner (Sieweke *et al.*, 1996). The ets family belongs to the winged-helix turn helix family of transcription factors (MacLeod *et al.*, 1992). There are many known members, and they all share the ets domain, which is a highly conserved DNA binding domain consisting of 3 α-helices and an antiparallel β-sheet. Crystallographic studies have shown that one of the helices of this motif recognizes the ets consensus sequence GGAA by fitting directly into the major groove of the DNA (Graves *et al.*, 1996).

The ets family can be split into two subgroups. The first is modeled on homology to v-ets, which was discovered to be the oncogene in the chicken transforming retrovirus E-Twenty-Six (Watson *et al.*, 1985). Members of this subgroup include c-ets-1 (Chen, 1985), c-ets-2 (Boulukos *et al.*, 1988), and PEA3 (Wasylyk *et al.*, 1989). Their ets domain is located at the carboxy terminus, and their DNA-binding activity is potentiated by phosphorylation (Fleischman *et al.*, 1993). Interestingly, this subgroup can synergize with the AP-1 transcription factors fos and jun (Gutman and Wasylyk, 1990). This synergism is not through direct protein contact between ets and fos or jun; their respective protein-interaction domains are hypothesized to mutually contact a third target protein (Gutman and Wasylyk, 1990).

The second subgroup consists of the TCFs (Ternary Complex Factors) (Shore *et al.*, 1996). Unlike the first group, their ets domain is located at the amino-terminus. Members include Elk-1 (Rao *et al.*, 1989), Sap-1 (Dalton and Treisman, 1992), and Sap-2 (Price *et al.*, 1995). They can form complexes with Serum Response Factor through a region called the B-box, which mediates protein-protein interactions (Shore *et al.*, 1996).

Ets protein involvement is documented in many processes. Best-studied is their role in growth and transformation; they have been implicated in being induced by growth stimuli, and activated by oncogenes (reviewed by Gutman and Wasylyk, 1990). Some exceptions are T-cells, where ets-1 expression is highest in quiescence (Bhat *et al.*, 1990), and macrophages, where differentiation induces ets-2 expression (Watson *et al.*, 1990). Ets-2 has also been implicated in the development of cartilage and bone; it is located on human chromosome 21 and is overexpressed in Down's Syndrome. The associated skeletal abnormalities characteristic of Down's Syndrome are believed to result from ets-2 overexpression (Sumarsono *et al.*, 1996).

c) C/EBP: C/EBPα was the first discovered member (Graves *et al.*, 1986, Johnson *et al.*, 1987). It was found to have an amino-terminal trans-activating domain and a carboxy-terminal basic DNA binding and leucine zipper dimerization (bZIP) domain (Johnson *et al.*, 1987). The bZip family is distinguished by the possession of a basic region known to be responsible for sequence-specific DNA binding, adjacent to a

"leucine zipper" motif. This motif allows members to heterodimerize with other leucine zipper-possessing transcription factors (Landschulz *et al.*, 1988). While C/EBPs are very highly conserved in their basic DNA-binding (DBD) and leucine zipper (LZ) domains, they are quite divergent in their amino-terminal halves, which house their transactivation domain (TD) and regulatory domains (Williams *et al.*, 1991). As a family of transcription factors, C/EBP is conserved across evolution; members have been seen in organisms as diverse as mammals, slime mold, and Arabidopsis (reviewed by Katz *et al.*, 1993).

The C/EBP family consensus binding sequence is T T/G N N G N A A T/G (Ryden and Beemon, 1989). To date there are five typical members and one divergent member, the aforementioned CHOP-10 (Ron *et al.*, 1992). C/EBPs always bind DNA as either homo or heterodimers, and all typical members discovered so far can dimerize with each other and bind identical sites. However, with the diversity in tissue and cell state-specific expression of each member, it is believed that there is enormous potential for regulation (Williams *et al.*, 1991).

i) C/EBP family members and isoforms

C/EBPα was first cloned in the rat (Johnson et al., 1987) and homologues have since been characterized in chicken (Calkoven *et al.*, 1997), mouse (Xanthopoulos *et al.*, 1989), Drosophila (Rorth and Montell, 1992), human (Antonson and Xanthopoulos, 1995), and frog (Chen *et al.*, 1994). In general, C/EBPα

expression is highest in tissues such as liver, fat, and intestine which are high-rate-metabolizers of lipid and cholesterol-related compounds (Birkenmeier *et al.*, 1989). The tissues which express high levels of C/EBPα tend to be differentiated and growth-arrested.

C/EBPα has more than one in-frame methionine in the open reading frame of its transcript, and the internal translation initiation sites produce truncated versions. The truncated versions generally lack portions of the transactivation domains of the full-length versions although DNA binding and dimerization domains are retained, believed to lead to either differing or repressive functions for these isoforms (Xanthopoulos *et al.*, 1989). 42 kDa, 38kDa, 30 kDa and 20kDa forms of C/EBPα have been seen in rat adipocytes and liver. The full-length 42kDa version functionally differs from the 30kDa form in that unlike its full-length counterpart, the shorter form is not antimitotic, nor can it induce adipocytic differentiation (Lin *et al.*, 1993). The 30kDa form is also much less transcriptionally active (Ossipow *et al.*, 1993).

C/EBPβ was the second family member to be discovered. It was cloned by several groups; as rat LAP (Liver Activating Protein) by Descombes and Schibler in 1991, as IL-6DBP (InterLeukin-6-DNA Binding Protein) by Poli *et al.* in 1990, mouse Crp2 (C/EBP-Related Protein-2) (Williams *et al.*, 1991), human Nuclear Factor-IL6 (Akira *et al.*, 1990), chicken Nuclear Factor Myeloid (NF-M) (Katz *et al.*, 1993) and

mouse AGP/EBP (α1-acid glycoprotein enhancer binding protein)(Chang *et al.*, 1990). C/EBPβ is involved in the differentiation of blood cell lineages, adipocytes and hepatocytes, and cytokine induction (Katz *et al.*, 1993, Descombes and Schilber, 1991, Williams *et al.*, 1991, Akira and Kishimoto, 1992).

C/EBPβ also generates different isoforms. In CEF, three isoforms of C/EBPβ have been seen: p42, p35, and p20, generated from three in-frame methionines (Sears and Sealy, 1994), while in rat liver, two forms have been seen: LAP (p35) and LIP(p20) (Descombes and Schilber, 1991). In liver, the LIP isoform acts as a dominant-negative form of C/EBPβ; it can attenuate transcription by the full-length form in a stoichiometric fashion, since it can bind DNA and heterodimerize but lacks a transactivation domain. The ratio of LAP:LIP increases as terminal differentiation in the rat liver proceeds, and this gradual difference in relative abundance is believed to correspondingly decrease LIP's inhibitory influence (Descombes and Schilber, 1991). In CEF, the isoforms act differently on target promoters. p35 and p42 repress the Rous Sarcoma Virus LTR 2-fold, while p20 can repress it 16-fold (Sears and Sealy, 1994). These alternate isoforms are likely to be fundamental in fine-tuning the transcription of C/EBP target genes.

C/EBPδ was first discovered in the mouse by Williams *et al.* (1991) and named Crp3. It was then cloned by Kinoshita *et al.* in 1992 as human NF-IL6β, and in the frog by Chen *et al.* (1994) as RcC/EBP2. In general, C/EBPδ has low-level

ubiquitous expression with induction during certain responses or high expression levels within selected tissues. In the human, C/EBPδ levels are low in normal tissues but are upregulated by LPS (lipopolysaccharide) or inflammatory cytokines (Kinoshita *et al.*, 1992). In mouse, however, C/EBPδ is most highly expressed in the lung, with strong expression as well in the liver (Williams *et al.*, 1991).

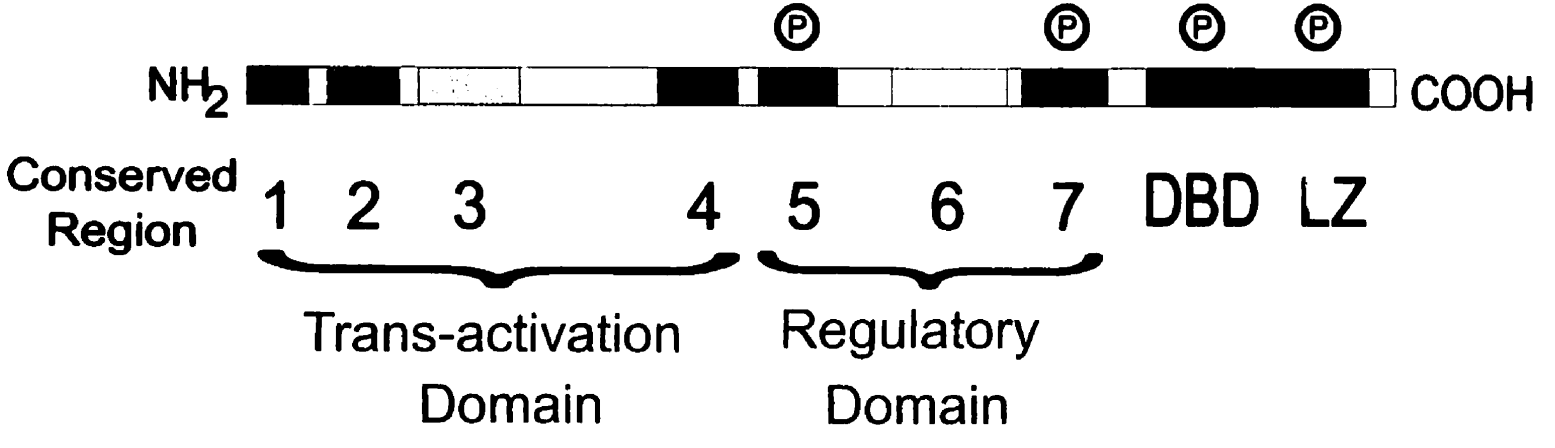
ii) Regulation of C/EBP members

C/EBPα and C/EBPβ both have CCAAT sequences in their promoters, and they are believed to be capable of autoregulation. As well, the C/EBPα promoter has several binding sites for myc which are believed to act in an inhibitory manner to silence C/EBPα expression during proliferation (Christy *et al.*, 1991, and Legraverend *et al.*, 1993, and Timchenko *et al.*, 1995).

Further structural analysis of C/EBP members has revealed several regulatory domains. C/EBPβ has been particularly well-studied in this manner. Alignment of C/EBPβ sequences from different species has revealed seven conserved regions (CR 1-7). They are separated by divergent amino acid stretches. The domain including CR1-4 is believed to correspond to the transactivation domain, and CR5 and CR7 are each separate regulatory domains (Kowenz-Leutz *et al.*, 1994).

Figure 3: C/EBPβ Protein Structure.

This diagram shows the conserved regions of the C/EBPβ protein. The conserved regions were deduced by aligning the amino acid sequences of C/EBPβ cloned from various species. The leucine zipper and DNA binding domains are highly conserved in all species, while the remainder of the protein shows high conservation in seven regions, marked CR1-7. CR1-4 correspond roughly to C/EBPβ's transcriptional activation domain, and CR5-7 correspond to its regulatory domain. The symbol "P" denotes regions of the protein known to be phosphorylated by various signaling kinases. These phosphorylations have a stimulatory effect on C/EBPβ activity. This figure was adapted from Kowenz-Leutz *et al.*, 1994.


P
P
P
P
NH2
COOH
Conserved Region
1 2 3 4 5 6 7
DBD
LZ
Trans-activation Domain
Regulatory Domain

C/EBPβ appears to be regulated in a unique manner; by derepression. It is believed that C/EBPβ generally exists in a repressed form with concealed activation potential. First characterized in chicken C/EBPβ, CR5 and CR7 are intramolecular repression domains which physically interact with the transactivation domain. C/EBPβ is believed to assume a tightly folded conformation in which its DNA-binding and transactivation domains are masked through interaction with its inhibitory domains CR5 and CR7. Deletion of either or both of CR5 and CR7 results in a constitutively active C/EBPβ (Twamley-Stein *et al.*, 1996). Their repressive effects may also be abrogated by their phosphorylation, which allows C/EBPβ to transactivate its target genes (Kowenz-Leutz *et al.*, 1994).

Phosphorylation of these inhibitory domains can be induced by several kinases. MAPK has been implicated in phosphorylating threonine-235 in human C/EBPβ (Nakajima *et al.*, 1993) and the corresponding serine-220 in CR7 of chickens (Kowenz-Leutz *et al.*, 1994). Trautwein *et al.* (1993) showed that TPA-induced stimulation of PKC results in phosphorylation of ser105 of the CR5 region, enhancing C/EBPβ's transcriptional efficacy (Kowenz-Leutz *et al.*, 1994).

A calcium-dependent phosphorylation site was characterized in the leucine zipper of C/EBPβ. This phosphorylation is believed to be performed by Calcium/calmodulin-dependent protein kinase II (CamKII). This phosphorylation

potentiates C/EBPβ's transactivating ability, but its mechanism of action is unknown (Wegner *et al.*, 1992).

There are several other oncogenic tyrosine kinases which can similarly phosphorylate and derepress C/EBPβ, although their specific phosphorylation sites are not yet known. Activation of v-erbB, PyMT, or raf derepresses C/EBPβ, depending on cell type. Many kinases converge on C/EBPβ, but it is likely that they are not functioning in a completely redundant manner; this may be a matter of tissue-specificity and the involvement of different signaling pathways. Regulatory effects seen in one cell type often cannot be duplicated in another, and the magnitudes of these effects can differ greatly (Twamley-Stein *et al.*, 1996, Williams *et al.*, 1995, and Kowenz-Leutz *et al.*, 1994). The phosphorylation-dependent regulation of C/EBPβ described above shows that C/EBPβ is the target of many signaling pathways. These complex regulatory mechanisms are probably a way of linking signaling to specific gene expression programs.

iii) C/EBP involvement in adipogenesis

The C/EBP family has a well-established role in adipocytic differentiation. Studies have been performed using preadipocytic cell lines and a sequence of events in adipogenesis has been assembled. By examining C/EBP expression patterns during the differentiation cascade, their relative roles have become clearer. When 3T3-L1 mouse preadipocytes in culture are exposed to glucocorticoid hormones, they begin the

process of adipogenesis. Hormone exposure is followed by a period of mitogenic clonal expansion which coincides with the transient expression of C/EBP β and C/EBP δ. This is followed by a growth arrest period which is probably mediated by C/EBPα-induction of p21. During this growth arrest period, C/EBPα becomes expressed together with PPARγ, which is another important transcription factor in adipocytes. Their appearance precedes the final phase of differentiation, the expression of adipocyte-specific genes (Mandrup and Lane, 1997).

In 3T3-L1 adipocytes in culture, the glucocorticoid hormones minimally required to allow full adipocytic conversion are dexamethasone (dex) and methylisobutylxanthine (mix). Dex and mix have been found to be direct inducers of C/EBPβ and C/EBPδ respectively (Yeh *et al.*, 1995). At this period of clonal expansion, C/EBPβ and C/EBPδ both play roles, although C/EBPβ appears to be more important. If the 3T3-L1 preadipocytes are lacking dex, adipogenesis can only occur at a very low frequency. If mix is withheld, there is much less of an inhibitory effect on the adipocytic program. Current thinking is that perhaps dex induces C/EBP δ which in turn induces C/EBPβ, this mechanism being possible because C/EBPβ has CCAAT sites in its promoter (Wu *et al.*, 1996). Other strong evidence of C/EBPβ's importance is that overexpression of the inhibitory 20kDa isoform of C/EBPβ (LIP) at this point strongly inhibits adipogenesis (Yeh *et al.*, 1995).

Following the period of clonal expansion, growth arrest occurs, and this coincides with the expression of C/EBPα and PPARγ. C/EBPα is believed to be largely responsible for the growth arrest occurring; ectopic expression of C/EBPα arrests a wide variety of fibroblastic and pre-adipocytic cell lines (Samuelsson *et al.*, 1991). Together with PPARγ, C/EBPα is responsible for much of the acquisition of the adipocytic phenotype (Mandrup and Lane, 1997). The PPAR family of transcription factors is expressed mainly in liver and adipose tissue and they transcriptionally activate enzymes important for lipid metabolism. C/EBPβ is believed to trans-activate PPARγ in adipose tissue (Wu *et al.*, 1995). C/EBPβ and C/EBPδ may also have a role in activating C/EBPα expression; C/EBPα has CCAAT sequences in its promoter. Once activated, C/EBPα and PPARγ appear to work together in being effectors of the adipocytic phenotype. Adipocyte-specific genes such as the fatty acid binding protein AP2 and phosphoenolpyruvate carboxykinase (pepck) possess both PPARγ and C/EBP binding sites in their promoters. In NIH 3T3 fibroblasts, ectopic expression of both PPARγ and C/EBPα results in a strong adipocytic phenotype, while expression of only one of them will result in only partial differentiation (reviewed by Mandrup and Lane, 1997).

iv) C/EBP involvement in the immune response

IL-6 is a cytokine which is central in the host immune response, and C/EBPβ is the principal transcriptional effector of IL-6 action (Poli *et al.*, 1990). When an

infection occurs, IL-6 is released by macrophages at the site of infection. IL-6 then travels to the liver, where it can induce acute phase protein synthesis. C/EBPβ is upregulated within 15 minutes of contact with IL-6, and this event directly precedes the transcriptional induction of acute phase proteins (apps), many of which have CCAAT sites in their promoters. In this situation, C/EBPβ transcribes apps such as C-reactive protein and α1-acid glycoprotein, G-CSF, and IL-8, and C/EBPβ rapidly redistributes from the cell bodies to the nucleus (Katz *et al.*, 1993). Therefore C/EBP β is very important to the liver's role in the acute phase reaction (Akira and Kishimoto, 1992).

v) C/EBP involvement in immune system differentiation

C/EBPβ is important for hematopoietic lineage commitment (Katz *et al.*, 1993). In the chicken, C/EBPβ is predominantly expressed in myeloid cells. Upon differentiation of multipotent progenitor cells to the myeloid lineage, C/EBPβ is upregulated. It is very important as a differentiation factor and inducer of cytokines. C/EBPβ transcribes many genes specifically induced in macrophages, such as IL-6, IL-1, IL-8, TNFα , G-CSF, and Nitric Oxide synthase. C/EBPβ-deficient mice are highly susceptible to bacterial and viral infection due to reduced functioning of their macrophages (Tanaka *et al.*, 1995, and Screpanti *et al.*, 1995).

vi) C/EBP involvement in growth arrest

There is ample evidence linking C/EBPα and growth arrest in many cell types. Ectopic expression of C/EBPα in most hepatocyte, adipocyte and fibroblast cell lines induces growth arrest which can be negated by the introduction of C/EBPα antisense (Freytag *et al.*, 1994, Diehl *et al.*, 1996). Indeed, this strong growth arrest upon C/EBPα overexpression is strong enough to impair the tumorigenicity of several liver carcinoma cell lines (Watkins *et al.*, 1996). In vivo, C/EBPα is elevated in differentiated, quiescent adipocytes and hepatocytes. This elevation is dependent on cell-cell contact in liver (Xanthopoulos *et al.*, 1989). As well, following partial hepatectomy, C/EBPα is transcriptionally repressed in regenerating liver. EGF-induced proliferation also represses C/EBPα mRNA (Mischoulon *et al.*, 1992).

C/EBPα's growth inhibitory properties are not mediated through p53 or Rb (Hendricks-Taylor *et al.*, 1995); they seem to be mediated through p21. C/EBPα overexpression can increase p21 levels through transcriptional and post-translational mechanisms and introducing p21 antisense RNA eliminates C/EBPα's ability to induce growth arrest. C/EBPα can induce p21 mRNA three-fold, while p21 protein is induced 12-20 fold. C/EBPα is believed to increase p21 transcription and perhaps transcribe a second gene whose product stabilizes p21 (Timchenko *et al.*, 1996).

C/EBPβ and C/EBPδ are mainly induced during proliferative phases of growth and are unable to inhibit growth when overexpressed in most cell types. For example, C/EBPβ is also important for transformation-induced autocrine growth in the chicken (Sterneck *et al.*, 1991). In chicken myeloid leukemia, following transformation by the kinase oncogenes v-myc or v-myb, chicken Myelomonocytic Growth Factor (cMGF) is secreted (Sterneck *et al.*, 1991). cMGF is the chicken homologue of the mammalian cytokines G-CSF and IL-6. cMGF is required in vivo for the outgrowth of chicken bone marrow-derived macrophages and granulocytes (Leutz *et al.*, 1984). However, in chicken myeloid leukemia, cMGF secretion sustains the autocrine growth characteristic of cancers. AP-1 and C/EBPβ-binding sites in the cMGF promoter were found to be essential for its expression (Sterneck *et al.*, 1991).

There have been exceptions in which C/EBPβ or C/EBPδ were not associated with proliferation; in rat HepG2 cells, C/EBPβ was found to have a strong antiproliferative effect. Its 20kDa truncated form did not arrest growth; as anticipated, it could antagonize the effects of the full-length isoform (Buck *et al.*, 1995). However, this effect of C/EBPβ has not been seen in any other tissue type or hepatoma cell line tested, and others were not able to duplicate these results (Hendricks-Taylor *et al.*, 1995, Ramos *et al.*, 1996, Diehl *et al.*, 1996). It is likely that C/EBP factor-specific processes such as induction of growth arrest are very complex, differing depending on tissue, cell line, or culture conditions.

In commaD mammary epithelial cells, C/EBPδ was induced by growth arrest conditions such as serum deprivation or contact-inhibition. This inducible arrest was significantly delayed by introduction of C/EBPδ antisense RNA. This effect seems specific to these cells; C/EBPδ has been correlated with proliferation and is decreased in growth arrested adipose, liver, and monocytes (O'Rourke *et al.*, 1997).

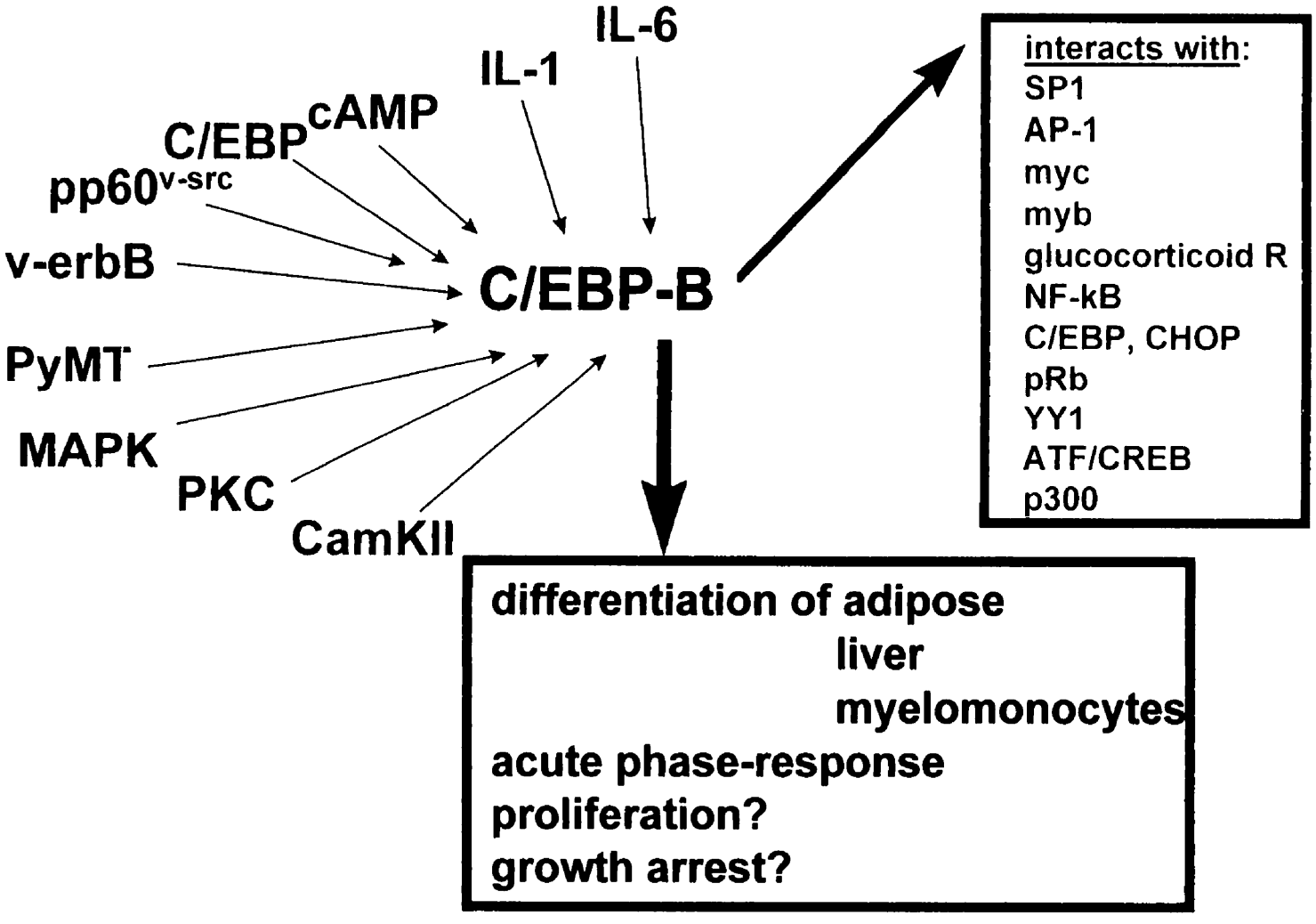
vii) C/EBP Protein Interactions

The C/EBP family has been found to interact by protein-protein interactions with several distinct families of transcription factors. In some cases they cross-dimerize with other bZip-bearing families, and in other cases the exact molecular interaction has not yet been elucidated. However, it is clear that the C/EBP family may be an integration point facilitating the convergence of a network of transcription factors.

C/EBPβ, fos, and jun can interact by virtue of their bZip domains, allowing the two families to regulate each other's DNA binding specificities. AP-1 factors can sequester C/EBPβ in order to prevent it from binding the usual sites which it binds as a homodimer. In turn, the 20kDa isoform of C/EBPβ (but not the full-length form) can also antagonize the binding of the AP-1 dimer to TREs. Interestingly, in liver, AP-1 is co-induced by IL-6 with C/EBPβ, allowing for the possibility of cross-talk between these transcription factor families in immune system functions (Hsu *et al.*, 1994).

Figure 4: Distinct signal transduction pathways converge on C/EBPβ, which is in turn involved in a variety of programs of gene expression.

This figure shows that C/EBPβ is a heavily regulated protein with many activators. C/EBPβ can be transcriptionally induced by treatment with cAMP, IL-1, or IL-6. Various kinases such as PKC, MAP Kinase, CamKII, v-erbB, and PyMT can potentiate C/EBPβ activity by phosphorylation. C/EBPβ is also involved in protein-protein interactions with the indicated transcription factor families. It seems that C/EBPβ may be central in mediating crosstalk between signaling pathways and gene expression programs. C/EBPβ is also crucial in several diverse processes outlined in the figure; its high level of regulation as well as its ability to interact with so many transcription factors may confer its pluripotency.


IL-6
IL-1
cAMP
C/EBP
pp60v-src
v-erbB
C/EBP-B
PyMT
MAPK
PKC
CamKII
interacts with:
SP1
AP-1
myc
myb
glucocorticoid R
NF-kB
C/EBP, CHOP
pRb
YY1
ATF/CREB
p300
differentiation of adipose
liver
myelomonocytes
acute phase-response
proliferation?
growth arrest?

The CREB/ATF bZip family can also interact with C/EBP factors by their leucine zippers. The CREB/ATF family binds cAMP-Response Elements (CREs), which are involved in modulating cAMP-responsive transcriptional induction. When C/ATF (C/EBP-related ATF) and C/EBPβ heterodimerize, they bind to asymmetric cAMP response elements such as those found in the pepck gene promoter. This is in contrast to the usual palindromic CREs bound by all CREB/ATF family homodimers (Vallejo *et al.*, 1993). A similar interaction was seen between C/EBPα and ATF-2, which co-immunoprecipitate in liver. ATF-2 affects C/EBPα-dependent transcription by transcriptional interference. It decreases transcription from C/EBP sites. However, C/EBPα confers on ATF-2 a different binding site specificity. The C/EBPα/ATF-2 heterodimer binds an asymmetric sequence composed of half sites corresponding to each monomer (Shuman *et al.*, 1997).

NFκB p65, p50, and rel and C/EBP α, β, and δ can interact and alter transcriptional consequences. The C/EBP protein's bZip domain is involved in interacting with NF-κB's N-terminal rel homology domain. This pairing results in an altered activation of promoters harbouring NF-κB motifs or C/EBP binding sites (Stein *et al.*, 1993). This heterodimerization is possibly facilitated by the frequent occurrence of adjacent NF-κB and C/EBP sites in several genes such as IL-6, IL-8/CEF-4, IL-12, G-CSF, and the acute phase proteins angiotensinogen and α1-acid glycoprotein (reviewed by Vietor *et al.*, 1996; Dehbi *et al.*, 1992). C/EBP factors in

combination with NFκB factors have been found to synergize in the transcription of these genes (Plevy *et al.*, 1997, Akira *et al.*, 1992).

Myc has an inhibitory effect on C/EBP-dependent transactivation. Freytag *et al.* (1992) showed that 3T3-L1 preadipocytes overexpressing myc could not differentiate, but compensatory C/EBPα overexpression could cancel this effect. In brown adipocytes, Antonson *et al.* (1995) demonstrated that myc overexpression represses endogenous C/EBPα expression. It should be noted that C/EBPα and C/EBPβ both have E boxes (myc consensus binding sites) in their promoters. Myc can supposedly inhibit C/EBPα and C/EBPβ in two ways; at a transcriptional level and at the protein level, although the mechanism of intramolecular interaction has not yet been elucidated (Mink *et al.*, 1996).

The transcription of rat cytochrome P450 was found to be activated by the combination of C/EBPβ and SP1. SP1 is a ubiquitous transcription factor which recognizes a "GC box" motif present in the promoters of many housekeeping genes. The site of interest in the cytochrome p450 promoter is a CCAAT sequence of weak consensus; SP1 is believed to recruit C/EBPβ to this low-affinity site and perhaps stabilize C/EBPβ's binding (Lee *et al.*, 1994).

c-Myb is the cellular homolog of v-myb, an oncogene transduced independently by two viruses: E26 and Avian Myeloblastosis Virus. It is important in

the regulation of differentiation of immature myeloid, erythroid, and lymphoid cells. In promyelocytes, myb was found to cooperate with C/EBPβ in the transactivation of the mim-1 gene, which harbours three myb sites (Ness *et al.*, 1993). The myb/C/EBP β interaction was recently found to involve a third binding protein, p300. p300 is a transcriptional co-activator known to link individual transcription factors via protein-protein interactions to the basal transcription machinery. It has been proven to be an essential co-activator for CREB, jun, myb, sap1a, fos, p53, myoD, NF-κB, and MEF-2. p300 was found to enhance myb and C/EBPβ's transcriptional activity individually as well as together. This is the first known instance of p300 directly linking two transcription factors (reviewed by Mink *et al.*, 1997).

Yin-Yang-1 (YY1) is a transcription factor which can activate or repress transcription, depending on the promoter context. In the Human Papillovirus (HPV)-type 18 promoter, there is a C/EBP site which can only be bound by C/EBPβ when it is interacting with YY-1. This site is contained within the upstream regulatory region of the viral promoter, which is believed to confer the ability of HPV to infect specific cell types (Bauknecht *et al.*, 1996).

The U937 large cell-lymphoma cell line differentiates to monocytes. During differentiation, Rb interacts directly through its SV40-Lt antigen binding domain with C/EBPβ. C/EBPβ can only bind the hypophosphorylated form of Rb prevalent in growth arrested cells. The consequence of this interaction is an enhancement of

C/EBPβ's binding activity to DNA. This interaction begins at the onset of differentiation and continues in terminally differentiated cells. The interaction with C/EBPβ appears to be transient and thus pRb may act as a chaperone to mediate the correct assembly and the optimal functional activity of C/EBPβ-containing complexes (Chen *et al.*, 1996).

In this study, we have investigated the role of C/EBPβ as a regulator of p20K's QRU. We show that the QRU's quiescence and proliferation-specific activities are dependent on C/EBP sites in the QRU, and that C/EBPβ is a component of C1, a quiescence-specific nuclear complex binding region A of the QRU. While C/EBPβ is necessary for activation of the QRU, it may not be sufficient. We postulate the existence of at least one additional quiescence-specific factor which cooperates with C/EBPβ to activate the QRU.

MATERIALS AND METHODS

Cell culture

Early passages of chicken embryo fibroblasts (CEF) were cultured at 41.5°C in Richter-improved minimal essential medium containing insulin and zinc (I+ medium, Irvine Scientific, Santa Ana, CA), 5% heat-inactivated enriched newborn bovine serum (BioMedia, Cansera, Rexdale, Ontario), 5% tryptose phosphate broth, and 1% glutamine, penicillin and streptomycin. CEF were starved in the complete absence of serum in Dulbecco's modified Eagle's medium (DMEM) containing 10% tryptose phosphate broth. To maintain a constant pH, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.4 was added routinely to the culture medium of starved cells. Cell lines expressing chicken C/EBPβ (NFM) from the retroviral vector RCASBP were made by transfecting early passage CEF in 60 mm dishes by calcium phosphate precipitation as described previously (Mao *et al.*, 1993; Graham and van der Eb, 1973).

Plasmid constructs and transient expression assays

The RCASBP-NF-M vector was constructed as described previously by Cabannes *et al.*, 1997. The deletion constructs of the 5' flanking region of the p20K gene were described previously (Mao *et al.*, 1993). Synthetic double-stranded oligonucleotides representing various sub-domains of the p20K's quiescence-responsive unit (QRU) were multimerized and inserted in the Hind III site of plasmid pJF CAT-TATA.

Plasmid pJFCAT-TATA includes a minimal promoter consisting of a TATAAAA box and the initiation start site of the human β globin gene. All transfections were done by calcium phosphate precipitation as described previously (Mao *et al.*, 1993; Graham and van der Eb, 1973). Briefly, cultures of normal CEF grown to approximately 80% confluence in 100mm dishes were transfected with 10 μg (micrograms) of test reporter plasmid, 2 μg of the lac Z containing plasmid pCH110 (Pharmacia), and 18μg of salmon sperm carrier DNA. The day after transfection, half of the cells were refed with serum-containing medium in order to stimulate growth and therefore maximize contact inhibition. The remaining cells were trypsinized and seeded at 1/6 of the original density in serum-containing medium. Cell lysates were prepared 40-48h (hours) after transfection and 24 hours after refeeding or trypsinization. The effect of C/EBP factors was examined in CEF co-transfected with 10 μg of the test plasmid and 2 μg of expression vector. The dominant negative mutant of C/EBPβ has been described by Kowenz-Leutz *et al.* (1994). For all transient expression assays, CAT activity was determined in lysates representing equal levels of β galactosidase activity. β galactosidase assays were performed as described by Miller (1972). CAT assays were performed as described by Mercota *et al.* (1985) and results quantified using an Instant Imager. All constructs were analyzed in duplicates in at least three separate experiments. The results of previous studies indicated that the SV40 enhancer/promoter in plasmid pCH110 is not regulated in our conditions of

proliferation and therefore could serve as internal standard to control for differences in transfection efficiency.

Electrophoretic Mobility Shift Assay (EMSA)

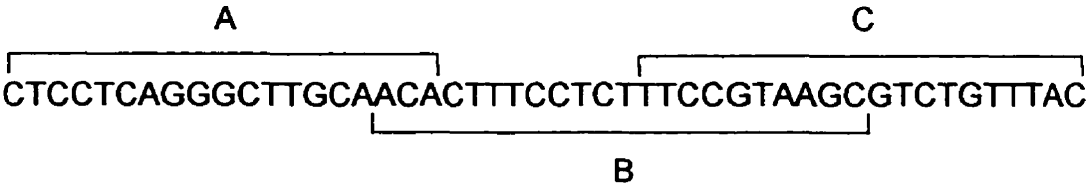
Nuclear extracts were prepared by a modification of the methods of Briggs *et al.*(1986) and Dignam *et al.* (1993). Routinely, 10^7 cells were washed once with cold PBS (phosphate-buffered saline), collected for 5 min in a microcentrifuge at 1,500 Xg (gravity) and resuspended in 500 µl (microlitres) of buffer A (10 mM (millimolar) HEPES; pH 7.9, 10 mM KCl, 0.5 mM DTT, 1.5 mM $MgCl_2$, and a cocktail of protease inhibitors containing 0.5 mM phenylmethyl sulfonyl fluoride [PMSF], 0.3 µg leupeptin per ml (millilitres), 0.3 µg antipain per ml, and 0.5µg aprotinine per ml. Nuclei were homogenized with a glass Dounce homogenizer (B pestle). The nuclear lysate was centrifuged for 20 min (minutes) at 12,000 Xg, and the nuclear pellet extracted with 1 ml of buffer C (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.42 mM KCl, 0.5 mM DTT (dithiothreitol), 1 mM EDTA (ethylenediamine tetraacetic acid), and protease inhibitors as mentioned above for 20 min at 4°C with continuous agitation. The nuclear lysate was centrifuged at 80,000 Xg for 1 h at 4°C and then partially purified by ammonium sulfate precipitation; 0.33g (grams) of ammonium sulfate was added slowly per ml of the resulting supernatant. The mixture was then incubated for 30 min at 4°C with gentle stirring and finally centrifuged at 21,000 Xg for 15 min. The supernatant was discarded and the pellet resuspended in

buffer D (20 mM HEPES pH 7.9, 20% glycerol, 0.1M KCl, 0.5 mM DTT, and 0.2 mM EDTA), and dialysed against the same buffer for 4h at 4°C. The dialysate from the purified nuclear extract was aliquoted and stored at -80°C until needed. Protein concentration was determined by the Bradford assay using bovine serum albumin as a standard. Synthetic DNA oligomers used for DNA binding analysis are shown in figure 5. Oligonucleotides were end-labelled (approximately 10 000 cpm/0.1 ng) (counts per minute; nanograms), and typically 20 000 cpm was used per binding reaction. Binding reactions containing 2 μg of nuclear extract were performed for 30 minutes at room temperature in a total volume of 20 ul of 1X binding buffer (10mM HEPES-KOH pH 7.9, 100 mM NaCl, 1 mM EDTA pH 8.0, 1 mM DTT, 7% glycerol) in the presence of 2 μg of poly dIdC (deoxyinosinic-deoxycytidylic acid) as a nonspecific competitor. Competition binding reactions were performed by pre-incubating the nuclear extract with an excess of unlabelled oligonucleotide for 15 minutes at 4 °C. Complexes were supershifted by preincubating nuclear extract with 2 μL of polyclonal antibody to C/EBPβ or preimmune serum for 2 hours at 4°C. Both were followed by addition of the probe and incubation for an additional 30 minutes at room temperature. The DNA-protein complexes were resolved on 4.8% non-denaturing polyacrylamide gels in 0.5X TBE (tris borate), and visualized by autoradiography.

Figure 5: QRU-derived Oligo Constructs Used in this Study.

This figure lists the various oligo constructs used in transient transfection and electrophoretic mobility shift assays. The top line reveals the location of consensus binding sites for candidate QRU transcriptional regulators. The second line shows the origin and relative placement of oligos A, B, and C in the QRU. Introduced mutations are indicated in lower case. All oligos with the exception of NFM are derived from the QRU. NFM was intended to represent a typical C/EBPβ binding site.

QRU



QRU-uAII
CTCCTCAGGGC**ac**GCAACACTTTCCTCTTTCCGTAAGCGTCTGTTTAC

QRU-uBII
CTCCTCAGGGCTTGCAACACTTTCCTCTTTCCGT**gt**GCGTCTGTTTAC

QRU-u2C/EBP
CTCCTCAGGGC**ac**GCAACACTTTCCTCTTTCCGT**gt**GCGTCTGTTTAC

QRU-mut A
CT**gg**TCA**aa**GCTTGCAACACTTTCCTCTTTCCGTAAGCGTCTGTTTAC

A
CTCCTCAGGGCTTGCAACA

A-uAII
CTCCTCAGGGC**ac**GCAACA

B
ACACTTTCCTCTTTCCGTAAGC

uB
ACACTTT**gg**TCTTT**gg**GTAAGC

C
TTCCGTAAGCGTCTGTTTAC

NFM
CACAATGAGGCAAC

Transcriptional Run-on Assay

Run-ons were performed according to the procedure of Cabannes et al (1997). 5 X 10^7 cells were scraped in PBS and spun down for 5 minutes at 4° C in a medifuge at 5000 rpm. They were resuspended in lysis buffer (15 mM NaCl, 60 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 14 mM β- mercaptoethanol, 0.5% Nonidet P-40, 0.15mM spermine, 0.5 mM spermidine, 10% sucrose, 15 mM Hepes pH 7.5) and lysed with a B pestle. Nuclei were purified through a 30% sucrose gradient in lysis buffer. The nuclei were then resuspended in transcription buffer (180mM Tris pH 8, 25mM NaCl, 0.3 mM EDTA, 0.1 M PMSF, 1.5 mM DTT, 700mM (NH4)2SO4, 8 mM MnCl2, 20mM Creatine phosphate, 200μg/mL creatine phosphokinase, 600U/mL Rnasine (Promega), 1mM each ATP, CTP, GTP, and 250 μCi [α^{32}P]UTP. Following treatments with Dnase RQ1 and proteinase K, run-on products were phenol-extracted and purified on a G-50 Sephadex column. They were then ethanol precipitated and suspended in hybridization buffer (10mM TES pH 7.5, 0.2% SDS, 1X Denhardt's, 10mM EDTA, 300mM NaCl, 250μg/mL yeast tRNA). Nytran membranes were prepared for hybridization by adhering 2μg each of linearized plasmids through a slot-blot apparatus. Run-on products were quantified using a scintillation counter before hybridization to the membranes for 60 hours at 65°C using 5 x 10^6 counts/mL. Membranes were then washed and analyzed by autoradiography.

cDNA library screen

A contact-inhibited CEF cDNA library was screened using a SacII fragment of chicken C/EBPα (provided by C. Calkoven) encompassing the DNA-binding and leucine zipper domains. Following secondary and tertiary screens of all clones picked, clones were excised using ExAssist helper phage protocol (Stratagene). Following excision, clones were analyzed by a combination of restriction analysis and sequencing followed by FastA database analysis (Pearson, 1990).

Immunoprecipitation

Polyclonal antibody to p20K was generated as described in Bédard *et al.*, 1987b. Immunoprecipitations were performed as described in Mao *et al.* (1993). Briefly, CEF were labeled in 35 mm dishes with 100μCi of [^{35}S]-methionine for 1 hour. The medium was collected and cells lysed in SDS (sodium dodecyl sulphate)-containing sample buffer. Incorporated counts were calculated using hot TCA (trichloroacetic acid) precipitation followed by quantification on a scintillation counter. Immunoprecipitations were carried out in RIPA buffer, and the results analyzed on a 10% polyacrylamide gel.

Northern

RNA samples were prepared and run on formaldehyde-agarose gels as described previously (Bédard *et al.*, 1987), and then blotted onto nitrocellulose (Schleicher and Schuell, BA85). Probes were prepared using the random priming method of Feinberg and Vogelstein (1983) using a commercial kit (New England Biolabs, Beverley, MA). Analysis was performed by autoradiography.

Western

Total cell extracts were subjected to SDS-polyacrylamide gel electrophoresis and blotted on nitrocellulose (Schleicher and Schuell, BA85). Polyclonal antibody to C/EBPβ was a gift of Karl-Heinz Klempnauer and was used at a dilution of 1:2000 in a 0.1% solution of Carnation milk in PBS (Johnson *et al.*, 1984). This was followed by incubation with a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) and a chemiluminescent reaction (ECL, Amersham).

Immunofluorescence

Immunofluorescence using C/EBPβ antibody was performed as described by Cabannes *et al.*, (1997). All steps were performed at room temperature, rinsing with PBS between each step, and all solutions were made in PBS. RCASBP-NF-M(C/EBPβ) and RCASBP infected cells were grown on coverslips and fixed in 3.7% formaldehyde for 15 min. This was followed by a 5 min treatment using 0.1% Triton X in order to

permeabilize the cells. Incubation with C/EBPβ/NFM antibody was performed at a dilution of 1:200 in 10% fetal calf serum (Cansera, Rexdale, Ontario). This was followed by incubation with a fluorescein-conjugated secondary antibody (Cappel Research Products, Durham, NC) at a 1:70 dilution in 10% fetal calf serum. Cells were then treated with a 2mg/ml solution of RNAse A in 0.3% Triton X-100, and stained for 1 hour in a 1 mg/ml solution of propidium iodide. Coverslips were then mounted in 70% glycerol and 0.1% phenylenediamine. Analysis of C/EBPβ/NFM localization was performed using the MRC-600 BioRad Confocal Microscopy System.

Lipid treatment and Oil Red O Staining

CEF were treated overnight by adding 200μM linoleate, palmitate, oleate, or diluent (ethanol) directly to their medium. Lipid droplet formation was analyzed by fixing the cells in 3.7% formaldehyde in PBS, followed by staining for 1 hour with a .25% solution of Oil Red O in ethanol (as outlined by Preece, 1965). Photographs of stained cells were taken on a Zeiss Telaval microscope.

RESULTS

1. Identification of functional domains of the QRU.

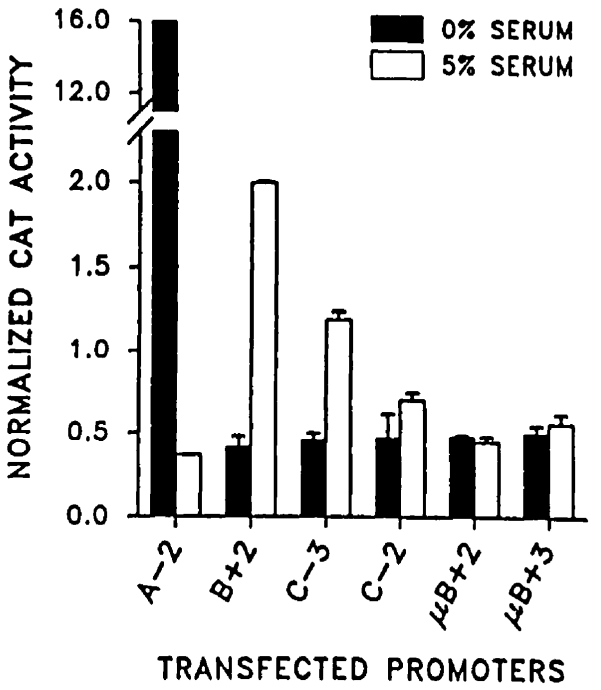
The region of the promoter required for activation of the p20K gene in quiescent cells was named the quiescence-responsive unit or QRU. The 48bp QRU confers quiescence-responsiveness to a heterologous promoter in conditions or serum starvation or contact inhibition, and is both necessary and sufficient for induction (Mao *et al.*, 1993). To begin the characterization of the QRU, we first identified potential binding sites for known transcription factors using the program Signal Scan (Prestige, 1991). The results of this analysis are seen in figure 2. Two consensus binding sites for the C/EBP and ets families are present within the QRU. A more divergent site for the Maf family of transcription factors is located at the 3' end of the QRU. A palindrome (CCTCAGG) with no significant homology with known regulatory elements is also found at the 5' end of the QRU. To identify functional domains within the QRU, we then arbitrarily defined three partially overlapping regions designated regions A, B, and C (please see figure 5) and analyzed their activity in transient expression assays. Region A includes the first C/EBP binding site and the palindrome located at the 5' end of the QRU. Region B contains the two potential ets-response elements and the second C/EBP binding site. Region C includes the second C/EBP binding site and the potential Maf recognition element. Synthetic double-stranded oligonucleotides corresponding to each region were multimerized,

inserted in proximity of a minimal promoter and investigated in proliferating and quiescent CEF. A construct containing two copies of Region A was strongly activated in quiescent cells with a 40-fold induction over the level observed in actively dividing cells (construct A-2 in figure 6). In contrast, constructs containing two or three copies of regions B or C did not respond to growth arrest and were in fact more active in proliferating cells. This proliferation-dependent activity was more obvious with region B than with region C but remained modest when compared to the strong activity of Region A in non-dividing cells. Therefore, the QRU is composed of both proliferation and quiescence-responsive elements. Region A contains a potent quiescence-responsive element.

Since several members of the Ets family have been implicated in gene activation in response to mitogens (Wasylyk *et al.*, 1989; Bhat *et al.*, 1990), we synthesized a modified version of the B oligo containing a mutation in both potential Ets-binding sites but affecting noncritical residues of the putative C/EBP binding site (constructs μB+2 and μB+3 in figure 6; Nye *et al.*, 1992; Thompson *et al.*, 1991). When investigated in transient expression assays, the mutation of the potential ETS-binding sites reduced the activity of region B in actively dividing cells suggesting that one or multiple proliferation-responsive elements are also present in the QRU. These weak proliferation-responsive elements of Region B remain to be characterized.

Figure 6: Regions A, B, and C of the QRU show growth state-responsive activity.

This experiment utilized multimerized copies of oligos A, B, and C, which comprise the QRU. μB oligos are identical to the Region B oligos except that they harbour mutations in the ets consensus sites. These oligos were inserted upstream of a minimal promoter and a CAT reporter gene. The oligos' orientation and copy number are indicated in the names of the constructs; "+" denotes a forward orientation whereas "-" represents a reverse orientation, and the copy number is specified by the number following the +/- symbol. These reporter constructs were transfected into CEF which were subsequently starved (0% serum) or allowed to actively divide (5% serum). Their activation in each condition was then quantitated. (Mao and Bédard, unpublished data).

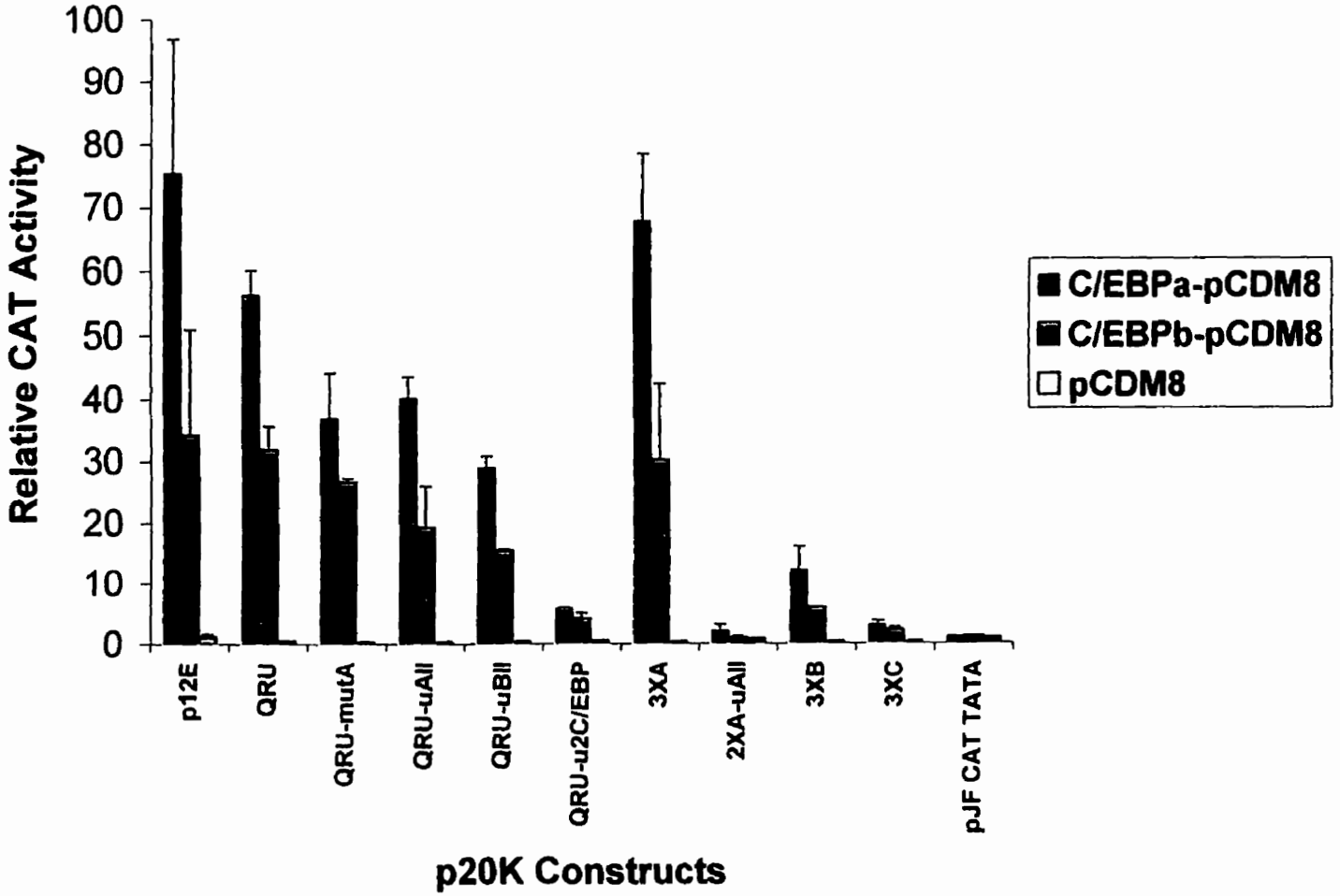

16.0
12.0
2.0
1.5
1.0
0.5
0.0
0% SERUM
5% SERUM
NORMALIZED CAT ACTIVITY
A-2
B+2
C-3
C-2
μB+2
μB+3
TRANSFECTED PROMOTERS

2. *Ectopic expression of C/EBP factors is sufficient to activate the QRU.*

The over-expression of several members of the Maf (c-maf I, c-mafII, mafB, mafF, mafG, and mafK) and ETS (C-ets-1, PEA3) families did not affect the activity of the QRU in CEF (data not shown). In contrast, members of the C/EBP family were potent activators of the QRU. Figure 7 shows the relative activation levels of p12E and of various QRU and QRU-derived constructs by the ectopic expression of C/EBP factors α and β (please refer to figure 5 for an outline of all oligo constructs used). Compared to controls, very strong activation is seen with p12E, QRU, and all derived QRU constructs except for μ2C/EBP. μ2C/EBP's relative lack of responsiveness reflects the mutations in its two C/EBP binding sites. Of the multimerized oligos, A shows the greatest induction (35-60 fold), and the mutations in 2XA-μAII which disable the CCAAT site abrogate this activation. B and C oligos also show some induction by C/EBP factors, although their levels (3-12 fold) are not as high as that seen by the A site. However, in the context of the entire QRU (i.e. using 48uAII and 48uBII) both CCAAT sites appear to be strong. The mutation of the palindrome of region A was also activated efficiently (QRU-mutA), which was expected as the palindrome does not overlap with the core sequence of the C/EBP binding site (Figure 5). The 2 X μB construct shows a reduced induction compared to 3 X B, but this may reflect the fewer number of inserts. It should be emphasized that the mutations in μB are meant to target the ets sites and do not affect critical residues in the CCAAT binding site of the B oligo. pJF CAT TATA, the parental promoter construct, did not

Figure 7: Ectopic expression of C/EBP factors activates p20K promoter constructs.

In these experiments, C/EBPα, C/EBPβ, or the empty expression vector pCDM8 was cotransfected with CAT reporter constructs containing the indicated portions of the p20K promoter. As a negative control, the empty CAT reporter construct PJF-CAT-TATA was also tested.

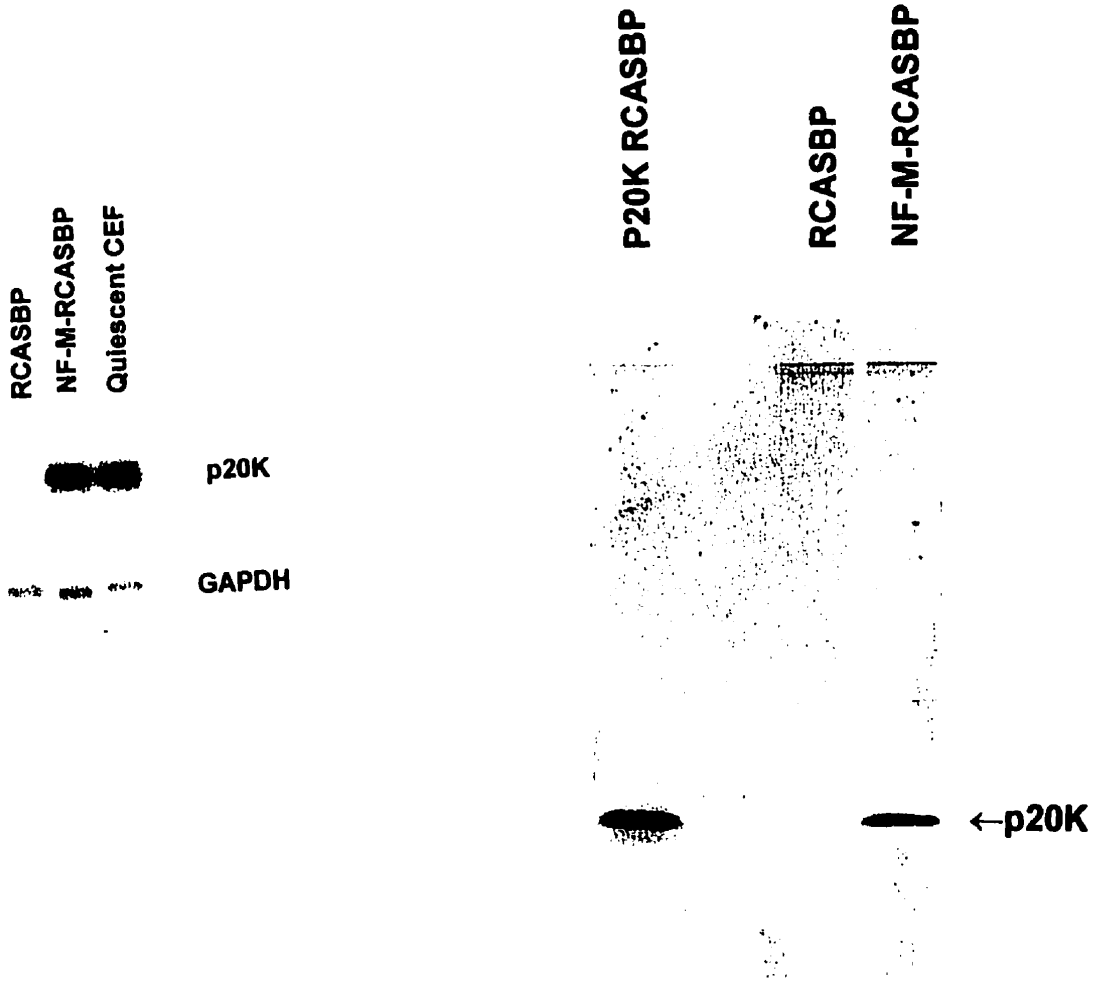

Relative CAT Activity
100
90
80
70
60
50
40
30
20
10
0
p12E
QRU
QRU-mutA
QRU-uAII
QRU-uBII
QRU-u2C/EBP
3XA
2XA-uAII
3XB
3XC
pJF CAT TATA
p20K Constructs
C/EBPa-pCDM8
C/EBPb-pCDM8
pCDM8

respond to over-expression of C/EBPα or β. Therefore we conclude that the QRU contains two C/EBP-responsive elements and is strongly transactivated by the α and β members of this family. In contrast, none of the QRU constructs was affected by CHOP-10 (also known as gadd153), when this C/EBP family pseudomember was expressed alone or in combination with C/EBPα or β (data not shown).

In figure 8, experiments were performed using CEF which were infected with a replication-competent retroviral construct, RCASBP-NFM(C/EBPβ), which expresses high levels of C/EBPβ. As a control, CEF were also infected with the parental virus, RCASBP. Infected cells were actively growing and cultured in conditions in which p20K is not normally expressed. In panel a, a Northern blot was performed using total RNA extracted from RCASBP-NFM(C/EBPβ)-infected CEF, RCASBP-infected CEF, and uninfected, contact-inhibited CEF. This northern demonstrates that levels of p20K RNA induction by quiescence and by overexpression of C/EBPβ/NFM are comparable. The immunoprecipitation in panel b shows that C/EBPβ/NFM induces p20K strongly at the protein level compared to the RCASBP-infected control. As a positive control, CEF were infected with a virus encoding p20K (P20K-RCASBP) and this sample was immunoprecipitated with p20K antibody in parallel. These results provide strong evidence that mere overexpression of C/EBPβ is sufficient to induce expression of p20K.

Figure 8: p20K is Induced in CEF infected with RCASBP-NFM (C/EBPβ).

CEF infected with the retroviral construct RCASBP-NFM(C/EBPβ) express very high levels of C/EBPβ. This figure shows p20K induction in these CEF as a result of this high C/EBPβ expression. In panel a, p20K mRNA induction by quiescence and by C/EBPβ ectopic expression is compared. RNA from CEF infected with the control virus RCASBP is included. To demonstrate that loading was equal in all lanes, GAPDH (Glyceraldehyde Phosphate Dehydrogenase) RNA levels are also shown. GAPDH is a housekeeping gene whose levels are not affected by growth conditions. Similarly, in panel b, an immunoprecipitation was performed which shows levels of p20K protein which are produced due to C/EBPβ overexpression. Endogenous p20K levels are seen in control CEF infected by RCASBP. As a positive control, p20K was also immunoprecipitated from CEF infected with RCASBP-p20K, a recombinant retrovirus harbouring the cDNA for p20K.3.



a. p20K mRNA

b. p20K Protein

3. C/EBP factors are involved in activating the QRU.

Figure 9 shows the induction of the p20K promoter and the QRU by quiescence when linked to the CAT reporter gene in transient transfection assays. p12E, QRU, and QRU-mutA have intact CCAAT sites and show strong induction by quiescence. This induction is abrogated by the mutation of either CCAAT site as seen in the cases of 48-uAII, 48-uBII, and u2-C/EBP. It is interesting that the site located in region B or C is essential when studied in the context of the QRU. This implies that the two sites cooperate in quiescence-specific activation of the QRU.

Figure 10 shows that transfection of Δ184, which is a dominant-negative form of C/EBPβ, significantly decreases quiescence-driven induction of p20K promoter constructs. Δ184 possesses an intact DNA-binding and dimerization domain, but it lacks a functional trans-activation domain (Kowenz-Leutz *et al.*, 1994). It is believed to be mediating its inhibitory effects by preventing endogenous C/EBP factors from binding the CCAAT sites of the QRU. The control vector pCDM8 does not have an effect. As a negative control, three copies of the AP-1 binding site were tested as well, and it can be seen that Δ184 did not affect their activation levels. These results strongly suggest that C/EBP factors play a major role in the QRU's activation in conditions of quiescence.

To identify a relevant C/EBP-related activator of the QRU, we screened a contact-inhibited CEF cDNA library with a DNA fragment corresponding to the conserved DNA binding and dimerization domain of avian C/EBPα. Thirty

Figure 9: QRU induction in quiescence is abrogated by mutation of its C/EBP sites.

This graph shows that the p20K promoter constructs p12E and QRU are induced strongly by conditions of quiescence. However, when either of the C/EBP sites is mutated, the ability of quiescence to induce the QRU disappears.

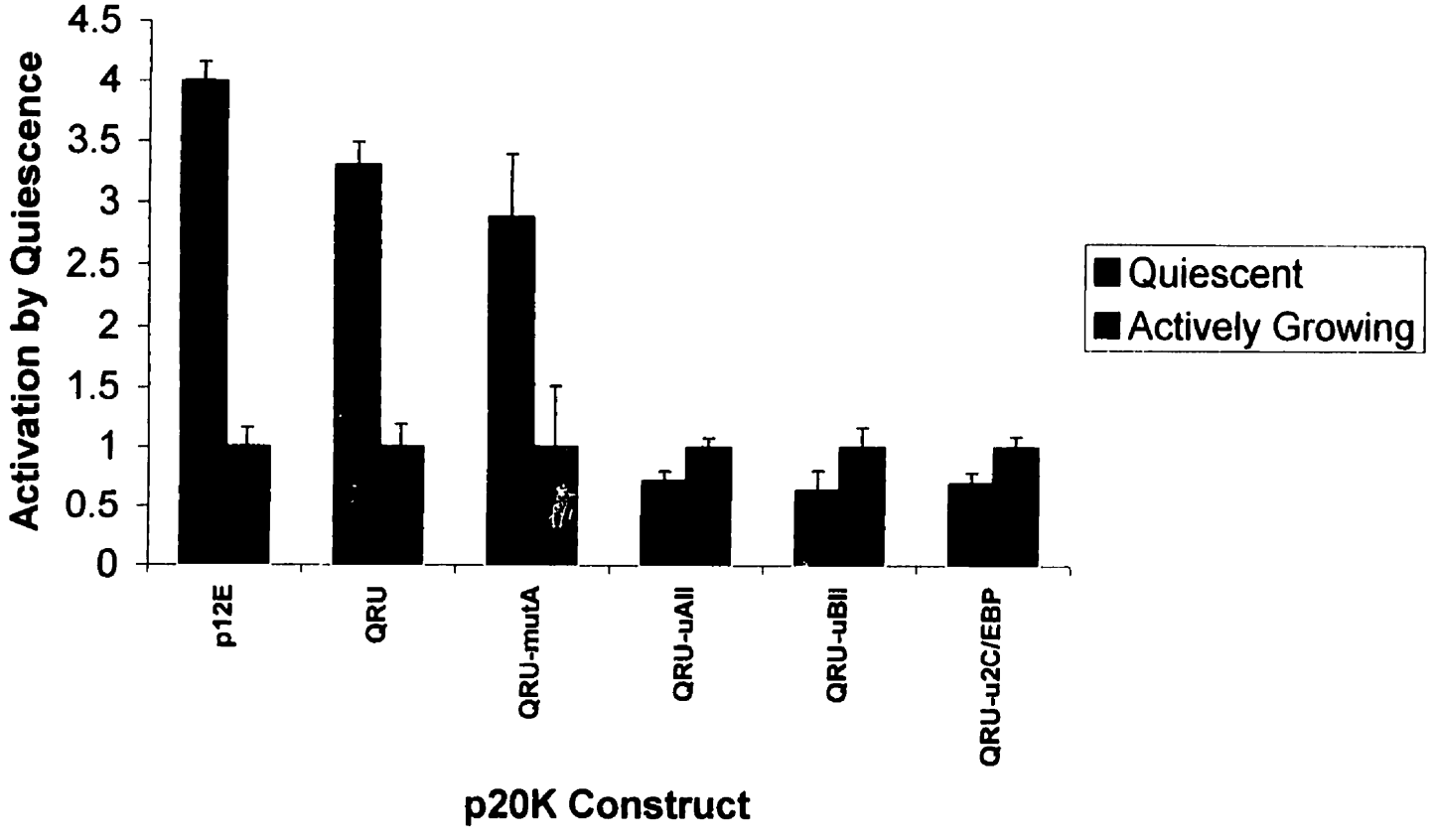
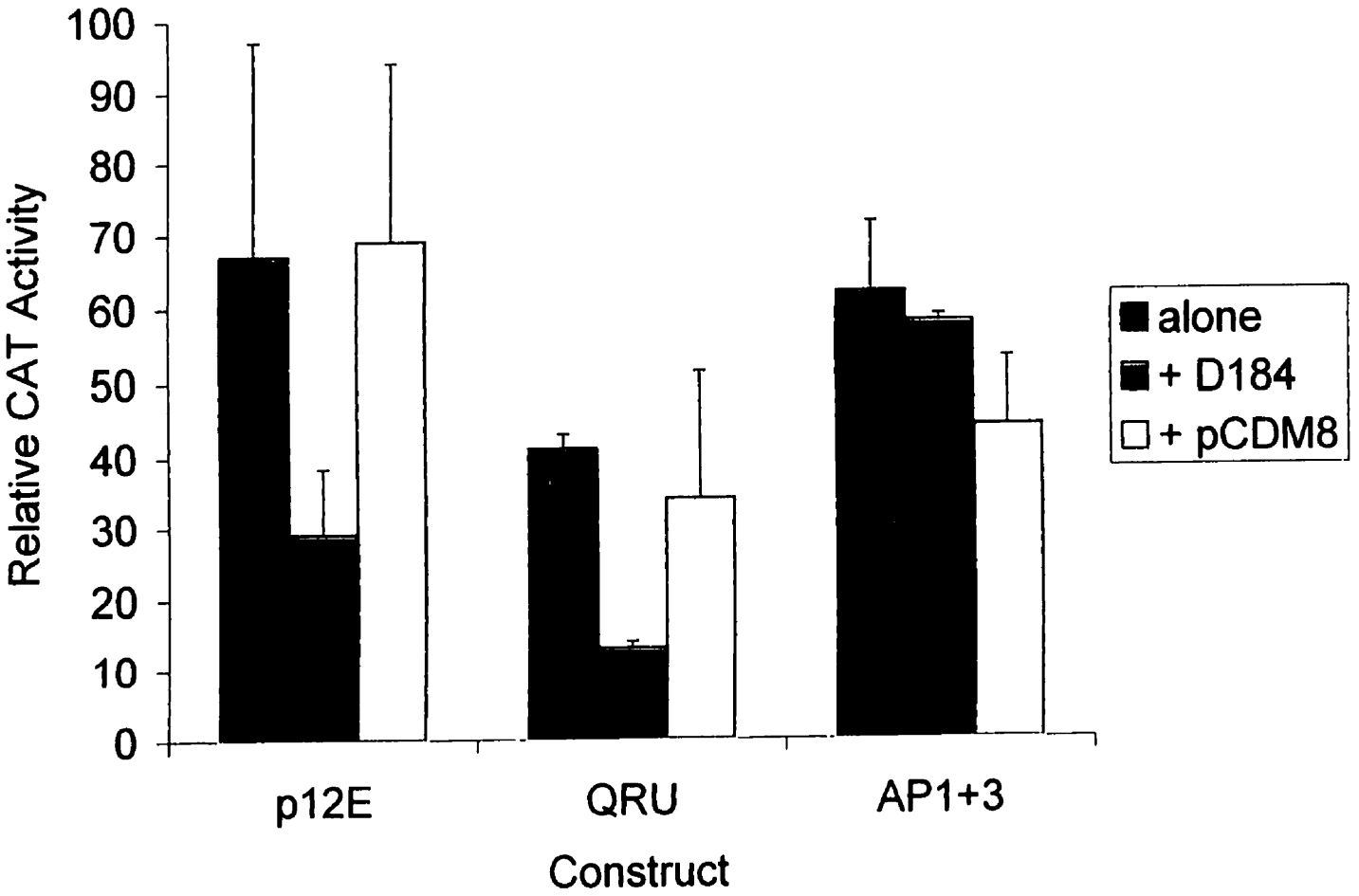

Activation by Quiescence
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
p12E
QRU
QRU-mutA
QRU-uAII
QRU-uBII
QRU-u2C/EBP
p20K Construct
Quiescent
Actively Growing

Figure 10: Dominant-negative C/EBPβ Decreases p20K Promoter Activation by Quiescence.

Δ184 is a dominant-negative version of C/EBPβ which lacks a functional trans-activation domain. This experiment demonstrates that cotransfection of Δ184 with p20K promoter constructs significantly decreases their activity in quiescence. As a negative control, Δ184 was cotransfected with a reporter construct containing three copies of the AP-1 binding site.

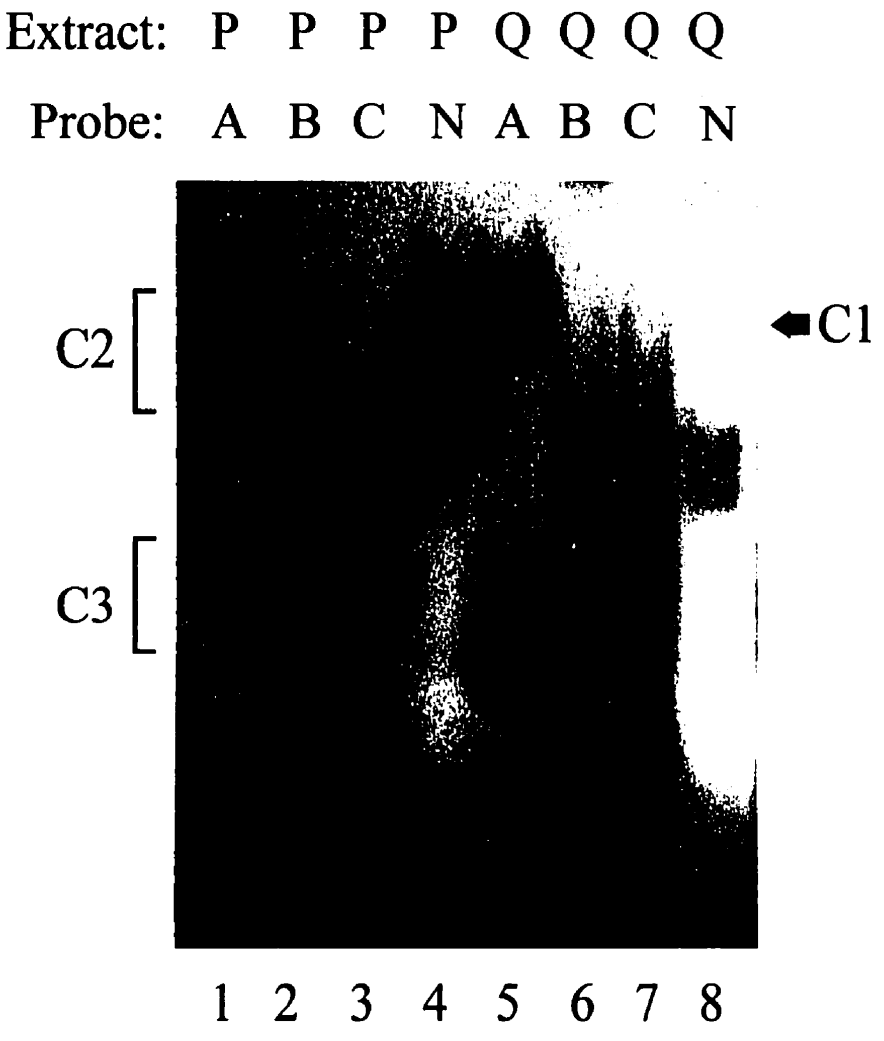

100
90
80
70
60
50
40
30
20
10
0
Relative CAT Activity
p12E
QRU
AP1+3
Construct
alone
+ D184
+ pCDM8

independent clones were isolated and were characterized by restriction digest and sequencing analysis. Twenty-one of the thirty clones encoded C/EBPβ, and four encoded C/EBPα, the only other C/EBP family member isolated in our screen (data not shown). This screen showed that C/EBPβ is the most frequently expressed C/EBP member in contact-inhibited CEF.

4. *Distinct nucleoprotein complexes are observed in quiescent vs. proliferating CEF.* In figure 11, radiolabelled oligos for the A, B, and C regions of the QRU as well as for an NF-M/C/EBP/β consensus binding site were used with nuclear extracts made from proliferating and quiescent CEF. Nucleoprotein complexes formed with regions B and C were not strikingly different when nuclear extracts were prepared from proliferating or quiescent CEF. In contrast, complexes of region A, designated C1 and C2, were quantitatively and qualitatively different (Lanes 1, 4, 5, and 8). In quiescent CEF, complex C1 is observed with the A oligo but not with the NFM oligo, implying that C1 cannot bind the NF-M oligo with high affinity. This insinuates that the A oligo CCAAT site has some specific characteristics which confer the ability of C1 to bind. Conversely, in proliferating cells, a complex named C2 is seen with the A oligo. When the NFM oligo is used instead of the A oligo, complex C2 is far more stable. This implies that the A oligo can also bind a growth-specific complex C2, albeit at a lower affinity compared to the NFM site. Taken together, these results indicate that the A oligo is central in mediating the growth state-specific binding of two distinct **Figure**

11: Distinct nucleoprotein complexes are seen in quiescent and proliferating CEF.

In this electrophoretic mobility shift assay, nuclear extracts isolated from proliferating (P) or quiescent (Q) cells were incubated with oligos A, B, C, or NFM (please see figure 5 for a list of their sequences). This electrophoretic mobility shift assay (EMSA) shows that the A oligo binds two distinctly migrating complexes: C1 in quiescent cells and C2 in proliferating cells. A large difference between C1 and C2 in terms of their affinities for the NFM oligo is also apparent.

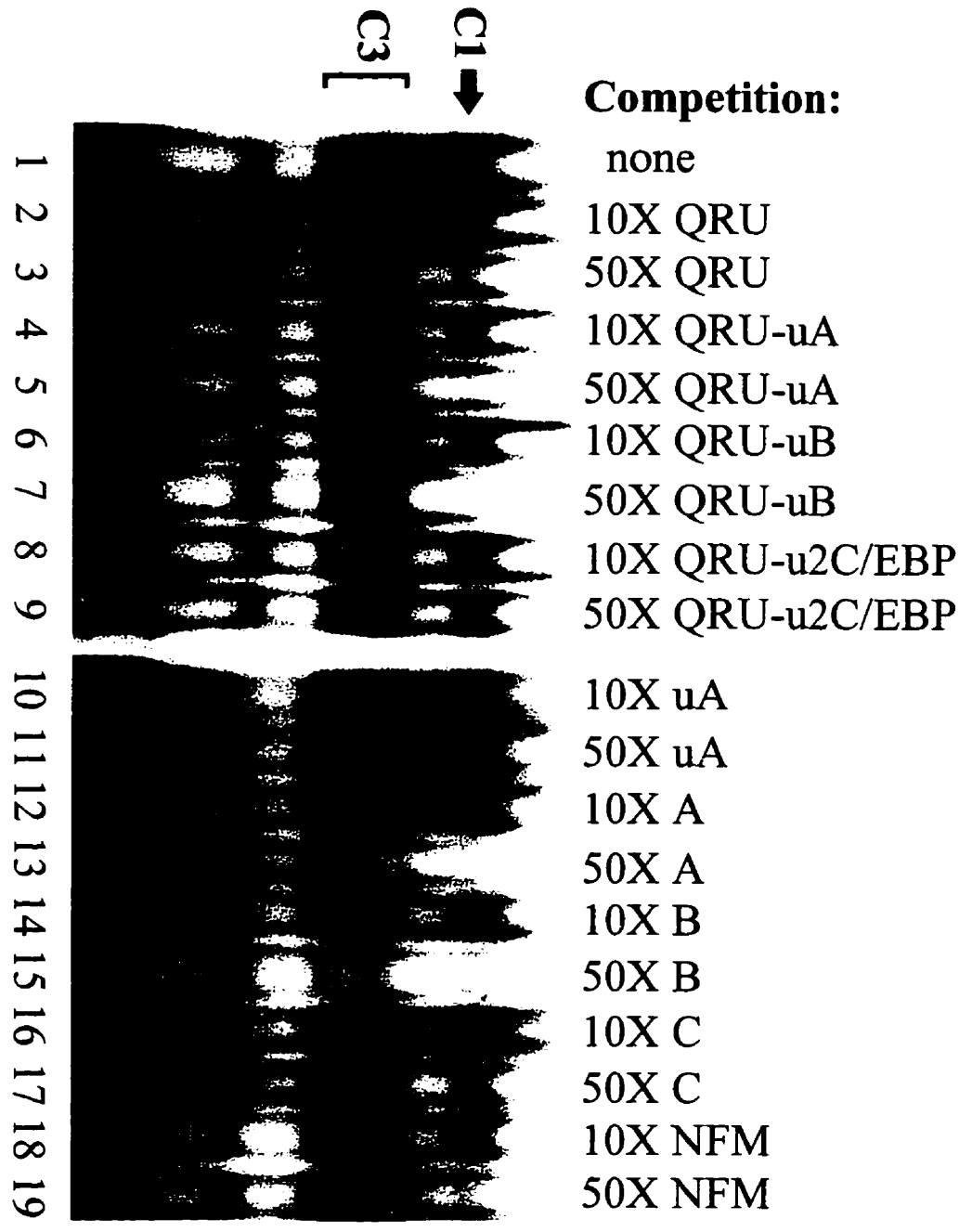

Extract: P P P P Q Q Q Q
Probe: A B C N A B C N
C2
C3
C1
1 2 3 4 5 6 7 8

nucleoprotein complexes. Other faster migrating complexes (labeled C3) were often observed with region A but their relative abundance varied with extracts prepared from different batches of contact-inhibited CEF. The significance of the C3 complexes is presently unknown.

In order to demonstrate the specificity of complex C1, competition EMSAs with unlabeled oligos were performed. Figure 12 shows that complex C1 can be competed by a 50-fold excess of QRU oligo, QRU-uA oligo, QRU-uB oligo (lanes 3, 5, and 7, respectively). These results were attributed to the fact that each of these oligos retains at least one intact CCAAT binding site. In contrast, QRU-μ2-C/EBP could not compete for C1 formation. Similarly, a 50-fold excess of the A oligo could efficiently compete for C1 formation (lane 13). In contrast, the μA oligo, which differs by two nucleotides critical for C/EBP binding, did not compete complex C1, even when present at a 50-fold molar excess (lane 11). A 50-fold excess of region B , C, or NFM oligos could also compete C1 formation, albeit less efficiently than region A (see lanes 15, 17 and 19, respectively). Taken together, these results show that C/EBP site-containing oligos can specifically compete complex C1. This indicates that the formation of the quiescence-specific complex C1 is dependent on nucleotides that are also critical for the binding of C/EBP.

Figure 12: Nucleoprotein Complex C1 is Competed by Oligos Containing C/EBP Sites.

Nuclear extracts from quiescent cells were incubated with an excess (10X or 50X) of various unlabeled oligos. Only competitors containing intact C/EBP sites were effective at competing complex C1.


C1
C3
Competition:
none
10X QRU
50X QRU
10X QRU-uA
50X QRU-uA
10X QRU-uB
50X QRU-uB
10X QRU-u2C/EBP
50X QRU-u2C/EBP
10X uA
50X uA
10X A
50X A
10X B
50X B
10X C
50X C
10X NFM
50X NFM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

5. *C/EBPβ is a component of both quiescence-specific and proliferation-specific nucleoprotein complexes.*

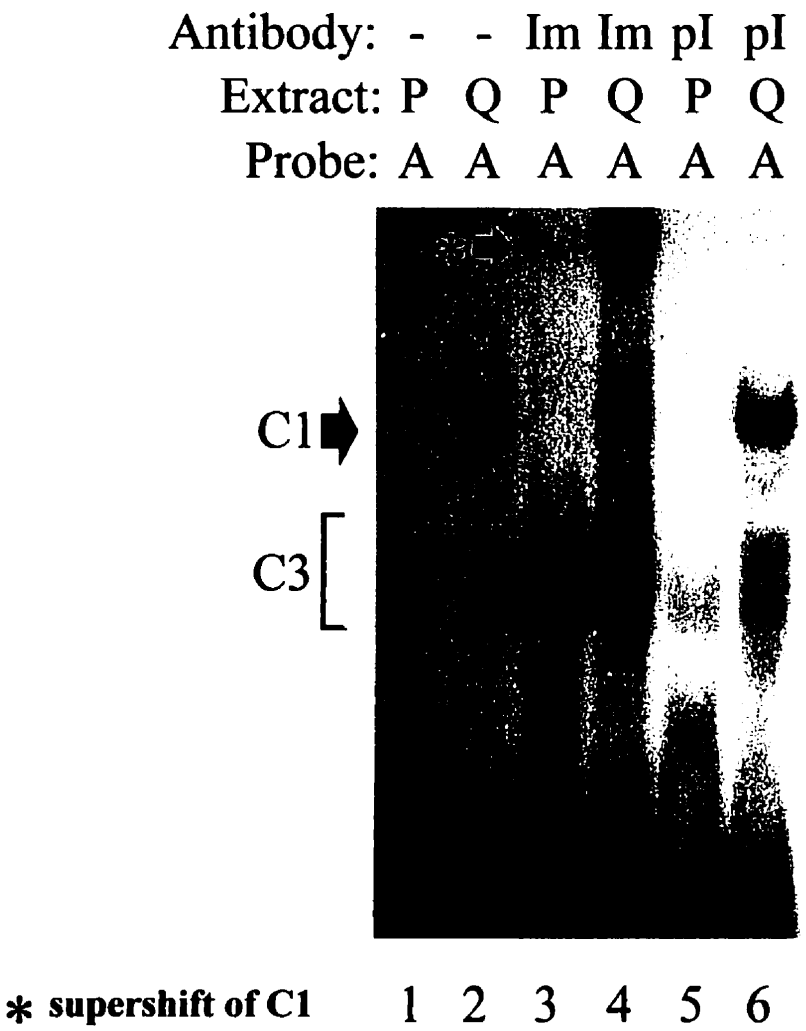
To characterize complexes C1 and C2, nuclear extracts prepared from quiescent or proliferating CEF were preincubated with a C/EBPβ antibody or with the corresponding preimmune serum and analyzed by EMSA. Figure 13 shows that most of complex C1 can be supershifted by preincubation with an antibody to C/EBPβ (lane 4). A less abundant C/EBPβ-containing complex was also supershifted by the antibody when region A was incubated with nuclear extract prerpared from actively-dividing CEF (lane 3). The results of other experiments indicate that this complex corresponds to the less abundant complex C2 (data not shown). Figure 14 shows that complex C2 can be similarly supershifted (lane 4). These results prove that both C1 and C2 contain C/EBPβ, implicating C/EBPβ in both quiescence and proliferation-driven aspects of QRU regulation.

6. *Levels of C/EBPβ increase in quiescent CEF.*

Figure 15 shows that C/EBPβ is induced both at the mRNA and protein level in quiescent CEF. The nuclear run-on assay reveals a 6-7 fold induction of C/EBPβ mRNA in quiescent cells. Similarly, the Western blot shows that C/EBPβ protein levels increase in quiescent cells. The cDNA library screen performed on quiescent CEF also indicated that C/EBPβ was the most common C/EBP factor present in

Figure 13: Nucleoprotein Complex C1 Contains C/EBPβ.

This EMSA shows that most of quiescence-specific complex C1 can be supershifted by preincubation with an antibody specific for C/EBPβ ("Im"; lane 4). A faint shift can also be seen using extract from proliferating CEF (lane 3). Preimmune serum (pI) had no effect.



* supershift of C1

Figure 14: Nucleoprotein Complex C2 Contains C/EBPβ.

In this EMSA, nuclear extracts from proliferating CEF are incubated with NFM probe, and complex C2 is seen. Competition of C2 with unlabeled NFM oligo (lane 2) eliminates the complex. Incubation of the nuclear extract with preimmune serum has no effect (lane3), while incubation with antibody specific to C/EBPβ results in a supershift of most of complex C2 (lane 4).

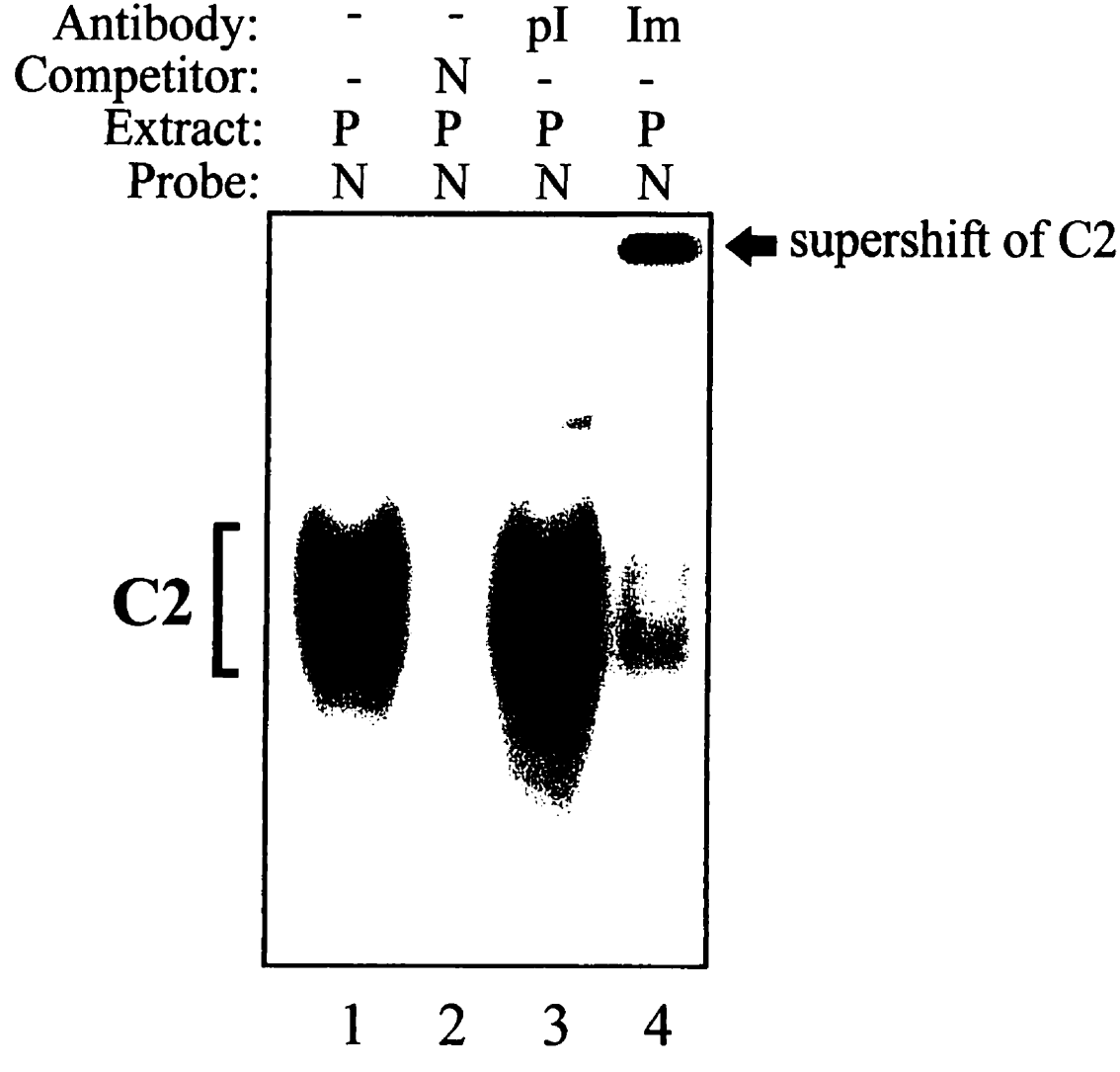
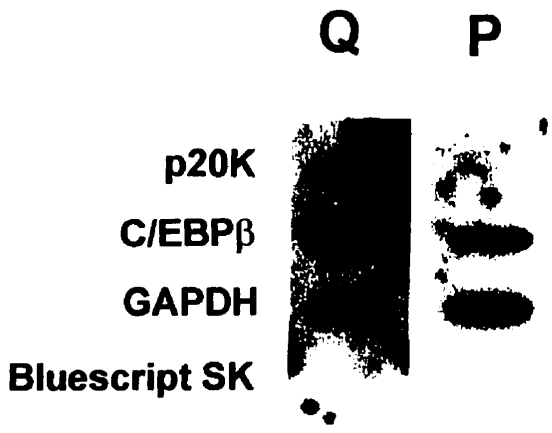

Antibody: - - pI Im
Competitor: - N - -
Extract: P P P P
Probe: N N N N
supershift of C2
C2
1 2 3 4

Figure 15: Levels of C/EBPβ are increased in quiescent CEF.

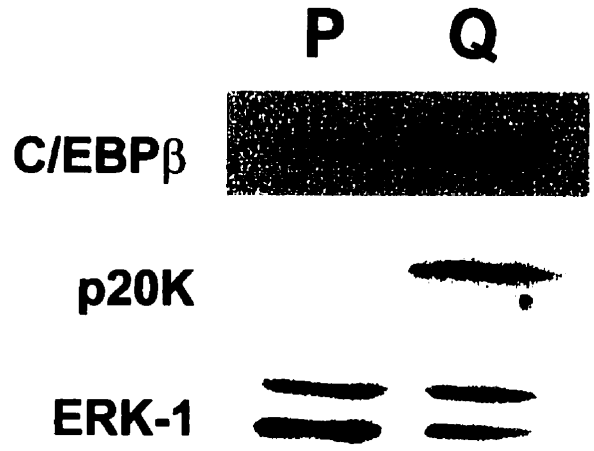
Panel a shows a transcriptional run on assay in which levels of transcription were compared between proliferating and quiescent CEF. C/EBPβ was induced 6-7 fold in quiescent CEF. Bluescipt SK and GAPDH, a housekeeping gene unaffected by the growth status of the CEF were included as controls.

Panel b shows a series of Western blots which show that C/EBPβ is also induced at the protein level in quiescent CEF. ERK-1, a constitutively expressed kinase whose levels are unregulated by growth, was included in as a control for protein loading.

a. Transcriptional Run-on



b. Western



P = Proliferating CEF; Q = Quiescent CEF

quiescent cells. Taken together, these results show that an induction of C/EBPβ is occurring in quiescent cells, providing support for the contention that C/EBPβ is involved in transcribing p20K in conditions of quiescence.

DISCUSSION

In figure 6, it can be seen that regions A, B, and C of the QRU have opposing responses to quiescence vs. active growth. These results imply that both proliferation and quiescence-responsive elements compose the QRU, attesting to the complexity of its regulatory mechanisms. This apparent duality of a growth arrest-responsive promoter segment is comparable to that seen in the gas1 promoter. In EMSAs, nucleoprotein complexes of differing sizes corresponding to extracts made from proliferating and quiescent cells were also seen. However, in footprinting analysis, identical patterns were observed in proliferating and quiescent cells. This implies that these complexes, despite differing in growth state-specificity, have an identical DNA binding specificity. This suggests that the gas1 promoter is similarly composed of elements which can respond to proliferation or quiescence (deMartin *et al.*, 1993).

Consensus binding sites for different families of transcription factors are outlined in figure 2. The ectopic expression of several Maf and Ets family members did not activate the QRU (data not shown). This is not surprising in the case of Maf members, which have been more heavily correlated with differentiated phenotypes than with growth states (Pouponnot *et al.*, 1995). In fact, all maf members discovered to date are unaffected by mitogens (Kataoka *et al.*, 1994b). However, we cannot rule out the possiblity that the QRU harbours a binding site of weak affinity for Maf factors. Indeed, Pouponnot and collaborators (1995) showed that the quiescence-

responsive domain of the neuro-retinal gene NR-1 binds a Maf factor which is competed by an excess of the p20K QRU in EMSA. It is not clear at this point if NR-1 is a typical growth arrest-specific gene since its expression is seen predominantly in the highly differentiated neuroretinal cells of the chicken.

There was some foundation for the hypothesis that Ets family members could be involved in regulating the QRU. As seen in figure 6, the proliferation-responsiveness seen by the B oligo which encompasses both Ets sites was abrogated when essential Ets sites were mutated. Furthermore, Ets transcription factors have a well-documented role in proliferation and transformation, since many members are induced by growth stimuli (reviewed by Gutman and Wasylyk, 1990). However, ectopic Ets expression using the members c-Ets-1 and PEA3 did not activate the QRU. Since it had been documented that Ets and Maf proteins can interact (Sieweke *et al.*, 1996), we also tried ectopically expressing Ets and Maf members in all possible combinations, but no induction of QRU activity was ever detected. It is possible that Ets has no role in activating the QRU, but it is equally possible that we have not tested enough Ets members to be able to state this with certainty. In the future, it would be interesting to test other Ets members in our system. C-Ets-1 and PEA3 both belong to the ets subgroup in which all members are homologous to v-Ets. Perhaps testing Ets members from the Ternary Complex Factor subgroup such as Elk-1 or Sap-1 would cause activation of the QRU.

In contrast to the results seen with maf and ets members, the ectopic expression of C/EBPα or C/EBPβ strongly activated all QRU constructs containing intact CCAAT sites (figure 7). These experiments show that both the A and B CCAAT sites can function well in this context. Since both the A and B sites perfectly match the consensus outlined by Ryder and Beemon (1989), it is unknown why 3XA is much stronger than 3XB or 3XC. It may be that flanking sequences are also important. B and C are both cut off at the 3' or 5' end of the CCAAT site respectively; perhaps essential residues required for high level C/EBP binding and activation are cut off in both oligos. In any case, multimerized oligos representing QRU segments may be a superficial way to analyze the strength of the two sites. It can be seen that in the context of the intact QRU, good activation by C/EBP ectopic expression is seen when either site is mutated, implying that both are high-affinity sites.

C/EBPα consistently gives stronger activation in ectopic expression than C/EBPβ. This probably does not reflect a higher affinity of C/EBPα vs. C/EBPβ for the two sites; it has been found that all C/EBP family members bind CCAAT consensus sites with virtually identical affinities (Williams *et al.*, 1991; Diehl and Yang, 1994). Rather, this reflects the fact that C/EBPα has a much stronger trans-activation domain than C/EBPβ (Williams *et al.*, 1995).

The A oligo CCAAT site also fits the consensus binding sequence of CHOP-C/EBP heterodimers (Ubeda *et al.*, 1996). Therefore, we tested whether ectopic

expression of CHOP could potentiate the activity of the QRU, either alone or by co-expressing it with C/EBP α or β. CHOP did not have any effect in either of these situations. A quiescent CEF cDNA library screen was also performed with the bZip domain of CHOP in an attempt to find CHOP-like sequences. None were found, implying that CHOP is not expressed in our conditions of quiescence. This is not surprising in light of the fact that CHOP is generally considered to be a stress-responsive gene (Chen *et al.*, 1996).

Figure 9 shows that mutating either C/EBP site abrogates quiescence-specific induction of QRU promoter constructs. This was surprising in light of the experiments performed in figure 6, which show that the A region of the QRU is quiescence-responsive, while B and C are more responsive to conditions of active growth (figure 6; Mao and Bédard, unpublished). These results would lead one to anticipate that QRU-μBII would retain quiescence-responsiveness. This was not the case, however. The fact that mutating either gives similar results implies that the two sites cooperate in quiescence-specific induction. The nature of the cooperation is unknown although given the abundance of examples of protein-protein interactions that C/EBP proteins have been implicated in, this is a plausible model (Hsu *et al.*, 1994; Shuman *et al.*, 1997; Mink *et al.*, 1997). For example, C/EBPβ can interact with p300, a transcriptional coactivator which can link individual transcription factors to each other or to the basal transcription machinery (Mink *et al.*, 1997). Perhaps a

protein similar to p300 can link the transcription factor complexes bound to the two C/EBP sites and thus mediate synergy between them.

Δ184 is a dominant-negative version of C/EBPβ which retains the DNA-binding and dimerization domains but lacks a functional trans-activation domain. Therefore, Δ184 can bind C/EBP sites and can dimerize, but it cannot activate transcription. As can be seen in figure 10, cotransfection of Δ184 with p20K reporter constructs significantly dampens quiescence-driven induction. This provides strong evidence that a C/EBP factor is involved in activating the QRU. It should be noted that Δ184 also decreases basal levels of QRU expression. That is, in actively dividing cells, p20K promoter activation is also reduced by Δ184 (results not shown). This implies that C/EBP factors are not only involved in quiescence-specific expression, but are also important for basal expression in actively dividing cells.

The electrophoretic mobility shift assays (EMSAs) show differential binding of nuclear proteins to region A of the QRU (figure 11). The supershift experiments reveal that both C1 and C2 contain C/EBPβ (figures 13 and 14). C2 is likely includes a C/EBPβ homodimer, since C/EBPβ/NF-M-RCASBP-infected cells show an identical pattern (results not shown). In these C/EBPβ/NFM overexpressing cells, C/EBPβ/NFM homodimers are expected to predominate. C1 certainly contains C/EBPβ, but its size difference from complex C2 as well as its different binding preference suggests the involvement of at least one other component.

It is interesting that C1 binds the NFM site with reduced affinity, while C2 binds it strongly. This implies that there is some specificity to the A site. But what sequence in the A oligo accounts for this specificity? Mutating core sequences in the A CCAAT site does not significantly affect activation levels in quiescence (results not shown). The mutations in the palindrome at the 5' end of the A oligo CCAAT site (figure 5) do not hinder quiescence-responsiveness. Further experiments will be needed to clarify this.

What is the identity of the binding partner in C1? There are several possibilities. The binding partner could be another C/EBP family member such as C/EBPα. C/EBPα would be a good candidate because it has been well characterized as being involved in growth arrest coupled to differentiation in adipocytes (reviewed by Mandrup and Lane, 1997). However, one would expect that C/EBPα would be more abundant in quiescent CEF; the results of our cDNA library screen did not show a high level of C/EBPα expression. As well, C/EBPα/C/EBPβ heterodimers do not differ in binding specificity compared to the respective homodimers (Williams *et al.*, 1991). We see that complex C1 cannot bind the NFM oligo at high affinity. Therefore, it is unlikely that another C/EBP factor could be conferring the specificity necessary for the binding of complex C1 to the A oligo.

Although C/EBP is induced in quiescent CEF as seen in figure 15, we do not believe that it is acting alone to induce growth state-specific transcription of the QRU. We postulate that there is a Quiescence-Specific Factor (QSF) which is the interaction

partner in C1. C/EBPβ is known to interact with several other transcription factor families; this makes a model possible in which a different interaction partner is involved in C1 formation. Overlapping with the palindrome in the 5' end of the QRU is the sequence TCA. This sequence is located four nucleotides upstream of the CCAAT sequence believed to be bound by C1. The sequence TCA is identical to the 3' end of the consensus sequence for the AP-1 and the ATF/CREB family of transcription factors. Could a member of one of these families interact with C/EBPβ in the formation of complex C1? Interestingly, the AP-1 binding site (TRE) can compete for complex C1 formation (results not shown). As well, C/EBPα has been known to coimmunoprecipitate in liver with ATF-2. The C/EBPα/ATF-2 heterodimer binds an asymmetric sequence composed of half sites corresponding to each monomer (Shuman *et al.*, 1997). Perhaps a similar situation is occurring in complex C1 with a member of the jun or ATF/CREB families. Experiments will be performed using a modified QRU in which the TCA sequence is altered. The mutations in the palindrome, as seen in oligo 48mutA (fig. 5) do not affect the TCA sequence.

YY1 interacts with C/EBPβ in regulating activation of the HPV-type 18 promoter; it contains a C/EBP consensus site which can only be bound by C/EBPβ when it is interacting with YY-1. This is a plausible mechanism to explain the specificity of complex C1; perhaps there is a limiting factor which is upregulated in quiescent CEF which provides C/EBPβ its binding specificity with the A oligo

(Bauknecht *et al.*, 1996). YY1 itself would not be considered a candidate interaction partner in this situation; an analysis of the sequences surrounding the HPV-type 18 promoter's CCAAT site reveals no homology to sequences surrounding the A oligo CCAAT site.

RCASBP-NFM/C/EBPβ-infected cells are known to express large amounts of C/EBPβ,as seen in two experiments. In figure 15 (lane 1), we can see greatly elevated levels of C/EBPβ protein in infected CEF. In transcriptional run-on assays, RCASBP-NFM/C/EBPβ-infected cells show a 60-fold induction in NFM transcription (results not shown). Figure 8 shows that overexpressing C/EBPβ(NFM) will force p20K expression in actively growing cells. In these situations, the CEF are likely to be saturated with large amounts of C/EBP homodimers. Therefore, a QSF is not necessary for p20K expression, although it is likely necessary for p20K's induced expression.

This study shows that C/EBPβ is pivotal in the expression of the growth arrest-specific gene p20K. There has never been a strong role in growth arrest demonstrated for C/EBPβ in the literature. In the majority of cases, C/EBPβ expression and activation has been correlated with proliferation. Furthermore, C/EBPβ is believed to be involved in the activation of genes by the $pp60^{v\text{-}src}$ oncoprotein (Gagliardi and Bédard, unpublished). This seeming paradox may be resolved by proposing that while C/EBPβ is essential for growth-regulated activation of p20K, it is not sufficient. There is a quiescence-specific factor which acts as an

interaction partner for C/EBPβ. This quiescence-specific factor may provide some or all of the binding specificity for region A of the QRU.

The data shown in appendix 1 show an interesting phenomenon occurring in CEF which also seems to be mediated through C/EBPβ. CEF were infected with RCASBP-NFM/C/EBPβ as a means by which to study p20K induction in this situation. These infected fibroblasts showed an interesting phenotype which implied that they were engaged in adipocytic differentiation in response to C/EBPβ overexpression (figure 16). The cells became larger (a phenotype typical of growth arrest) and they also produced lipid droplets very characteristic of adipocytes. In the 3T3-L1 mouse fibroblast system, C/EBPβ overexpression was found to be sufficient for complete adipogenesis only when supplemented with hormones important in adipocytic differentiation (Cao *et al.*, 1991). We have not sufficiently characterized our infected CEF to determine the extent of acquisition of the adipocytic phenotype; what we see may only represent partial conversion. Howevever, this phenotype is interesting for several reasons. It is paradoxical that C/EBPβ controls the expression of p20K, a marker of quiescence which may be induced by serum-starvation, and simultaneously C/EBPβ activates a program characteristic of energy storage, namely adipogenesis. This is seemingly irreconcileable in consideration of the fact that p20K is a starvation-responsive gene and should not be produced in cells with an energy surplus.

We believe that these RCASBP-NFM/C/EBPβ infected CEF are receiving conflicting signals due to this artificially induced C/EBPβ overexpression. In vivo, CEF can probably respond to signals for adipocytic differentiation or for attainment of quiescence, and C/EBPβ is likely to be involved in both processes. The implication is that C/EBPβ is central in mediating diverse pathways in response to environmental cues, but that its overexpression causes the activation of all of these programs in the same cell at the same time. Thus, C/EBPβ may be acting as a "master switch" between the phenotypes of reversible growth arrest and differentiation.

Further experiments imply that induction of adipogenesis in fibroblasts through the activation of the PPARγ nuclear receptor is incompatible with p20K expression. PPARγ is an important mediator of adipogenesis which is activated downstream of C/EBPβ in the adipogenesis program (reviewed by Mandrup and Lane, 1997). Fatty acids are known to be ligands for PPARγ, which, following their binding, proceeds to activate adipocytic differentiation. The greater the length of the carbon chain and the greater the number of unsaturated bonds, the more effective the fatty acid is as a ligand for PPARγ (reviewed by Mandrup and Lane, 1997). In figure 18, CEF were contact-inhibited in order to maximize p20K expression. This was followed by overnight treatment with the fatty acids linoleate, palmitate, or oleate. Accordingly, linoleic acid showed the greatest adipogenic potential (fig. 17), followed by oleate and palmitate (results not shown). In an immunoprecipitation, linoleate could suppress p20K expression induced by contact inhibition (figure 18). Therefore, PPARγ, a

downstream effector of C/EBPβ and fatty acids, may act as a repressor of p20K, a marker of reversible growth arrest. Further studies are needed to confirm that PPARγ is indeed a direct or indirect regulator of p20K.

In summary, p20K was coinduced with the adipogenic program in RCASBP/NFM/C/EBPβ-infected CEF. A second way of inducing adipogenesis, activation of PPARγ, suppressed p20K, implying that p20K expression *in vivo* may normally be incompatible with energy storage. These data bring to light the possibility that C/EBPβ may be very important as a mediator in regulating diverse processes. This system is useful in that it may provide a means by which to study the phenomenon of C/EBPβ as a pluripotent switch.

The broader implications are that C/EBPβ may be central in many crosstalking signal transduction pathways as well as gene expression pathways. This is certainly a plausible contention; C/EBPβ has already shown itself to be a central player in many diverse processes such as differentiation and immune system activation. As seen in figure 4, it is regulated by many diverse signal transduction pathways as well as being an interaction partner for several diverse regulatory proteins. Its interaction shown with p300 opens the door for its indirect interaction with many other transcription factors such as Sap1a, p53, MyoD, and MEF-2, with which p300 may act as a bridging protein (Mink *et al.*, 1997).

Further studies are necessary to clarify the interesting mechanisms by which p20K is regulated transcriptionally. The most obvious question involves the identity

of the QSF. A consensus binding site of the QSF may be defined by dissecting the region just upstream of the A oligo CCAAT site. Since we postulate that the QSF interacts with C/EBPβ, a protein-protein interaction screen using C/EBPβ as the target may allow identification of the QSF.

In summary, the results of this study are significant in light of the fact that so little is known about the transcriptional inducers of the growth arrested state. C/EBPβ has not been previously implicated as being essential for the transcriptional regulation of a growth arrest-specific gene, so this study breaks new ground by demonstrating its necessity. The present study is the first characterization of a promoter controlled by contact inhibition-induced reversible growth arrest. p20K, as a marker of the growth arrested state, provided a tool with which to further our understanding of this neglected field. In addition, the existence of proliferation-responsive domains in the QRU and the role played by C/EBPβ in the activation of the QRU suggests an evolutionary relationship between p20K, a marker of reversible growth arrest, and genes of the proliferative response.

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Appendix

Figure 16: The Phenotype of RCASBP-NFM (C/EBPβ)-Infected CEF.

In this experiment, RCASBP-NFM(C/EBPβ)-infected CEF (panels c and d) are compared with a control, RCASBP-infected CEF(panels a and b). Immunofluorescence was performed using C/EBPβ antibody (panels a and c). Cells were also stained for propidium iodide (panels b and d).

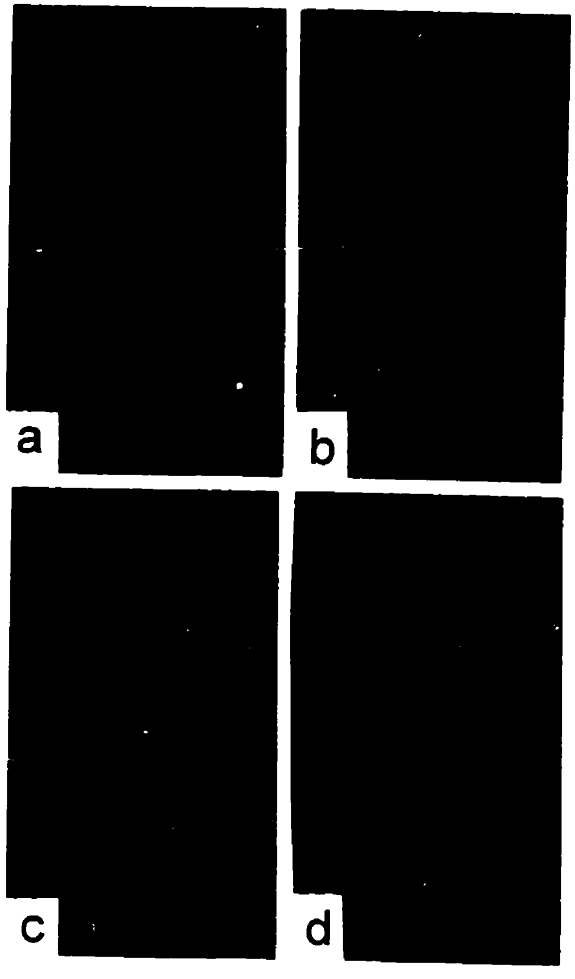
a
b
c
d

Figure 17: Lipid Droplet Accumulation in Linoleate-treated CEF.

CEF were treated overnight with a control (panel a) or 200uM linoleate (panel b), after which these cells were fixed and stained with the lipophilic dye Oil Red O.

a

b

Figure 18: Suppression of p20K Expression in Linoleate-Treated CEF.

This is a p20K immunoprecipitation performed on quiescent CEF which were treated with 200uM palmitate, 200uM linoleate, diluent control (an equal volume of ethanol), or given no treatment. A strong reduction in p20K levels is seen only with the linoleate-treated CEF.

Diluent

Palmitate

Linoleate

None