Characterization of 32b1, a partial cDNA encoding novel sequence having high similarity with the immediate early gene, Cyr61

by

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ABSTRACT

The 32b1 cDNA clone was isolated from a λgtll library made from a Jurkat T lymphocyte cell line during a search for a transactivating factor for the HIV-LTR that was responsible for Ras responsiveness. The clone was 1.3kb in length and the full-length clone, postulated from Northern blot experiments done in human tissues, was estimated to be approximately 2.6 kb. This led to a comprehensive search of different libraries of human blood leukocytes, lung and brain cell lines to find the missing 5'end. Although the original segment of cDNA clone was isolated many times, novel sequence was never found experimentally. A search in the NCBI database revealed new 5' end sequence as well as evidence linking 32b1 with wound repair. Further examination of the gene induction kinetics were carried out via Northern Hybridizaiton analysis. Unexpectedly, the gene was not found in peripheral blood leukocytes and none of the cell lines that the cDNA clone was isolated from was seen to express it. The only cell line that did express it was a Rat-2 fibroblast cell line where a message having high sequence similarity with 32b1 was seen to be induced by serum, EGF but not by the phorbol ester, PMA. The stability of the rat mRNA transcript was also studied from induction patterns after actinomycin D treatment with and without cycloheximide. It was found to have an transient half life of less than 30 minutes. The induction of the 32b1 mRNA message was prolonged after treatment with cycloheximide which implicated that translation had an effect on its mRNA stability. These results allowed certain conclusions to be made about 32b1 that can be a basis in further research into its function in cell proliferation. The focus of this research was to complete the cDNA

sequence, find the missing 5′ end of the cloned cDNA and to characterize the expression

of the gene.

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LIST OF ABBREVIATIONS

ActD	Actinomycin D
AIDS	Acquired Immunodeficiency Syndrome
BSA	Bovine Serum Albumin
CEF	Chicken Embryo Fibroblasts
CCN	CEF-10/cyr61, CTGF and nov
CTGF	Connective Tissue Growth Factor
DAG	1,2-Diacylglycerol
depc	diethyl-pyrocarbonate
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Disodium ethylene diamine tetraacetate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK1/ERK2	Extracellular-signal-related kinases
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
fisp-12	fibroblast-inducible secreted protein-12
GDP	Guanine diphosphate
GTP	Guanidine triphosphate

LIST OF ABBREVIATIONS

Hepes	N-2-hydroxyethylpiperazine-N^1-2-Ethanesulfonic acid
HIV-1	Human Immunodeficiency Virus type-1
HUVE	Human umbilical vein endothelial cells
MEK	MAPK/ERK kinase
MAPK	Mitogen-Activated Protein Kinase
MOPS	3-(N-morpholino)propanesulfonic acid
MW	Molecular Weight
nov	nephroblastoma-overexpressed gene
O.D.	Optical Density
OLB	Oligolabelling buffer
PDGF	Platelet Derived Growth Factor
PCR	Polymerase chain reaction
PLC	Phospholipase C
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
PVP	Polyvinylpyrollidone
RBE	Ras-responsive binding element
RBF	Ras-responsive binding factor
RTK	Receptor Tyrosine Kinase
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate

LIST OF ABBREVIATIONS

ssDNA	Salmon sperm DNA
TBE	Tris borate EDTA
TEMED	N^1,N^1,N^1,N^1-tetramethylethylenediamine
TGF-β	Transforming Growth Factor- beta
TPA	phorbol 12-tetradecanoate 13-acetate
Tris	Tris(hydroxymethyl)aminomethane
tsg	Twisted gastrulation
UTR	Untranslated Region

UNITS OF MEASUREMENT

Ci	curie
cpm	counts per minute
Da	Dalton
dpm	disintegrations per minute
g	gram
k	kilo
l	liter
M	Molar
mol	mole
m	milli
μ	micro
n	nano
p	pico
pfu	plaque forming units
%	percent
U	units

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Literature Review

1. The discovery of 32b1

The human immunodeficiency virus type-1 (HIV-1) has been previously identified as the causative agent for the Aquired Immunodeficiency Syndrome (AIDS). Within its nucleoid core, there are two single stranded RNA (ssRNA) molecules encoding its genome with a reverse transcriptase attached to one end of each. Once transmitted into the human host through bodily fluids, it finds its receptor, the CD4 molecule on the surface of Helper T Lymphocytes and monocytes and becomes internalized into the cell through binding and endocytosis. Here, the reverse transcriptase will transcribe the ssRNA molecules into double stranded DNA (dsDNA) which then migrates into the nucleus and integrates intself into the human host's genome as a latent provirus. It remains in this state for an undetermined amount of time before it is reactivated by the binding of cellular transcription factors to its promoter, the 5' Long Terminal Repeat (LTR). After this, the virus retranscribes its mRNA which then travels back into the cytoplasm where it is translated into viral proteins during assembly into a viable virion which then lyses the cell or buds out to infect other cells (Greene et al., 1991).

Extensive research has been carried out in the area of cellular activation of HIV-1 from latency. Various cytokines have been found to effect HIV-1 gene expression both positively and negatively (Garcia et al., 1994). A cytokine activated pathway that has been implicated in the signal transduction of extracellular signals into the nucleus is the Ras/ERK pathway (Baldari et al., 1992).

Recently, four binding sites on the HIV-LTR have been defined as responsible for Ras responsiveness of the promoter, referred to as Ras-responsive binding elements I - IV (RBE I - IV) (Bell et al., unpublished). These sites are dispersed throughout the HIV-LTR and bind to two transcription factors, Ras responsive binding factors-1 and 2 (RBF-1 and RBF-2). This discovery led to Southwestern screening of a Jurkat T lymphocyte λgtll cDNA library using oligonucleotide A (comprising -159 to -138 of the HIV-LTR and encompassing RBE IV) to find the presumptive transcription factor, RBF-1, that bound to this site. This resulted in the isolation of six partial cDNA clones, the largest of which was 32b1. The cDNA fragment, 32b1, was cloned into the vector pGEM5fz(+) and analysis revealed that it contained novel DNA sequence. A sequence homology search uncovered resemblance to members of an immediate early gene family, referred to as CCN, named after members of the family. Immediate early genes are the primary genes induced during the transition from G_0 to the G_1 state of the cell cycle.

2. Cell growth

In vivo or in culture, the cell is always found in one of two stages, either growing or quiescent. Growing cells are always increasing in mass and travelling through the cell cycle which eventually leads them to cell division. The cell cycle is categorized into four stages, seen in Figure 1: G_1, the gap between nuclear division and DNA synthesis; S, the period of DNA synthesis; G_2, the gap between DNA duplication and nuclear division, and M, the period of mitosis, during which the cell becomes two daughter cells (Darnell et al, 1990).
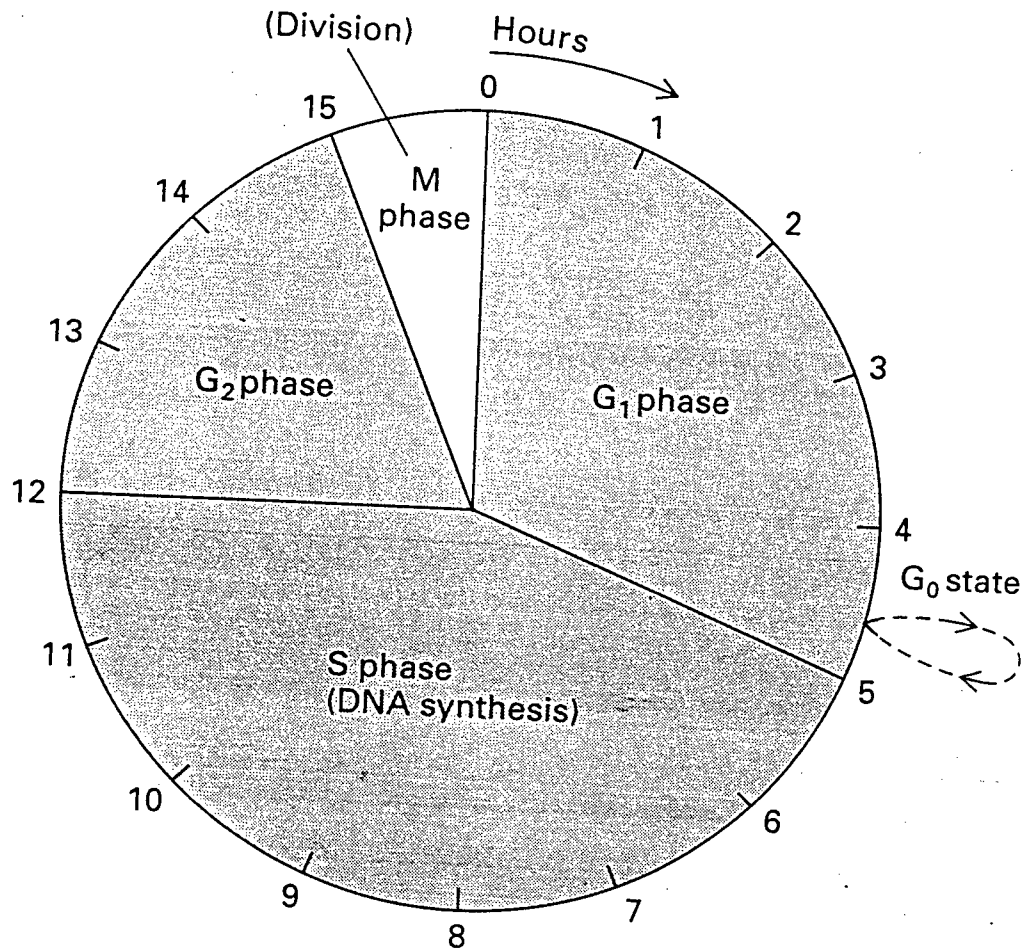
Figure 1. The cell cycle in a mammalian cell having a generation time of 16 hours. The cycle is made up of four stages: the G1 (the first gap) phase; the S (synthetic) phase, the G2 (second gap) phase and the M (Mitosis) phase (Darnell, 1990).

In contrast, quiescent cells are in a suspended state as they do not pass through the cell cycle nor do they increase in cell mass. They are in a state referred to as G_0 (see Figure 1). In cell culture, such cells can be induced to reinitiate growth by the addition of fresh serum. Serum is thought to supply the cells with trace amounts of growth factors and hormones which signal each cell to start events leading to cell growth and division. These ligands bind to specific receptors found on the cell suface to commence signal cascades leading to transcription of immediate early genes in the nucleus which mediate cell proliferation (Hill and Triesman, 1995). Many growth factors, such as EGF and PDGF belong to a structurally diverse class of ligands that bind to receptors referred to as receptor tyrosine kinases (RTK). These specific receptors consist of an extracellular domain which binds the ligand, a single transmembrane domain, and an intracellular (cytoplasmic) domain with tyrosine kinase activity. RTKs are involved in many functions including the signaling of cell proliferation and differentiation depending on where they are expressed (Marshall, 1995). Figure 2 shows how these receptors link into two known pathways, the phosphatidylinositol signaling pathway and the Ras pathway (Berridge, 1993).

Once a specific growth factor has bound to its RTK, the receptor becomes activated through dimerization at which time one receptor phosphorylates a tyrosine residue on the other in a process termed "autophosphorylation". This phosphorylated tyrosine acts as a docking site for phospholipase C-γ1 (PLC-γ1) and adapter proteins, such as Grb2, which bind with high affinity through their src-homology-2 (SH2) domains. In the phosphatidylinositol pathway, the RTKs activate PLC-γ1 by phosphorylating it on specific residues which allow it to hydrolyse phosphatidylinositol
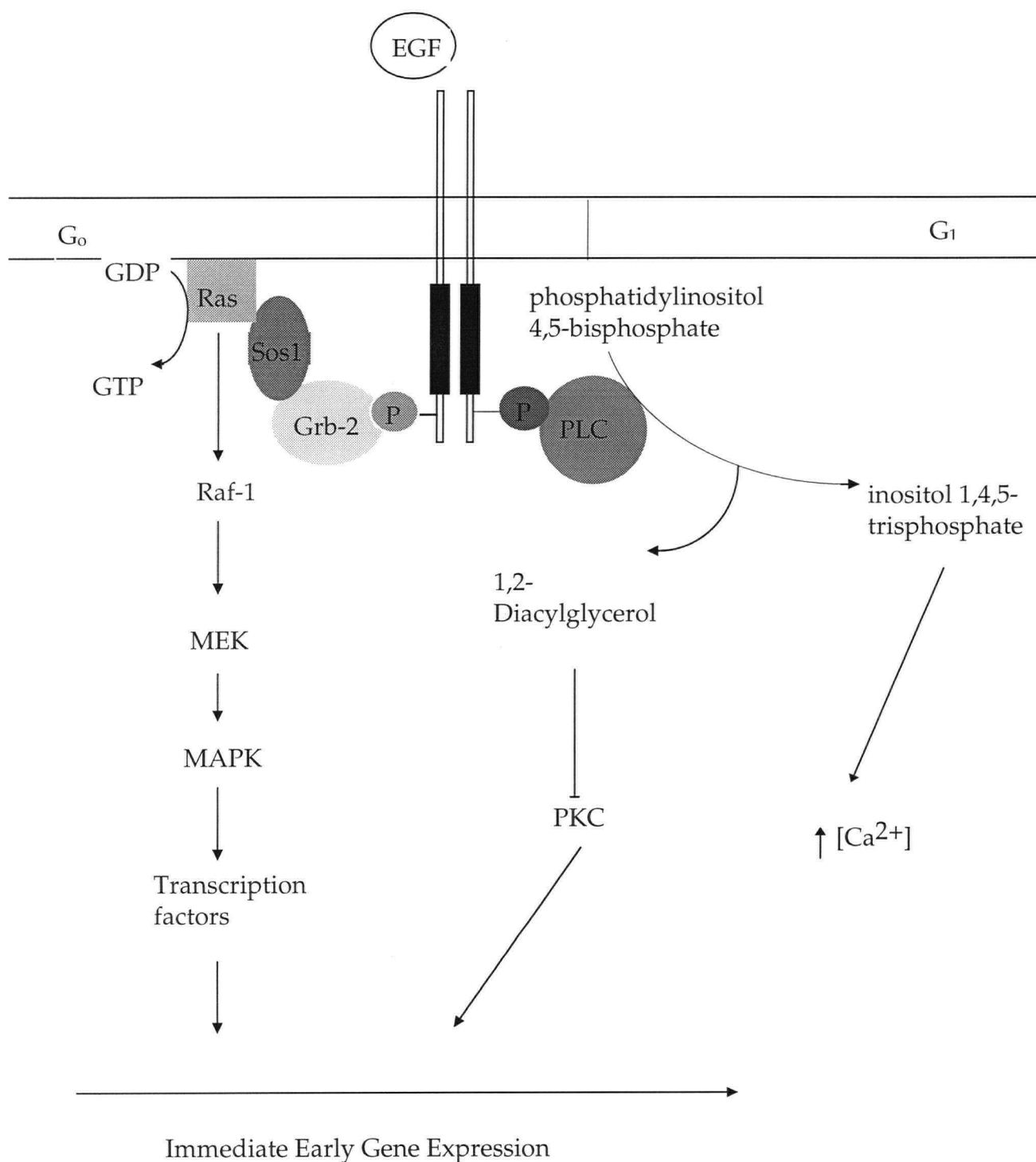
Figure 2 Summary of two pathways initiated by EGF ligand binding of receptor protein tyrosine kinases. EGF has been found to initiate two pathways, phosphatidylinositol and ras signal transduction pathways. EGF, Epidermal Growth Factor; PLC, phopholipase C; PKC, Protein Kinase C; GDP, Guanine diphosphate; GTP, guanine triphosphate.

4,5-bisphosphate and create the products 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. Inositol trisphosphate then plays a role in the increase of intracellular calcium (Ca^{2+}) while the primary function of diacylglycerol is to activate a family of plasma membrane protein kinases called protein kinase C (PKC). PKC also requires Ca^{2+} ions which cause it to bind to the cytoplasmic side of the plasma membrane where it is then activated by DAG. PKC plays an important role in the mediation of growth in many cells (Darnell, 1990). PKC has also been found to phosphorylate the EGF receptor to decrease its affinity for EGF and thus down-regulates EGF's growth-stimulating ability. The tumor promoters, phorbol esters such as PMA activate PKC by acting as a DAG analog (Borner et al., 1995). Through PKC, PMA regulates the activity of many transcription factors to alter the expression of cellular genes (Huang et al., 1995). Different PKC isoforms show different susceptibility to this treatment seen as a function of the cell type it is expressed in (Bi and Mamrack, 1994; Borner et al., 1995). Prolonged treatment with phorbol esters lead to eventual downregulation of the PKC pathway due to proteolysis (Bi and Mamrack, 1994; Borner et al., 1995).

In the Ras/ERK MAPK pathway, the Grb2 protein which binds to the phosphotyrosine on the RTK is complexed with the Sos1 protein, a common guanine exchanger through its src homology 3 (SH3) domains which have an affinity for proline rich sequences found on Sos1 (Songyang and Cantley, 1995). The binding of Grb2 brings SoS1 in close vicinity to Ras, a guanine nucleotide-binding protein localized on the inner membrane, allowing them to interact. SoS1 activates the latter by catalysing exchange of the GDP bound to Ras with GTP (Figure 2). This GTP-bound Ras binds to the N-terminus of a Raf family member bringing it to the membrane. At this point, it is

thought that an as yet unidentified protein activates Raf through phosphorylation. Raf then phosphorylates kinases such as MEK1/MEK2 which in turn phosphorylate and activate ERK1/ERK2, types of MAP kinases. A fraction of these kinases translocates into the nucleus and phosphorylates transcription factors. Some MAPKs phosphorylate other protein kinases which then migrate into the nucleus and also phosphorylate transcription factors (Hunter, 1995). Another kinase referred to as MEK kinase (MEKK) also requires Ras for activation but works on the same level as c-Raf in a parallel cascade leading to MEK activation (Cano and Mahadevan, 1995; Crews and Erikson, 1993).

3. Immediate Early Genes

As previously mentioned, the first genes induced as part of the rapid response through signaling cascades following stimulation with growth factors have been termed immediate early (IE) genes (Lau and Nathans, 1987). Their expression is rapidly activated within minutes to a few hours following the initial signal without requiring de novo protein synthesis. Immediate early genes have three defining characteristics: (1) many mRNA products of IE genes are nearly undetectable in quiescent cells but are usually present and functional at low levels allowing them to be rapidly accumulated within one hour of stimulation by growth factors (Kelly and Siebenlist, 1995; Lau and Nathans, 1991); (2) their induction occurs without de novo protein synthesis and (3) they are transcriptionally activated upon growth factor stimulation (Lau and Nathans, 1991). Once induced, immediate early gene expression is attenuated by rapid degradation and a cessation of transcription within a short time, causing the mRNA

expression to be transient (Lau and Nathans, 1987). This built-in negative feedback loop is carried out by the induced proteins encoded by the immediate early genes (Kelly and Siebenlist, 1995). These proteins downregulate the primary activating signalling pathways at different levels as seen with the activation of IκB-α, the inhibitor of NF-κB immediately upon induction of the NF-κB gene (Kelly and Siebenlist, 1995). As well, this negative regulatory system is seen when FOS represses transcription of its own c-fos gene leading to message degradation (Herschman, 1991). These immediate early genes are also often superinduced in the presence of protein translation inhibitors such as cycloheximide (Simmons et al., 1989). This superinduction is thought to occur for two reasons. First, transcription is prevented from being shut off, and secondly, mRNA stability increases (Almendral et al, 1988). The expression of these early genes can lead to a variety of outcomes, depending on the distinct cellular environment that receives the signals (Kelly and Siebenlist, 1995). These responses may include differentiation, shape changes, locomotion and proliferation (Rozengurt, 1986). A subset of these genes may also be involved in regulation of complicated differentiation and wound healing processes involving cell division (Ryseck et al, 1991). Due to their numerous functions, immediate early genes can be divided into three categories according to their function and their pattern of induction upon stimulation by serum. The first set of genes, group I, is detectable within 5 minutes of growth factor stimulation, peak in concentration levels within 30 - 60 minutes and then become undetectable within approximately 2 hours. Identified genes of this group have been found to encode various transcription factors, such as c-fos and Jun-B (Lau and Nathans, 1991; Almendral et al, 1988). Group II mRNAs appear somewhat later and reach peak levels approximately two hours after

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stimulation. These genes decay much slower and become undetectable between 4 to 8 hours after mitogen induction. These genes encode for proteins such as c-myc and JE. Finally, the last category, group III, have extremely long half-lives and encode many structural proteins (Lau and Nathans, 1991).

4. CCN Family and its characteristics

In recent years, a group of immediate early genes containing related sequences has been discovered. Collectively, these are referred to as the "CCN" family, named after members of the family: CEF-10/ cyr61, CTGF and nov (Bork, 1993). The protein products of these genes contain a putative secretory signal at their amino termini suggesting that they are secreted proteins. The most prominent characteristic of this family is that each member contains 38 completely conserved cysteine residues, making up approximately 10% of the total sequence. These cysteines are dispersed throughout the amino and carboxy termini whereas the central region contains a divergent, acidic segment lacking any cysteines. Some members of the "CCN" family exhibit a new set of immediate early gene induction kinetics. Certain genes of this family accumulate their mRNA for as long as 8 hours but the mRNA itself only has a half life of 10 - 20 minutes (O'Brien et al., 1990) indicating that the mRNA is constantly transcribed. The members of this family have four conserved domains that have homology with other known proteins (Figure 3A). Domain 1 encodes a low-molecular-weight insulin-like growth factor-binding protein (IBP) consensus motif, GCGCCXXC, in the amino terminus of these family members. Insulin-like growth factor functions as a growth-stimulating peptide with mitogenic properties and travels in plasma tightly bound to

these specific IBPs (Brinkman et al., 1988). Domain II has identity with a von Willebrand factor type C repeat (VWC) region which is thought to participate in oligomerization. Domain III, first found in thrombospondin (type 1 repeat) (TSP1), has previously been involved in binding to both soluble and matrix macromolecules. Finally, domain IV, the C-terminal domain (CT module), has resemblance to the *slit* gene in *Drosophila* which may be involved in dimerization or receptor binding (Bork, 1993; Xin et al., 1996) . The family members are found in many species which implies that the members of this family play an important cellular function. Figure 3B shows a representative amino acid sequence alignment of the CCN family. Figure 4 shows a dendrogram displaying amino acid divergence among the family members.

CEF-10 was the first member of this family to be discovered (Simmons et al., 1989). It was found to be induced at the permissive temperature in chicken embryo fibroblasts infected with the Rous sarcoma virus containing a temperature-sensitive mutation for v-src. CEF-10 was considered an immediate early gene because it exhibited characteristics similar to other IE genes such as expression as a rare transcript in control cells, increase of mRNA expression within an hour of mitogen stimulation, and superinduction in the presence of cycloheximide (Simmons et al., 1989). Comparison of the amino acid sequence of CEF-10 with a more recently found murine clone called cyr61 revealed approximately 80 per cent similarity suggesting that cyr61 may be a mouse homologue of CEF-10 (O'Brien et al., 1990). The cyr61 cDNA was isolated from quiescent mouse 3T3 fibroblasts induced with serum or PDGF. An identical cDNA clone termed βIG-M1 was also independently cloned from a cDNA library constructed with RNA extracted from a mouse embryo cell line that was treated

A

variable

B

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         cyr61   M--SSSTFRTLAVAVTLLHL--TRLALST-CPAAC--HCPLE-APKCAPGVGLVRDGCGCCKVCAK   58
         CEF10   M--GSAGARP-ALAAALLCL--ARLALGSPCPAVC--QCPAA-APQCAPGVGLVPDGCGCCKVCAK   58
         Fispl2  M--LASVAGPISLA-LVLLALCTRPATGQDCSAQC--QCAAEAAPHCPAGVSLVLDGCGCCRVCAK   61
         CTGF    M--TAASMGPVRVAFVVLLALCSRPAVGQNCSGPC--RCPDEPAPRCPAGVSLVLDGCGCCRVCAK   62
         Nov     METGGGQCLPVLLLLLLLLRPCEVSGREAACPRPCGGRCPAEP-PRCAPGVPAVLDGCGCCLVCAR   65

         Cyr61   QLNEDCSKTQPCDHTKGLECNFGASSTALKGICRAQSEGRPCEYNSRIYQNGESFQPNCKHQCTCI   124
         CEF10   QLNEDCSRTQPCDHTKGLECNFGASPAATNGICRAQSEGRPCEYNSKIYQNGESFQPNCKHQCTCI   124
         Fispl2  QLGELCTERDPCDPHKGLFCDFGSPANRKIGVCTAK-DGAPCVFGGSVYRSGESFQSSCKYQCTCL   126
         CTGF    QLGELCTERDPCDPHKGLFCDFGSPANRKIGVCTAK-DGAPCIFGGTVYRSGESFQSSCKYQCTCL   127
         Nov     QRGESCSPLLPCDESGGLYCDRGPEDGGGAGICMVL-EGDNCVFDGMIYRNGETFQPSCKYQCTCR   130

         Cyr61   DGAVGCIPLCPQELSLPNLGGPNPRLVKVSGQCCEEWVCDEDSIK-DSLDD-QDDLLGLDASEVEL   188
         CEF10   DGAVGCIPLCPQELSLPNLGGPSPRLVKVPGQCCEEWVCDESKDALEELEGFFSKEFGLDASEGEL   190
         Fispl2  DGAVGCVPLCSMDVRLPSPDCPFPRRVKLPGKCCKEWVCDEPKDRTAVGP-------ALAAYRLED   185
         CTGF    DGAVGCMPLCSMDVRLPSPDCPFPRRVKLPGKCCEEWVCDEPKDQTVVGP-------ALAAYRLED   186
         Nov     DGQIGCLPRCNLGLLLPGPDCPFPRKIEVPGECCEKWVCDPRDEVLLGGF-------AMAAYRQEA   189

         Cyr61   TRNNELIAIGKGSSLKRLPVFGTEPRVLFNPLHAHGQKCIVQTTSWSQCSKSCGTGISTRVTNDNP   254
         CEF10   TRNNELIAIVKGG-LKMLPVFGSEPQ----SRAFENPKCIVQTTSWSQCSKTCGTGISTRVTNDNP   251
         Fispl2  T-----------------FGPDP-------TMMRANCLVQTTEWSACSKTCGMGISTRVTNDNT   225
         CTGF    T-----------------FGPDP-------TMIRANCLVQTTEWSACSKTCGMGISTRVTNDNA   226
         Nov     T-----------------LGIDV-------SDSSANCIEQTTEWSACSKSCGMGFSTRVTNRNQ   229

         Cyr61   ECRLVKETRICEVRPCGQPVYSSLKKGKKCSKTKKSPEPVRFTYAGCSSVKKYRPKYCGSCVDGRC   320
         CEF10   DCKLIKETRICEVRPCGQPSYASLKKGKKCTKTKKSPSPVRFTYAGCSSVKKYRPKYCGSCVDGRC   317
         Fispl2  FCRLEKQSRLCMVRPCEADLEENIKKGKKCIRTPKIAKPVKFELSGCTSVKTYRAKFCGVCTDGRC   291
         CTGF    SCRLEKQSRLCMVRPCEADLEENIKKGKKCIRTPKISKPIKFELSGCTSMKTYRAKFCGVCTDGRC   292
         Nov     QCEMVKQTRLCMHRPCEN-EEPSDKKGKKCIQTKKSMKAVRFEYKNCTSVQTYKPRYCGLCNDGRC   294

         Cyr61   CTPLQTRTVKMRFRCEDGEMFSKNVMM-IQSCKCNYNCPHPNEASFRLY--SLFNDIHKFRD   379
         CEF10   CTPQQTRTVKIR.FRCDDGETFTKSVHM-IQSCRCNYNCPHANEA-YPFY--RLVNDIHKFRD   376
         Fispl2  CTPHRTTTLPVEFKCPDGEIMKKN-HMFIKTCACHYNCPGDNDIFESLYYRKMYGDMA   348
         CTGF    CTPHRTTTLPVEFKCPDGEVMKKN-HMFIKTCACHYNCPGDNDIFESLYYRKMYGDMA   349
         Nov     CTPHNTKTIQVEFRCPQGKFLKKP-MMLINTCVCHGNCPQSNNAFFQPLDPMSSEAKI   351
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Figure 3. **Characteristic features of the CCN family members.** A, The four modules found in the CCN family showing sequence similarity with other proteins. The numbers indicate amino acid positions where the domains are found in cyr61. IBP, Insulin like growth factor binding protein, VWC, Von Willebrand factor type C repeat, TSP1, thrombospondin (type 1 repeat), CT, C-terminal domain. B, An amino acid alignment showing representative members of the CCN family. An asterisk is placed above each of the 38 conserved cysteines. Numbers at the right of the sequences refer to the last amino acid of that row. (O'Brien and Lau, 1992)

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Figure 4. Dendrogram of the amino acid sequences from representative members of the CCN family. This tree was generated using the PILEUP program and is a representation of the sequence similarity seen among the CCN family members reported from published papers (Ying and King, 1996)

with TGF-β and cycloheximide (Brunner et al., 1991). Their results showed that cyr61/βIG-M1 can be expressed when cells are stimulated to divide with serum, as well as when cells become growth arrested after treatment with TGF-β. Both the CEF-10 and cyr61 3' region contained at least two copies of the octanucleotide sequence TTATTTAT found to confer instability in mRNA transcripts (Shaw and Kamen, 1986) as well as exhibiting similar induction kinetics after treatment with serum (Simmons et al., 1989; O' Brien et al., 1990). Despite this, a significant difference in induction patterns was seen after treatment of the cell lines with the phorbol ester, phorbol 12-tetradecanoate 13-acetate (TPA). The mRNA for CEF-10 was immediately induced at time 0 but disappeared within one hour. Within two hours following initial exposure, the mRNA levels rebounded to show steadily increasing mRNA amounts beyond twenty-four hours. This time course correlated with the translocation, activation, and degradation pattern of protein kinase C (PKC) after activation suggesting that PKC may somehow repress the expression of CEF-10 (Simmons et al., 1989). Instead of being repressed like CEF-10, cyr61 was induced by TPA (O'Brien et al., 1990). These results suggest that although cyr61 and CEF-10 are considered homologues, they may perform some different functions that cause dissimilar induction patterns after treatment with various mitogens.

Further studies performed with cyr61 revealed that in actively growing cells, its protein was secreted and bound to both the extracellular matrix (ECM) and the cell surface with a half life of greater than 24 hours. Alternatively, in quiescent cells that were treated with serum to induce passage from G_0 to G_1 stage, cyr61 associates with the cell surface and has a short half life of 30 minutes (Yang et al., 1991). The cyr61

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protein has similar functions to Wnt-1 (Yang et al., 1991). Like many growth factors, it has a chemotactic function but unlike growth factors, it also promotes cell attachment and spreading. From these and other results it was suggested that cyr61 may be an extracellular matrix signaling molecule. Other experiments also show that although it does not have detectable mitogenic activity on its own, it plays a role in enhancing the effect of other growth factors on fibroblasts and thus may play a regulatory role in cell proliferation and differentiation during development (Kireeva et al., 1996). Further clues to cyr61 function were seen when cyr61 was one of 12 genes isolated from murine Swiss 3T3 cells during a search for genes encoding critical inflammatory regulators which were attenuated by glucocorticoids as part of its anti-inflammatory effect. The product of these genes were thought to play a part in the mediation of intercellular interactions. The protein products of these genes were thought to function mostly in a paracrine manner and have functions that are intercellular or extracellular rather that within the cell (Smith and Herschman, 1995).

During the isolation of βIG-M1 (cyr61), a related gene was found to be induced simultaneously and was referred to as βIG-M2 (Brunner et al., 1991). The same gene was isolated by another reseach group in serum-induced mouse fibroblasts and was referred to as fibroblast-inducible secreted protein-12 (fisp-12) (Ryseck et al., 1991). The gene's predicted protein has approximately 65% sequence identity to CEF-10/cyr61 (Ryseck et al., 1991) and 94% similarity to Connective Tissue Growth Factor (CTGF) (Bradham et al., 1991). The CTGF was isolated from human umbilical vein endothelial cells (HUVE) as a secreted competitor for one of the platelet-derived growth factor (PDGF) receptors. Lke PDGF, CTGF functions as a mitoattractant in the endothelial

media but contains very little sequence homology with the PDGF dimers (Bradham et al., 1991). Further studies showed that in primary cultures of human foreskin fibroblasts, TGF-β induced autocrine production of CTGF which was not seen after stimulation with other growth factors. TGF-β must regulate CTGF through a unique pathway not used by the growth factors tested here: PDGF, EGF, FGF (Igarashi et al., 1993). CTGF has also been found to substantially increase during the response of scleroderma fibroblasts to TGF-β1, a protein previously found to be important in scleroderma progression (Kikuchi, K. et al., 1995). As well, CTGF distribution was increased in tissue sections from patients diagnosed with systemic and localized sclerosis as well as various other fibrotic skin disorders in comparison to normal tissue (Igarashi, A. et al., 1995; Igarashi, A et al., 1996). CTGF expression was also induced after scrape wounding of quiescent high density green monkey renal epithelial cells and found throughout the entire culture (Pawar, S. et al., 1995). Calcium oxalate monohydrate crystal treatment of the same cells also stimulated CTGF expression. The presence of these crystals is a symptom of hyperoxaluria, a disease which lead to fibrosis, implicating CTGF as a causative agent (Hammes, M. et al., 1995). Together, the results from these experiments suggest that CTGF is an important factor in wound repair. Experiments done with cyr61 similar to those seen with CTGF imply that although they have 45% sequence identity, they act in completely different ways, CTGF as a growth factor and Cyr61 as an extracellular matrix signaling molecule (Kireeva et al., 1996).

A proto-oncogene was also found to be related to the "CCN" family. An infectious retroviral strain of myeloblastosis-associated virus (MAV) type-1 was isolated

that induced formation of nephroblastomas after injection into chickens (Joliot et al., 1992). Upon progression of these polyclonal kidney tumors, a highly expressed novel gene called 'nov' (nephroblastoma-overexpressed gene) was discovered (Joliot et al., 1992). Unlike other members of the CCN family, the mRNA of this gene did not contain the octanucleotide sequence (TTATTTAT) in its 3' UTR that promotes mRNA instability. Rather, it was found to associate with the extracellular matrix (ECM) (Perbal, 1994) and an amino terminus truncated form of nov was seen to induce morphological transformation in cells (Joliot et al., 1992). The human, mouse and *Xenopus laevis* orthologs have all been previously found and referred to as novH, novM and xnov respectively (Martinerie et al., 1994, Martinerie et al., 1996, Ying, Z. and M. King, 1996). The novH promoter is negatively regulated by WT-1 proteins and nov, as well as the other family members, are considered by this research group to be a novel type of insulin like growth factor binding protein (IBP) (Martinerie, C. et al., 1996, Xin et al, 1996; Kiefer et al., 1991). Previously identified IBPs did have similar features with the CCN family such as the 12 conserved cysteines within the first 100 base pairs which align with the members; as well, the middle domain lacked homology or cysteine residues and the last domain which also contained six conserved cysteine residues (Kiefer et al., 1991). NovH also participated in experiments similar to CTGF for characteristic comparative (Xin et al., 1996). Results indicated that their promoter sequences were completely lacking similarity with each other which suggested that the two genes were regulated in different ways (Xin et al, 1996). Evidence suggests that novH is a negative regulator of cell growth while CTGF has mitogenic properties (Xin et al., 1996)

A *Drosophila* gene encodes a protein referred to as "twisted gastrulation" (tsg) (Mason et al., 1994). It functions as one of seven genes that act as determinants to specify the fate of dorsal cells in *Drosophila* embryos. It contains a motif similar to that found in the CCN family where the cysteine residues can be completely aligned. It spans the region encompassing amino acid residues 26 to 78 in the CCN family members, which is in the same vicinity as domain I that has sequence similarity to insulin-like growth factor binding protein (IBP). The significance of this similarity is unknown. As well, domain II of cyr61 was shown to have sequence similarity with another *Drosophila* gene called short gastrulation which plays a role in dorsal patterning (Francois, V. et al., 1994, Kireeva et al., 1996).

Experimental results seen with this family of immediate early genes suggest that although they have similar sequences and all seem to play a role in mediating cell differentiation, they have distinct patterns of induction and functions in different cellular environments.

2.1 Vectors

The 32b1 cDNA insert was cloned into the vector, pGEM-5Zf(+) (New England Biolabs) previous to my arrival in the lab. The vector contains 3003 bp and encodes a binding site for m13 forward sequencing primer, M13 reverse sequencing primer, T7 promoter and SP6 promoter. It also contains multiple cloning sites. The 32b1 cDNA insert was cloned into the NOT I site.

2.2 Oligonucleotides

Oligonucleotides were designed and synthesized for use in manual sequencing and the RACE protocol (Further explained in Section 2.6 in Materials and Methods below). They are listed in Table 1 in the order of synthesis.

2.3 Manual and Automated DNA Sequencing

Cloned cDNAs of interest were sequenced in both directions using a Sequenase^{TM} kit (United States Biochemistry Corporation) based on the dideoxynucleotide chain termination method from Sanger et al. (1977). The enzyme used was a modified form of bacteriophage T7 DNA Polymerase (version 1.0). Potential cloned cDNAs were also sequenced using an Applied Biosystems (ABI) Model 373 Stretch Automated Sequencer found in the Nucleic Acid Service Laboratory at UBC.

Table 1. Specific oligonucleotides made for Sequencing and RACE methods. The oligonucleotides are listed according to their order of synthesis. Orientation refers to the direction in which the primer was made with respect to the universal priming sites.

Oligo name	Orientation	Use	Sequence
CC1	Reverse	Sequencing	5′ TAG TAT TGG AGC ACA TGT TA 3′
CC2	Forward	Sequencing	5′ ACA CCT GAT TTC TGT TTG ACA 3′
CC3	Reverse	Sequencing	5′ GGG ATA TCA CTA GTG CGG CC 3′
CC5	Reverse	Sequencing	5′ AGT ACT GCG GTT CCT GCG TG 3′
CC6	Forward	Sequencing	5′ CTT AAA ACA CCT GAT TTC TG 3′
CC7	Reverse	Sequencing	5′ CCT GAA GGG GGA CAC TCC AT 3′
CC8	Forward	Sequencing	5′ AAG CAG TAA CAT GTG CTC CAA 3′
CC9	Forward	Sequencing	5′ CAA CTC CAC AAG CTC CAA AA 3′
GSP1	Reverse	RACE	5′ GGCGGCACTCAGGGTTGTCATTGGTAACTCG 3′
GSP2	Reverse	RACE	5′ GGCCTTGTAAAGGGTTGTATAGGATGCGAGG 3′
RACE 3′	Forward	RACE	5′ CCTCGTGTACAATGACGAAGTAAAACCTCG 3′
UAP	Forward	RACE	5′ CTACTACTACTAGGCCACGCGTCGACTAGTAC 3′
CC10	Forward	Sequencing	5′ GAA CAA ACA GAT CCA CAC GG 3′
CC11	Forward	Sequencing	5′ CCC AGC TCC CCC GAA TTC ATC 3′
CC12	Reverse	Sequencing	5′ TGT GCC CTG GAA ACC CAG GT 3′
CC13	Reverse	Sequencing	5′ AGT CGT ATT AAT TTC GAT AA 3′
CC14	Reverse	Sequencing	5′ ATT TCT TGG TCT TGC TGC ATT T 3′

This method employs Taq DNA polymerase in ABI's Amplitaq DyeDeoxy[TM] Terminator Cycle Sequencing chemistry.

The cDNA templates were prepared according to the protocol given with the 373 DNA Sequencer, *"Preparation of plasmid DNA: A modified mini alkaline-lysis/PEG precipitation procedure."* This procedure was followed by cycle sequencing on a *PERKIN ELMER* DNA Thermal Cycler according to the protocol with the 373 DNA Sequencer, *"Sequencing dsDNA from plasmid using Taq Terminator chemistry"*, using 6 pmol of primer in each reaction. The tubes were placed in a thermal cycler preheated to 96 ^{O}C and the following cycle was carried out:

Rapid thermal ramp to 96 ^{O}C, 96 ^{O}C for 15 sec
Rapid thermal ramp to 50 ^{O}C, 50 ^{O}C for 1 sec,
Rapid thermal ramp to 60 ^{O}C , 60 ^{O}C for 4 minutes,
 25 cycles total.
Rapid thermal ramp to 4 ^{O}C and hold.

Before the sample was loaded on the gel, it was extracted with phenol/chloroform according to the 373 Sequencer protocol to remove excess, unincorporated dideoxy terminators. The pellet was applied to a polyacrylamide gel using the Applied Biosystems Automated DNA Sequencer in the NAPS unit. A hard copy or disk copy of the DNA sequence was returned for further analysis. Some DNA sequencing was completely performed by the Nucleic Acid Service Laboratory at UBC after submission of the miniprep DNA and specific primers. Nucleotide and amino acid sequence analyses were carried out using Dnasis Pro Version 3.2.

Sequencing done on the LICOR automated synthesizer followed procedures in the protocol *"Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP"* (Amersham Life Science).

2.4 Library Screening

2.4.1 Touchdown PCR

A λgt11 cDNA library of a U937 monocyte cell line treated with PMA for 3.5 days and an additional λgt11 Jurkat T cell library (Clontech) as well as libraries (from I. Sadowski or R. MacGillivray) derived from the Hela cell line, human lung tissue and human brain tissue were searched. The method used for screening required annealing temperatures from 65 °C to 55 °C in the "touchdown" PCR reactions as described by Don et al (1991). The template was first denatured for 5 minutes at 94 °C and then 2 cycles at each annealing temperature were carried out for 1 min with an immediate denaturing at 94 °C (1 min) before and extension at 72 °C (2 min) afterwards. At the final annealing temperature, 20 cycles were carried out. The reaction finished with a 10 minute extension at 72°C. Each screening used a 1:10 dilution of the library (stock: 1-9 x 10^9 pfu/ml when the concentration was known) and was amplified using Vent DNA polymerase (New England Biolabs). Each PCR reaction contained 1 x Vent polymerase buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH8.8,@ 25 °C), 2mM $MgSO_4$, 0.1% Triton X-100) , 30mM dNTPs, 100mM of each primer, 100mM $MgSO_4$, 100 µg/ml

BSA, 2 U vent polymerase. Each reaction used a vector-directed primer found on either

side of the cloning site (λgt11 arms forward or reverse) and a gene specific primer.

2.4.2 DNA fragment purification

Upon completion of each reaction, the PCR products were separated by

electrophoresis on a 0.7%-1% agarose gel. Distinct bands were purified on a NA45

DEAE membrane (Schleicher & Schuell) (S&S NA45) using a method based on an

application update from Schleicher & Schuell. Once the desired sized band was

specified, a slit was placed before and after the band into the agarose. The NA45 DEAE

membrane was placed into these slits using sterile tweezers. The gel was then placed

back into the gel apparatus and run for 5 minutes at 150V or 20 minutes at 120V. The

NA45 DEAE membrane below the band into which the DNA had run was placed in a

microfuge tube and 300μl of NET buffer was added. The tube was incubated at 65 ^{O}C

for 30 minutes. Any ethidium bromide was extracted with 750 μl water-saturated n-

butanol by vortexing and brief centrifugation. Following this, the membrane was

removed from the tube using tweezers. After centrifugation, the solution in the tube

separated into two layers, the lower layer containing DNA and the NET buffer. This

lower layer was transferred to a new microfuge tube and the DNA was precipitated by

addition of 750 μl of 95% ethanol and placement at -70 ^{O}C for 30 minutes or -20 ^{O}C

overnight. The precipitated DNA was then pelleted via centrifugation for 10 minutes at

high speed. The pellet was washed with 70% ethanol and dried under vacuum. The

pellet was resuspended in 20 μl of distilled water. A small amount of the DNA solution

(usually 2 µl) was applied and run on an agarose gel for estimation of the amount of DNA recovered.

2.4.3 Ligation and Digestion

Purified "blunt-end" DNA fragments were ligated into a vector in a reaction containing: the treated vector (cut with either *Hinc II* or *Sma I* and dephosphorylated) (Maniatis et al, 1992), the purified fragment (2 µl of a concentrated DNA miniprep), T4 DNA ligase (1 µl, New England Biolabs), 1x ligase buffer (supplied by the manufacturer containing 50mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1mM ATP, 50 µg/ml BSA) and 4% PEG 8000 (Sigma). Following an overnight ligation at 15 °C, the reaction was transformed into competent DH5α cells and grown overnight on an LB ampicillin plate. Drug resistant colonies were picked and then the plasmid DNA was extracted according a protocol found in Maniatis et al (1992). Plasmids containing the desired insert were found by restriction enzyme digestion on either side of the cloning site. Digestions contained 1 µl (20U) of each enzyme in appropriate 1x buffer (supplied by the manufacturer) with the miniprep DNA (2 µl). The reaction was then incubated at the recommended temperature for at least 1 hour before electrophoresis on a 0.7-1% w:v agarose gel. Each gel was run in 1x TBE (0.89 M Tris, 0.89 M boric acid, 1 mM EDTA (pH 8.0)) containing 0.1 µg/ml ethidium bromide. Gels were run by electrophoresis at 10V/cm with a size marker, λ DNA digested with *Hind III* (Sigma). After separation, bands were detected by UV illumination and photographed. DNA sequence of positive clones was determined allowing further analysis to be carried out.

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2.5 Southern Blots

PCR products and digests of putative cloned cDNAs were separated on a 0.7% w:v agarose gel (as described in 2.4.3) and then transferred onto a piece of nylon membrane (Zeta-Probe GT Genomic Tested Blotting Membrane; BIO-RAD) using a vacuum blotter, *trans-Vac TE 80* (Hoefer Scientific Instruments). The method employed for transfer was specified by the manufacturer. Each blot was then exposed to 300 mJ UV irradiation in a GS Gene Linker^{TM} UV Chamber (BIO-RAD) to crosslink the DNA to the blot. Blots were prehybridized for 4 hours in hybridization solution (50% formamide, 6X SSC, 0.1% polyvinylpyrollidone, 0.1% ficoll 400), 50mM sodium phosphate (pH 6.5), 0.5 % sodium dodecylsulfate (SDS), 100 μg/ml salmon sperm DNA (ssDNA)) at 42 ^{O}C. The ssDNA was denatured in 0.15 M NaOH at 37 ^{O}C for 5 minutes and neutralized with one equivalent of acetic acid immediately prior to addition to the hybridization solution. Following prehybridization, overnight hybridization was carried out in the same conditions after a change to 20 ml of fresh solution. The probe was added to the ssDNA to be denatured simultaneously before addition to the hybridization solution. The blot was washed in approximately 250 ml of 0.1 X SSC, 0.1% SDS for three washes at 30 minutes each at 50 ^{O}C. The blot was then wrapped in saran wrap and exposed to film (Kodak Scientific Imaging Film X-OMAT AR 8X10).

2.6 Using the 5′ RACE System

An alternative method was used to search for the missing fragment of the 32b1 cDNA clone using a RACE protocol. A kit called the *5′ RACE System for Rapid*

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Amplification of cDNA ends was purchased from GIBCO BRL. An overview of its protocol is shown in figure 5. The method used for screening followed the protocol given with the kit. The RNA used in this procedure was extracted using method described in section 2.8.

2.7 Cell Culture and Mitogenic Stimulation

The Jurkat T cell line, U937 promonocytic cell line (human histiocytic lymphoma) and Hela (epitheloid carcinoma of the cervix) cell line were cultured in RPMI-1640 medium (with L-glutamine and without sodium bicarbonate; Gibco BRL). HL-60 cells (promyelocytic leukemia cell line from peripheral blood) were grown in Iscove's modified Dulbecco's medium (IMDM with L-glutamine, with 25mM Hepes buffer, without sodium bicarbonate; Gibco BRL). The Rat-2 fibroblast cell line was grown in Dulbecco's modified Eagle medium (D-MEM with low glucose, with L-glutamine, with 110 mg/L sodium pyruvate, without sodium bicarbonate). Each medium used was supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% streptomycin (10000 μg/ml) and penicillin (10000 U/ml) (Gibco BRL).

Before induction, the Rat 2 fibroblasts and CNA7 cells were rendered quiescent through density arrest as described by Jahner et al (1991). The various stimulants were used in the following final concentrations: epidermal growth factor (EGF; Calbiochem, receptor grade), 20ng/ml; phorbol 12-myristate 13-acetate (PMA; Gibco BRL), 100ng/ml; fetal bovine serum (FBS; Gibco BRL), 20%; actinomycin D (Calbiochem Corporation), 1μg/ml; cycloheximide (Sigma), 10μg/ml. The agents were dissolved in media and then added directly to the cell culture.

mRNA 5' ——————————————————(A)$_n$-3' ⇐GSP1

Anneal first strand primer, GSP1, to mRNA.

cDNA 5' ——————————————————(A)$_n$-3'

Copy mRNA into cDNA with SUPERSCRIPT II RT.

··——(A)$_n$-3'

Degrade mRNA template with RNase H.

Purify cDNA with GLASSMAX Spin Cartridge

3'-(dC)$_n$

Tail purified cDNA with dCTP and TdT.

Anchor Primer

3'-(dC)$_n$ ⇐GSP2

Amplify dC-tailed cDNA by PCR using the Anchor Primer and nested GSP2.

PCR product ready for:
agarose gel electrophoresis/size enrichment
nested primer amplifications
UDG cloning into vector pAMP1

Figure 5. Protocol for the 5' Race System for Rapid Amplification of cDNA ends. This procedure is based on reverse transcription of a sample of RNA from a specific cell line shown to express the desired message. A gene specific primer (GSP1) is used as a start site for the reverse transcriptase and once completed, the cDNA fragment is purified and a 3' poly (dC) tail is added. The fragment is then amplified using polymerase chain reaction (PCR) with an anchor primer with affinity for the dC tail. From here, theoretically, one can clone and sequence the 5' fragment and religate it to the original clone. (figure from protocol with RACE kit)

2.8 Extraction of RNA

Total RNA was isolated from the various cell lines according to the protocol included with the "Ultraspec RNA Isolation System" (I. D. Lab Inc.). The method is based on the procedure described in Chomczynski et al (1987). Caution was used during each extraction procedure. Gloves were worn at all times and separate freshly autoclaved tips and tubes were used for RNA only. The solutions used, (eg. the deionized distilled water) were treated with diethyl-pyrocarbonate (depc) and autoclaved. Tissue culture cells were grown in the specified media for several days until a total cell count reached 5-10 million cells/ml for cells grown in suspension or 1.5 to 2.3 million cells per plate of monolayer cells. Cells grown in suspension were sedimented by centrifugation at 12,000 x g for 5 minutes. The cell pellet was suspended in 1 ml of Ultraspec RNA solution and transferred to a 1.5 ml tube. For plated cells, the growth medium was discarded, 1 ml of Ultraspec RNA solution was added, the cells were scraped off the plate and the cell suspension was transferred to a 1.5 ml microfuge tube. After homogenization, the cells were stored at 4 °C for 5 minutes to allow complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per ml of Ultraspec RNA solution used. The mixture was vigorously mixed for at least 15 seconds, stored on ice at 4 °C for 5 minutes, and centrifuged at 12,000 x g at 4 °C for 15 minutes. The upper aqueous phase containing the RNA was transferred to a fresh tube. An equal amount of isopropanol was added and the samples were stored for no more than 10 minutes at 4 °C. Tubes were then centrifuged at 12,000 x g at 4 °C for 10 minutes. The RNA formed at the bottom of the tube as a white pellet. The supernatant was removed and the pellet covered with 1 ml of cold 70% ethanol followed by

centrifugation at high speed for 5 minutes at 4 °C. The wash solution was then carefully removed using a sterile tip and the tube left open to air dry for 10 minutes. Pellets were resuspended in 50 µl of depc-treated ddH$_2$O and the RNA concentration of each sample was quantified by UV spectroscopy and stored at -70 °C.

2.9 Northern Blots

Samples containing 20µg of RNA were separated by electrophoresis in a 1% agarose gel containing 5% formaldehyde and 1x MOPS EDTA buffer (20mM MOPS pH 7.0, 5mM sodium acetate, 1 mM EDTA; depc-treated and autoclaved). Each RNA sample was adjusted to a volume of 5 µl with depc-treated deionized distilled water and 25 µl of RNA loading buffer (56% formamide, 1x MOPS EDTA buffer, 2.2 M formaldehyde, 7.5% glycerol, 0.3% bromophenol blue) was added. The samples were heated at 70 °C for 15 minutes before addition of 1 µl of ethidium bromide (1 mg/ml). The samples were then loaded and the gel was run from 50V to 100V in 1x MOPS-EDTA buffer until the bromophenol blue dye was approximately 1 cm from the bottom. Visibility on a UV box after electrophoresis ensured intact RNA.

Overnight transfer onto nylon paper (Zeta-Probe GT Genomic Tested Blotting Membrane; BIO-RAD) was done using capillary action in 10X SSC. The membrane was irradiated with UV and hybridized as for the Southern blot explained previously.

Following detection of a message, the Northern blots were also stripped by immersing the nylon membrane in approximately 500 ml of boiling 0.1% SDS solution for 20 minutes. Complete stripping was ensured by reexposure of the blot to film

overnight to detect any remaining signal. The stripped blots were then reprobed with GAPDH, a control, to ensure equal loading of RNA.

2.10 Radioactive Labelling of the Probe

DNA to be labelled was purified by electrophoresis on a 0.7% agarose gel and the appropriate band cut out. The gel slice was placed inside an autoclaved 0.7 ml tube containing a plug of siliconized glass wool. A hole was pierced through the bottom of the tube using a 21G needle. This smaller tube was placed within a larger 1.5 ml tube and the assembly spun at high speed for 5 minutes. The liquid that flowed through to the larger tube contained template DNA for labelling.

Randomly labelled probes were used in the Northern and Southern hybridizations. Approximately 600 ng (estimated by visualization on a gel stained with ethidium bromide under UV light) of DNA was added to distilled water to a final volume of 68μl. The DNA was denatured at 100 $^{\circ}$C for 10 minutes and then immediately placed on ice for 1 to 5 minutes. The following reagents were added: 20 μl oligolabelling buffer (Pharmacia), 10 μl [α ^{32}P]-dCTP label (100 μCi), 3 μl DNA polymerase I (Klenow fragment) (3.3U/μl). This reaction was placed in a 37°C water bath for at least 1 hour. The probe was then precipitated at -20 $^{\circ}$C for 4 to 24 hours after addition of an equal volume of 5M ammonium acetate and 4 volumes of 95% ethanol at -20 $^{\circ}$C. The labelled probe was recovered by centrifugation at high speed in a microfuge for 10 minutes and the supernatant removed. The pellet was resuspended in 50 μl dH$_2$O and 1 μl was counted to ensure adequate labelling. If the specific activity was judged to be too low, the probe was relabelled by repeating the above protocol.

Results

I. The 32b1 sequence

a. Analysis of the 32b1 sequence

The 32b1 insert was cloned into the pGem5fz(+) vector prior to my arrival in the lab. It was partially sequenced by another technician but the entire sequence was repeated and checked for any discrepancies. The majority of the 32b1 clone was sequenced manually but sections were also sequenced using a LICOR automated sequencer for increased accuracy. Figure 6A is a map showing the sequencing strategy used (refer to sections 2.2 and 2.3 in Materials and Methods for primer sequences and sequencing methods). The insert in the 32b1 cDNA clone was largely sequenced in both directions. A section of DNA at the 5′ end spanning 245 bp as well as a small section at the 3′ end were sequenced numerous times, but only in one direction. Because of the numerous times it was repeated, the DNA sequence is most likely accurate. Also, careful inspection of chromatograms from the automated sequencers ensured that only clear sharp peaks were used in the sequencing anaylsis. Figure 6B shows the DNA sequence of 32b1 translated into its amino acid sequence. Another indication of the accuracy of 32b1 was the ability to translate the DNA sequence without encountering stop codons in the coding region which resulted in 91% similarity with the amino acid sequence of cyr61 (Figure 7).

The sequence of the 32b1 cDNA contains an open reading frame (ORF) encoding a predicted protein with cysteines at positions characteristic of the "CCN" family

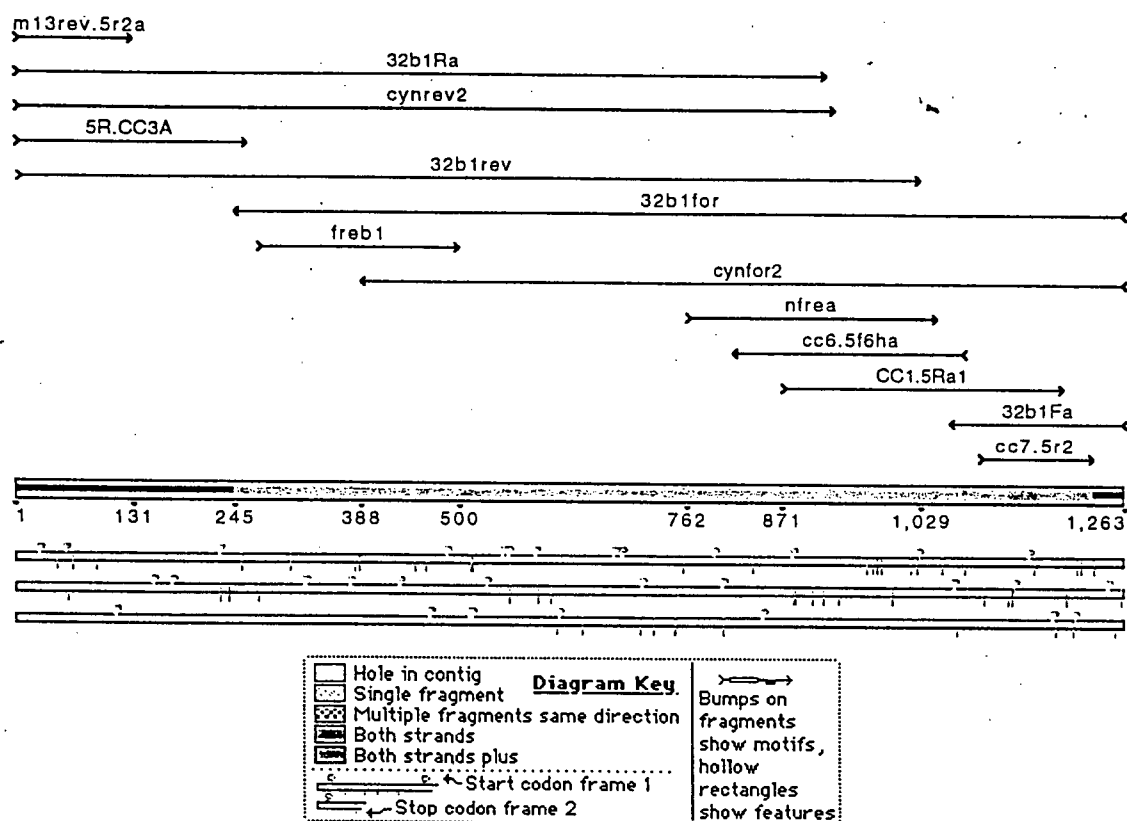**Figure 6A Map of the sequencing strategy for the 32b1 cDNA.** Manual and automated sequencing techniques were used to sequence the cloned 32b1 cDNA. In addition to m13univ and m13rev primers binding to the vector, specific oligonucleotides were designed and synthesized to sequence the rest (refer to Materials and Methods section 2.2 and 2.3 for oligonucleotide sequences and sequencing procedures).

```
   1 AATTCGGGGGAGCTGGGATTCGATGCCTCCGAGGTGGAGTTGACGAGAAACAATGAATTG   59
   1 AsnSerGlyGluLeuGlyPheAspAlaSerGluValGluLeuThrArgAsnAsnGluLeu   20

  60 ATTGCAGTTGGAAAAGGCAGCTCACTGAAGCGGCTCCCTGTTTTTGGAATGGAGCCTCGC  119
  21 IleAlaValGlyLysGlySerSerLeuLysArgLeuProValPheGlyMetGluProArg   40

 120 ATCCTATACAACCCTTTACAAGGCCAGAAATGTATTGTTCAAACAACTTCATGGTCCCAG  179
  41 IleLeuTyrAsnProLeuGlnGlyGlnLysCysIleValGlnThrThrSerTrpSerGln   60

 180 TGCTCAAAGACCTGTGGAACTGGTATCTCCACACGAGTTACCAATGACAACCCTGAGTGC  239
  61 CysSerLysThrCysGlyThrGlyIleSerThrArgValThrAsnAspAsnProGluCys   80

 240 CGCCTTGTGAAAGAAACCCGGATTTGTGAGGTGCGGCCTTGTGGACAGCCAGTGTACAGC  299
  81 ArgLeuValLysGluThrArgIleCysGluValArgProCysGlyGlnProValTyrSer  100

 300 AGCCTGAAAAAGGGCAAGAAATGCAGCAAGACCAAGAAATCCCCCGAACCAGTCAGGTTT  359
 101 SerLeuLysLysGlyLysLysCysSerLysThrLysLysSerProGluProValArgPhe  120

 360 ACTTACGCTGGATGTTTGAGTGTGAAGAAATACCGGCCCAAGTACTGCGGTTCCTGCGTG  419
 121 ThrTyrAlaGlyCysLeuSerValLysLysTyrArgProLysTyrCysGlySerCysVal  140

 420 GACGGCCGATGCTGCACGCCTCAGCTGACCAGGACTGTGAAGATGCGGTTCCGATGCGAA  479
 141 AspGlyArgCysCysThrProGlnLeuThrArgThrValLysMetArgPheArgCysGlu  160

 480 GATGGGGAGACATTTTCCAAGAACGTCATGATGATCCAGTCCTGCAAATGCAACTACAAC  539
 161 AspGlyGluThrPheSerLysAsnValMetMetIleGlnSerCysLysCysAsnTyrAsn  180

 540 TGCCCGCATGCCAATGAAGCAGCGTTTCCCTTCTACAGGCTGTTCAATGACATTCACAAA  599
 181 CysProHisAlaAsnGluAlaAlaPheProPheTyrArgLeuPheAsnAspIleHisLys  200

 600 TTTAGGGACTAAATTGCCTACCTGGGTTTCCAGGGCACACCTAGACAAACAAGGGAGAAG  659
 201 PheArgAsp***                                                  203

 660 AGTGTCAGAATCAGAATCATGGAGAAAATGGGCGGGGGTGGTGTGGGTGATGGGACTCAT  719
 720 TGTAGAAAGGAAGCCTTGCTCATTCTTGAGGAGCATTAAGGTATTTCGAAACTGCCAAGG  779
 780 GTGCTGGTGCGGATGGACACTAATGCAGCCACGATTGGAGAATACTTGCTTCATAGTATT  839
 840 GGAGCACATGTTACTGCTTCATTTTGGAGCTTGTGGAGTTGATGACTTCTGTTTTCTGTT  899
 900 TGTAAATTATTTGCTAAGCATATTTTCTCTAGGCTTTTTTCCTTTTGGGGTTCTACAGTC  959
 960 GTAAAGAGATAATAAGATTAGTTGGACAGTTTAAAGCTTTTATTCGTCCTTTGACAAAA 1019
1020 GTAAATGGGAGGGCATTCCATCCCTTCCTGAAGGGGGACACTCCATGGAGTGTCTGTGAG 1079
1080 ACGGCAGTCTATCTGCACTCTAAACTAAACTGCAAACAGAAATCAGGTGTTTTAAGACTG 1139
1140 AATGTTTTATTTATCAAAATGTAGCTTTTGGGGAGGGAGGGGAAATGTAATACTGGAATA 1199
1200 ATTTGTAAATGATTTTAATTTTATATCAGTGAAAAGATTTTATTTATGGAATTAACCATT 1259
1260 TAATACCCGAATTCC                                              1274
```

Figure 6B. The partial cDNA sequence of the cloned 32b1 cDNA. This sequence encompasses 1.3kb. The open reading frame was found after comparison to the cyr61 DNA coding sequence and careful inspection of experimental data.

```
SCORES        Init1: 820 Initn: 985 Opt: 992
              91.0% identity in 201 aa overlap

                                               10        20        30
20a022                              NSGELGFDASEVELTRNNELIAVGKGSSLKRLPV
                                    ||:|||||||||||||||:|||||||||||
Cyr6_M  RLVKVSGQCCEEWVCDEDSIKDSLDDQDDLLGLDASEVELTRNNELIAIGKGSSLKRLPV
        150        160       170        180       190       200


                  40        50        60        70        80        90
20a022  FGMEPRILYNPL--QGQKCIVQTTSWSQCSKTCGTGISTRVTNDNPECRLVKETRICEVR
            ||  |||:|:|||   :|||||||||||||||:|||||||||||||||||||||||||||
Cyr6_M  FGTEPRVLFNPLHAHGQKCIVQTTSWSQCSKSCGTGISTRVTNDNPECRLVKETRICEVR
        210       220       230       240       250       260


                100       110       120       130       140       150
20a022  PCGQPVYSSLKKGKKCSKTKKSPEPVRFTYAGCLSVKKYRPKYCGSCVDGRCCTPQLTRT
        ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||  |||
Cyr6_M  PCGQPVYSSLKKGKKCSKTKKSPEPVRFTYAGCSSVKKYRPKYCGSCVDGRCCTPLQTRT
        270       280       290       300       310       320


                160       170       180       190       200
20a022  VKMRFRCEDGETFSKNVMMIQSCKCNYNCPHANEAAFPFYRLFNDIHKFRD
        |||||||||||| |||||||||||||||||||:|||:|::|:|||||||||
Cyr6_M  VKMRFRCEDGEMFSKNVMMIQSCKCNYNCPHPNEASFRLYSLFNDIHKFRD
        330       340       350       360       370
```

Figure 7 Amino acid sequence similarity between 32b1 and cyr61. The amino acid sequence was aligned and calculated to have a 91% sequence similarity.

allowing it to be aligned with other members. The cloned cDNA contained 1274 nucleotides and from comparison with cyr61, the sequence with which it is most similar, the coding sequence contained 203 amino acids ending at nucleotide #617. The amino acid sequences of cyr61 and 32b1 have a 91% similarity and their DNA sequences are 84% identical. The remaining 646bp of 32b1 was considered to be the 3' UTR. According to a cyr61 sequence comparison, 32b1 is still missing 175 amino acids of the peptide sequence which includes an amino terminus similar to cyr61 and part of an acidic highly diverse central portion.

b. Search for the 5' end

The cloned 32b1 cDNA was considered to be missing its 5' end because in comparison to the full-length cDNA encoding cyr61, approximately 500 basepairs were missing. As well, no initiation codon was found for the translated version of 32b1 that was similar in position to that in cyr61. In Northern blots of RNAs from human tissues, the sizes of the RNAs hybridizing to a probe for 32b1 were approximately 4.4kb and 2.6kb (see Figure 9). These sizes are much larger than that of the probe itself (1.3 kb). In order to characterize 32b1 accurately, it was important to find the full length cDNA. Conventional hybridization techniques used to rescreen the original Jurkat T lymphocyte λgt11 library yielded three 200 bp cDNA fragments making them unattractive to clone and sequence. Touchdown PCR techniques used to screen two Jurkat T cell libraries revealed that the 5' end of 32b1 was undetectable here (refer to Materials and Methods section 2.4 for details). Cloned cDNAs identical to the cloned

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32b1 cDNA were isolated numerous times but no additional sequence information was acquired.

In another effort to isolate the extreme 5′ end of the 32b1 cDNA, RACE was attempted (see section 2.6 in Materials and Methods) but was also unsuccessful in this Jurkat T cell line. In exploring various reasons for this result, Northern Hybridization was performed in an attempt to assess the quality of the T cell mRNA used in this procedure. Although the total RNA looked intact in a stained gel, probing a blot with a labelled 32b1 cDNA probe revealed that there was no detectable message being expressed in this cell line. Accordingly, the search for the 5′ end was extended to other libraries. A library derived from U937 monocytes treated with PMA for 3.5 days (Section 2.4.1 in Materials and Methods) was found to contain fragments that hybridized to the 32b1 cDNA clone in a Southern blot analysis. DNA sequencing determination of the largest fragment revealed that it contained a cDNA sequence identical to that in the 32b1 cDNA but not any novel sequence. Northern blot analysis revealed similar results to that of the Jurkat T cell library. Although the 32b1 cDNA clone could be found using PCR, Northern blot analysis could not detect expression of 32b1 mRNA in this cell line with or without PMA induction. The repeated isolation of 32b1 in the leukocyte derived libraries using PCR suggested that perhaps a mitogen was required to stimulate significant expression in these cell lines. Thus, various cultures of Hela cells, HL-60 cells, Jurkat T cells and U937 cells were stimulated with the phorbol ester PMA for 30 minutes and 2 days and probed with a labelled 32b1 cDNA in a Northern blot. There was no detection of a hybridizing species. As well, serum and DMSO added to the selected cell culture media for various amounts of time did not

induce 32b1 mRNA expression (refer to section 2.7 in Materials and Methods for details). Other libraries made from human lung tissue, Hela cell lines and human brain tissue were probed but no sequence was found that even allowed binding of the gene specific primer to create a PCR product.

c. The discovery of novel 5′ end sequence of 32b1 from Fasta search

A FASTA search revealed numerous matches with 32b1 from various sources. Most matches were fragments of DNA derived from the WashU-Merck EST project (Hillier, L. et al., 1995). Figure 8A shows representative DNA sequences found, referred to here by their accession numbers in the nucleotide database (NCBI) for convenience. Some representative DNA sequences found within the known 32b1 sequence were: T92167 and Y09859 (Hillier, L. et al, 1995, Pilarsky, C. et al, 1996). Three other sequences: W51901, N86328 and Z50168 were found to align with 32b1 and reveal novel sequence in the 5′ end (Genini, M, et al., 1996, Hillier, L. et al, 1995, Liew, C.C., 1996) (Figure 8A). The largest 5′ fragment obtained from the search, Z50168, was 304 bases long and overlapped with 32b1 with a 100% match aligning 85 bases. The remaining 221 bases was novel sequence from the 5′ end of 32b1. This z50168 sequence was connected with 32b1 (referred to as z5-32b1 for convenience) and translated in frame to produce an amino acid sequence lacking any stop codons and containing three cut sites (Figure 8B). The novel amino acid sequence aligned with 32b1 is shown in figure 8C. In an alignment with Cyr61, there are still approximately 100 amino acids missing.

A

z50168

n86328

w51901

32b1fini

t92167

y09859

1 89 161 305 391 632 960 1,135 1,270 1,495

B

DraII (187)   XmaIII (637)   HindIII (1209)
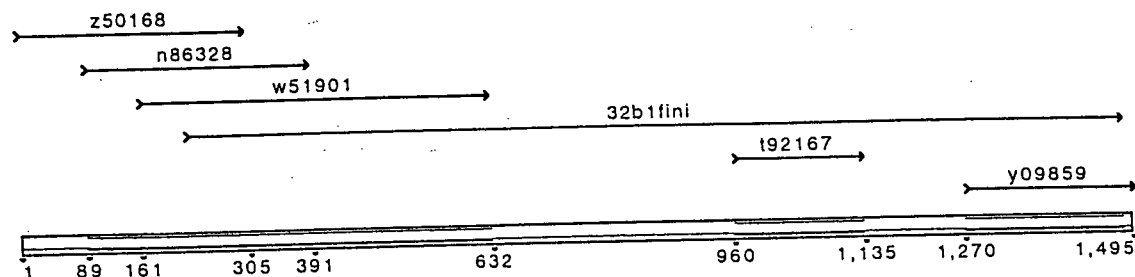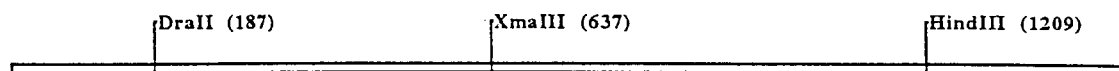
Mapping all cutsites.

Cutters : DraII, HindIII & XmaIII

Non-Cutters : ApaI, BamHI, Bsp106, BstXI, EcoRI, EcorV, KpnI, NotI, PstI, SacI, SacII, SalI, SmaI, SpeI, XbaI & XhoI

Figure 8 Representative DNA sequences showing high sequence similarity with 32b1 found in a FASTA search. A, The sequences found are referred to here by their accession number in the NCBI nucleotide database for convenience. B, The sequence that aligned with 32b1 revealing new 5′ end sequence was checked for new cut sites.

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
HSA33210-5'   CCAAAACGGGGAAAGTTTCCAGCCCAACTGTAAACATCAGTGCACATGTA
   #1
   ───

               CCAAAACGGGGAAAGTTTCCAGCCCAACTGTAAACATCAGTGCACATGTA
                Q   N   G   E   S   F   Q   P   N   C   K   H   Q   C   T   C   I

HSA33210-5'   TTGATGGCGCCGTGGGCTGCATTCCTCTGTGTCCCCAAGAACTATCTCTC
   #51
   ───

               TTGATGGCGCCGTGGGCTGCATTCCTCTGTGTCCCCAAGAACTATCTCTC
                 D   G   A   V   G   C   I   P   L   C   P   Q   E   L   S   L

HSA33210-5'   CCCAACTTGGGCTGTCCCAACCCTCGGCTGGTCAAAGTTACCGGGCAGTG
   #101
   ───

               CCCAACTTGGGCTGTCCCAACCCTCGGCTGGTCAAAGTTACCGGGCAGTG
                P   N   L   G   C   P   N   P   R   L   V   K   V   T   G   Q   C

HSA33210-5'   CTGCGAGGAGTGGGTCTGTGACGAGGATAGTATCAAGGACCCCATGGAGG
   #151
   ───

               CTGCGAGGAGTGGGTCTGTGACGAGGATAGTATCAAGGACCCCATGGAGG
                 C   E   E   W   V   C   D   E   D   S   I   K   D   P   M   E   D

HSA33210-5'   ACCAGGACGGCCTCCTTGGTAAGGAGCTGGGATTCGATGCCTCCGAGGTG
32b1fini   GGAGCTGGGATTCGATGCCTCCGAGGTG
   #201
   ───

               ACCAGGACGGCCTCCTTGGTAAGGAGCTGGGATTCGATGCCTCCGAGGTG
                 Q   D   G   L   L   G   K   E   L   G   F   D   A   S   E   V

HSA33210-5'   GAGTTGACGAGAAACAATGAATTGATTGCAGTTGGAAAAGGCAGCTCACT
32b1fini   GAGTTGACGAGAAACAATGAATTGATTGCAGTTGGAAAAGGCAGCTCACT
   #251
   ───

               GAGTTGACGAGAAACAATGAATTGATTGCAGTTGGAAAAGGCAGCTCACT
                E   L   T   R   N   N   E   L   I---A   V   G   K   G   S   S   L

Figure 8C The alignment of 32b1 with the novel 5′ end sequence found
from the FASTA search and its traslated amino acids. The three DNA
sequences showing novel 32b1 5′ end were connected with 32b1 forming
z5-32b1.

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2. Expression of 32b1 mRNA

a. Tissue distribution of 32b1 mRNA in adult humans

A Northern blot was performed using the cloned 32b1 cDNA insert to probe different human tissues in order to find out where the mRNA was expressed for comparison with other CCN family members. It was also helpful in estimating the approximate size of full-length 32b1 mRNA in humans. A blot containing 2.0 µg of poly A$^+$ RNA extracted from one of 8 different human tissues in each lane was probed with 32b1 (refer to Materials and Methods section 2.9). Figure 9 shows that a major RNA species of 2.6 kb was detected in 7 of 8 tissues. The 2.6 kb mRNA was expressed at the highest levels in the ovary and colon (Figure 9, lanes 5 and 7). Moderate amounts of mRNA were found in the small intestine, testis, prostate, and spleen (Figure 9, lanes 6, 4, 3 and 1). A low level of 32b1 was transcribed in the thymus (figure 9, lane 2). The only tissue that did not express the 2.6 kb mRNA was peripheral blood leukocytes (Figure 9 lane 8). An additional 4.4 kb species was most prominent in RNA extracted from the spleen (figure 9, lane 1) although it was seen in all the tissues shown to express the 2.6 kb mRNA (figure 9, lane 1-7). There was also a dark smear seen in the RNA extracted from the colon tissue and a faint smear in the spleen RNA implying that 32b1 may also be found in smaller sizes in these areas or imperfections in the purchased Clontech blot.
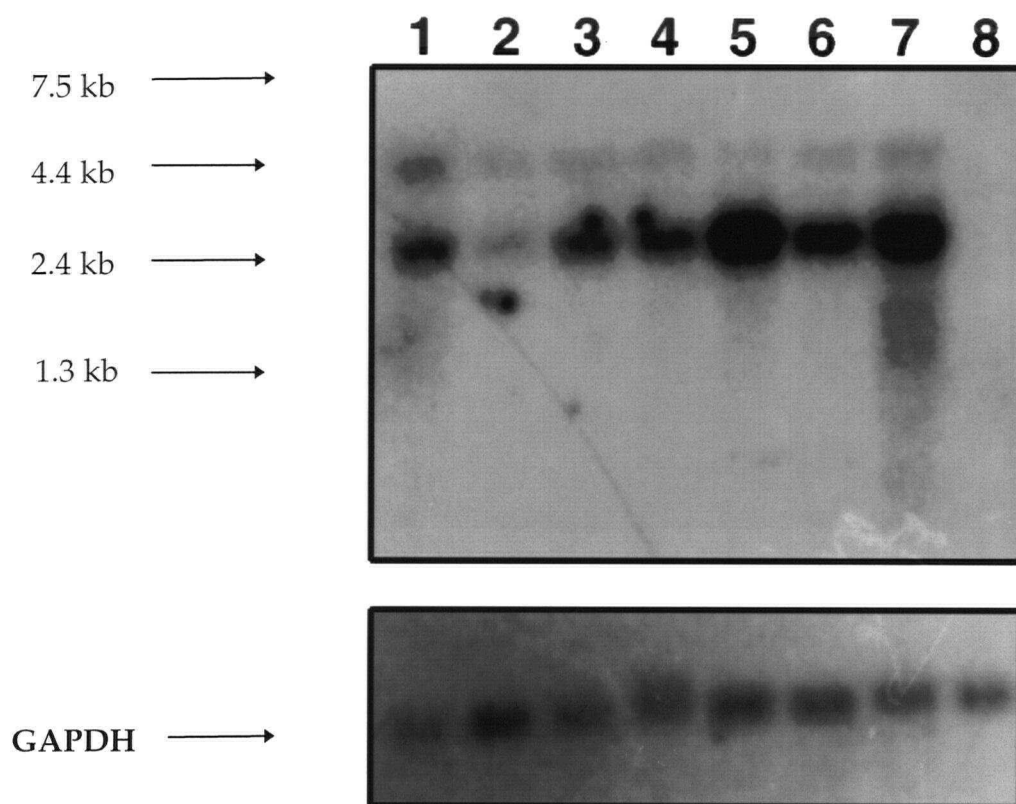
Figure 9. Human Tissue Blot probed with 32b1. In order to examine where the 32b1 gene was expressed, a Human Tissue blot was purchased from Clontech and was probed with 32b1 as described in section 2.9 of Materials and Methods. Each lane contained 2 μg of poly A+ mRNA. The blot was stripped and reprobed with labelled GAPDH as a control to ensure equal amounts of RNA was loaded in each lane. The lanes contained RNA extracted from the following tissues: 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, peripheral blood leukocyte.

b. Effect of mitogenic stimulation on expression of 32b1 mRNA in different cell lines

The induction kinetics of the 32b1 mRNA expression in different cell lines were deduced in order to acquire additional information about when and where expression of the gene was stimulated in an immediate early gene manner and thus allow comparison with other CCN family members. Figure 10 shows a representative blot of total RNA from cell lines probed with 32b1 cDNA. A number of cell lines of human lymphocyte origin were examined for their ability to express the putative 32b1 mRNA under various conditions. Figure 10 lanes 1-3 shows that cells grown for at least 2 days in serum including Jurkat T cells (lane 1), U937 (lane 2) and HL-60 (lane 3) failed to express the 2.6 kb mRNA. These cell lines were subjected to a number of treatments in an attempt to induce expression of 32b1 mRNA: treatment with PMA for 30 minutes or 2 days; DMSO for 2 days; and starvation followed by serum treatment. In no case was the 2.6 kb mRNA detected. (data not shown). Unexpectedly, this 32b1 probe hybridized with a 2.6 kb transcript in Rat 2 fibroblasts that is induced in cells treated with epidermal growth factor (EGF) (Figure 10, lanes 4-6). A 2.6 kb message appears after 30 minutes of induction (Figure 10, lane 5) but is less intense after 90 minutes of induction (Figure 10, lane 6).

3. Analysis of the Induction Kinetics for 32b1 mRNA through Northern Hybridization

The results from the previous Northern hybridization experiment showed that a 2.6 kb species with high sequence identity to the 32b1 gene was transiently induced after stimulation with EGF in Rat-2 cells. This experiment identified a gene displaying

41
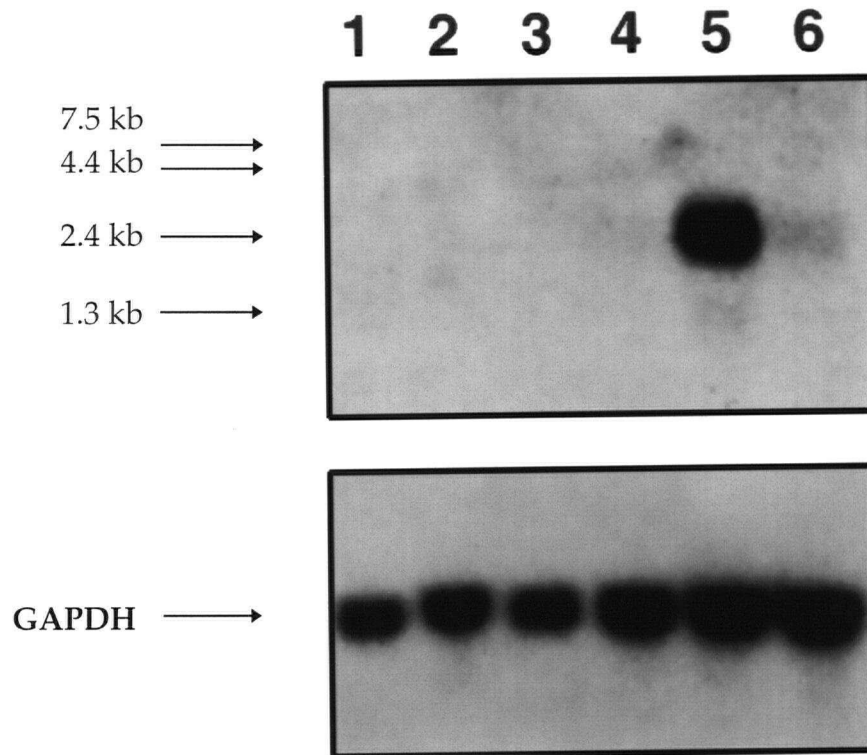
Figure 10. Induction of 32b1R. 20 µg of total RNA was loaded in each lane of a formaldehyde gel, separated electrophoretically, blotted to a nylon membrane, and then probed with labelled 32b1 cDNA, as in section 2.9 in Materials and Methods. The RNA in the first three lanes was extracted from cells growing in serum for approximately 2 days. RNA in the last three lanes was extracted from Rat-2 fibroblasts that were rendered quiescent (as described in section 2.7 in Materials and Methods) and then treated with 20 ng/ml EGF for various lengths of time. The cell lines and treatments are as follows: 1, Jurkat T cell; 2, U937; 3, HL-60; 4, Quiescent Rat-2 fibroblasts; 5, 30' EGF induction of quiescent Rat-2 fibroblasts; 6, 90' EGF induction of quiescent Rat-2 fibroblasts.

immediate early gene induction characteristics in rodent fibroblasts and suggested that further study of its induction kinetics would be necessary to characterize its role in cell proliferation. To prevent confusion, the Rat-2 hybridizing species will be referred to as 32b1R for "rat".

a. Time Course of 32b1 induction in Rat-2 fibroblasts treated with Fetal Bovine Serum

In order to define the kinetics of induction of 32b1R mRNA with serum, medium containing 20% fetal bovine serum (FBS) was added to Rat 2 cells which were rendered quiescent (refer to section 2.7 in Materials and Methods). The data in Figure 11 show that a 2.6 kb mRNA appeared after 45 minutes and reached a peak concentration at one hour (Figure 11, lanes 4 and 5). The size of the message became slightly smaller at 90 minutes (Figure 11, lane 6) and completely disappeared within 4 hours.

b. Time course of 32b1 induction in Rat-2 cells treated with Phorbol 12-myristate 13-acetate (PMA)

Phorbol esters were added to density arrested Rat-2 fibroblasts to determine whether the expression of 32b1R mRNA could be induced. After PMA addition, RNA was extracted at various time points and analysed on a Northern blot. Unexpectedly, a 2.6 kb RNA could not be detected (Figure 12A). This experiment was repeated several times with the same result. As a control, the same blot was probed with c-fos (Figure 12B). The c-fos mRNA was detected within 30 minutes following induction but was no longer detectable 60 minutes following induction.

Figure 11. Time Course of Rat-2 fibroblasts treated with Fetal Bovine Serum (FBS) and probed with 32b1. The Rat-2 fibroblasts were grown in DMEM media supplemented with 10% Fetal Bovine Serum and 1% antibiotics. They were rendered quiescent after confluency through density arrest and then induced with 20% FBS. RNA was extracted at various time points and analysed by Northern Hybridization (Refer to sections 2.7, 2.8, and 2.9 in Material and Methods for details). The RNA extractions were organized on the gel in the following order: 1, Quiescent cells; 2, 15 min; 3, 30 min; 4, 45 min; 5, 1h; 6, 90 min; 7, 2h; 8, 4h; 9, 8h. The same blot was stripped and reprobed with a control DNA probe, GAPDH, to ensure that a consistent amount of RNA was loaded in each lane.
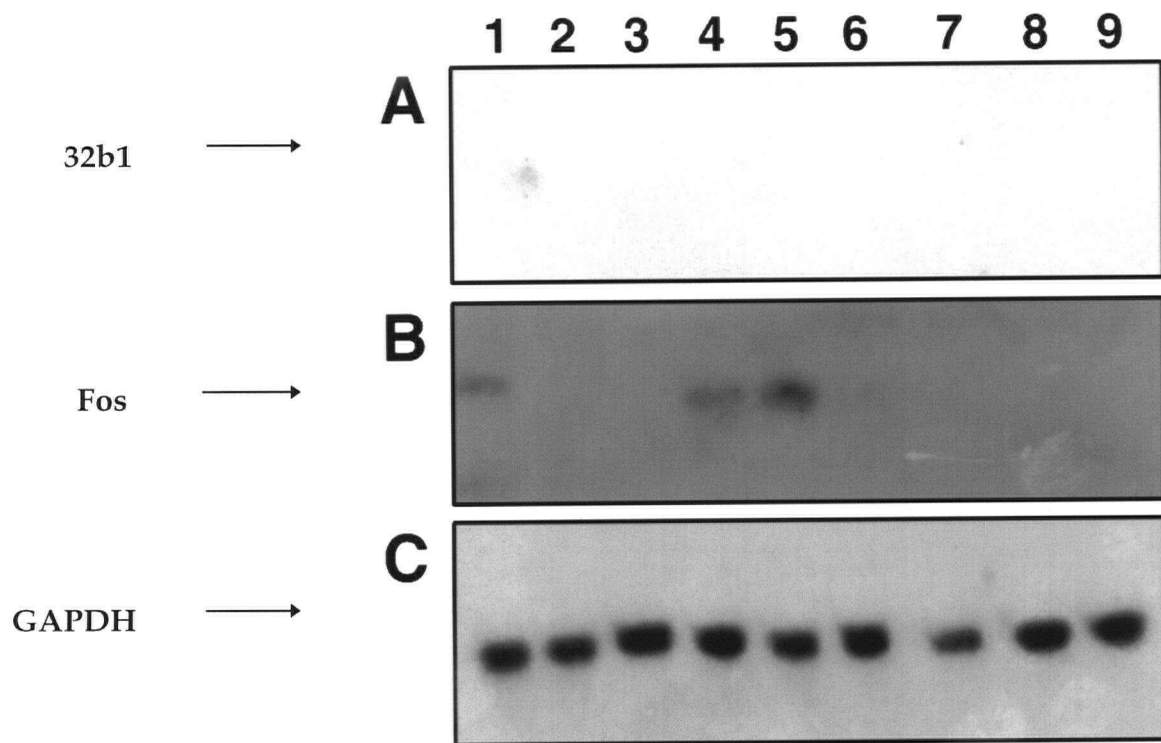
Figure 12. Time Course of induction of 32b1 RNA accumulation in Rat-2 fibroblasts treated with Phorbol 12-myristate 13-acetate (PMA). The Rat-2 fibroblasts were grown in DMEM medium supplemented with 10% fetal bovine serum and 1% antibiotics. They were rendered quiescent after confluency through density arrest and then induced with 100 ηg/ml of PMA (Section 2.7 in Materials and Methods). Cells were harvested at appropriate times and total RNA was extracted (section 2.8, Materials and Methods). Samples containing 20 μg of RNA were denatured, resolved by electrophoresis, and probed with a 32b1 cDNA (section 2.9, Materials and Methods). A, RNA extraction followed PMA addition at the following time points: 1, Quiescent cells (0 min); 2, 15 min; 3, 30 min; 4, 45 min; 5, 1h; 6, 90 min; 7, 2h; 8, 4h; 9, 8h. B, The same Northern blot was stripped and reprobed with a c-fos probe. C, The same blot was stripped and reprobed with a control probe, GAPDH, to check the loading of RNA in each lane.

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c. Induction of 32b1 mRNA in Rat-2 cells with EGF

A time course was done in which Rat-2 cells were treated with 20 ng/ml of EGF at time 0 and RNA was extracted at various time points thereafter. This experiment was attempted in order to obtain a more detailed picture of the kinetics of induction by EGF with a higher number of time points over a shorter period of time (compared to Figure 10). A Northern blot probed with a 32b1 cDNA revealed that EGF induced an RNA of 2.6 kb, which was detectable only 30 minutes after treatment (Figure 13, lane 3).

4. Effect of transcription and translation inhibitors on stability of 32b1 mRNA

a. Time course of decay of 32b1 mRNA in Rat-2 fibroblasts treated with Actinomycin D

In order to estimate the half-life of 32b1 mRNA, Rat-2 fibroblasts were induced with serum for 1 hour and then treated with actinomycin D, an inhibitor of transcription. RNA was extracted subsequently at 30 minute intervals. A 2.6 kb mRNA was detected at the 30 minute time point (Figure 14A, lane 2), as was a smaller species at 60 minutes (Figure 14A, lane 3). No hybridizing species was detectable at 90 minutes (Figure 14A, lane 5).

b. Time course of 32b1 decay in Rat-2 fibroblasts treated with Actonomycin D and Cycloheximide

Cycloheximide, an inhibitor of translation was added with Actinomycin D in order to qualitatively measure the stability of 32b1 mRNA. Cycloheximide was added
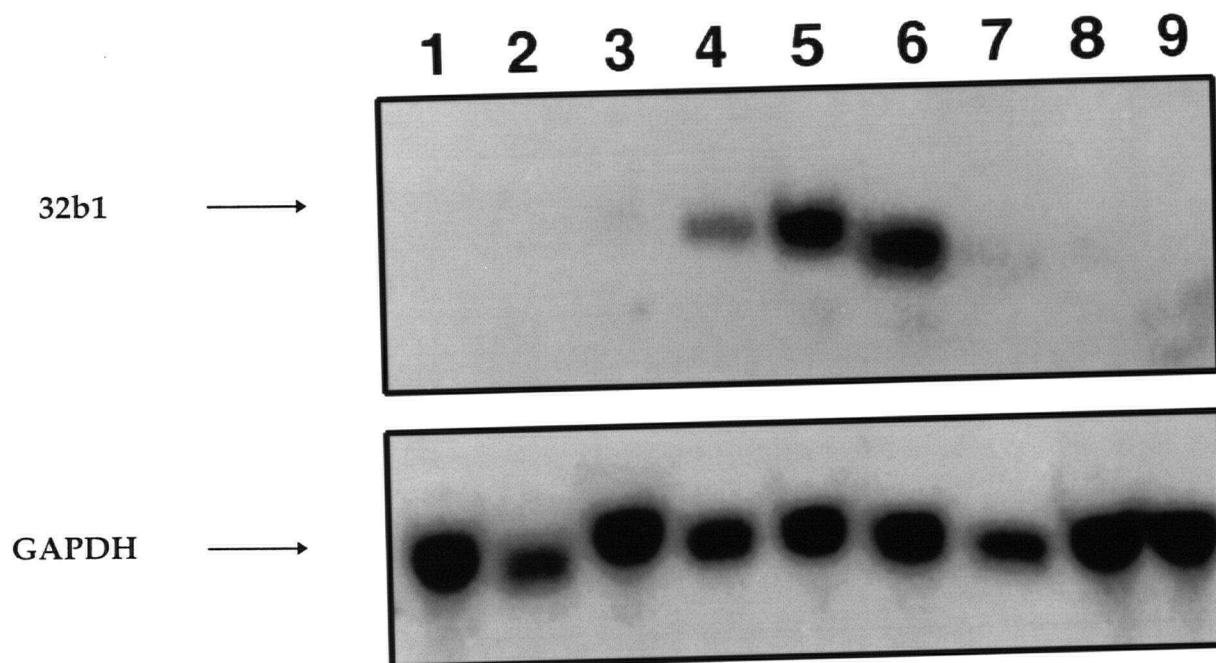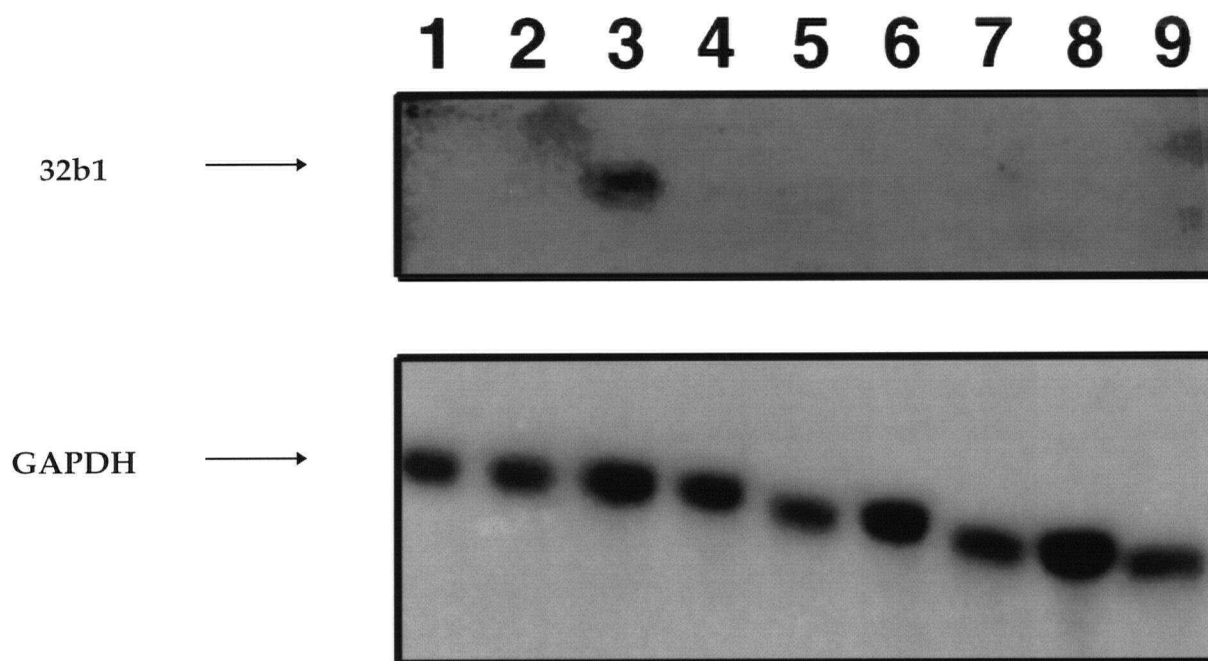
Figure 13. **Time Course of Rat-2 fibroblasts treated with Epidermal Growth Factor (EGF) and probed with 32b1.** The Rat-2 fibroblasts were grown in DMEM media supplemented with 10% Fetal Bovine Serum and 1% antibiotics. They were rendered quiescent after confluency through density arrest and then induced with 20ng/ml EGF (Section 2.7 in Materials and Methods). Cells were harvested at appropriate times and total RNA was extracted (section 2.8, Materials and Methods). Samples containing 20 ug of RNA were denatured, resolved by electrophoresis, and probed with a 32b1 cDNA (section 2.9, Materials and Methods). RNA extraction followed EGF addition at the following time points: 1, Quiescent cells; EGF was added and RNA extraction followed at these time points: 2, 15 min; 3, 30 min; 4, 45 min; 5, 1h; 6, 90 min; 7, 2h; 8, 4h; 9, 8h. The same blot was stripped and reprobed with a control probe, GAPDH, to check the loading of RNA in each lane.
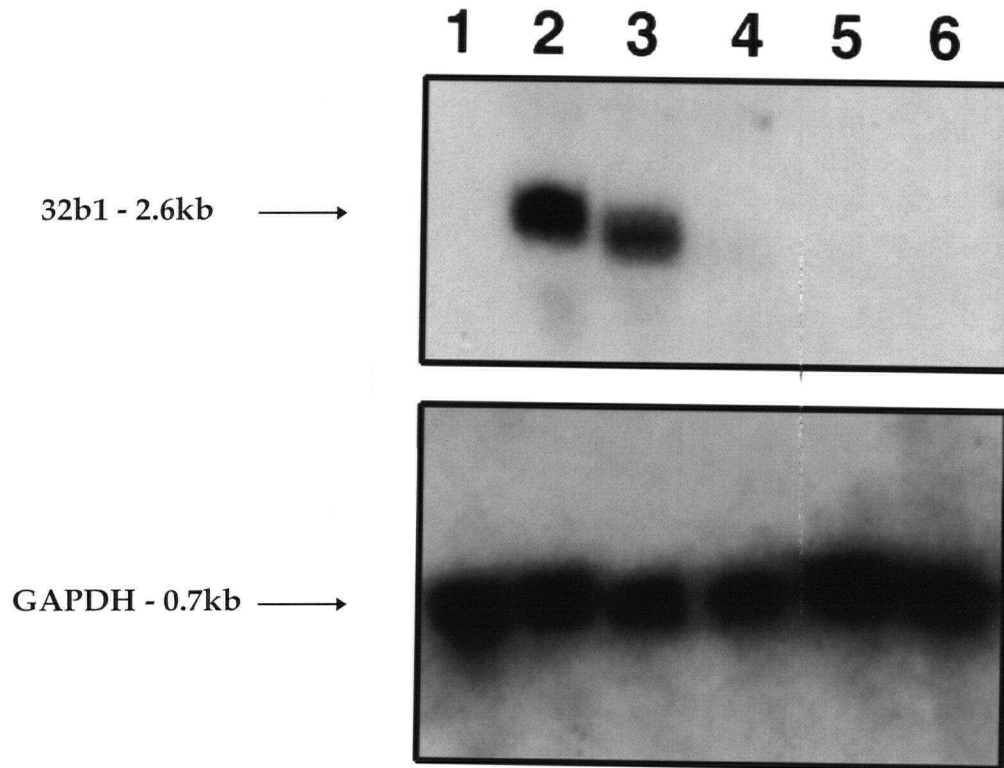
Figure 14A. A time course of decay of 32b1 mRNA in Rat-2 fibroblasts following treatment with Actinomycin D. Rat-2 fibroblasts were rendered quiescent via density arrest and treated with 20% FBS for 1h (Section 2.7 in Materials and Methods). Cells were then treated with actinomycin D (1ng/ml) and samples were taken for RNA extraction (Section 2.8, Materials and Methods). 20 µg of each RNA sample was separated in each lane, transferred to nylon blotting membrane and then probed with a 32b1 cDNA probe (section 2.9, Materials and Methods). Lane 1, quiescent Rat-2 fibroblasts; 2, quiescent Rat-2 cells treated with FBS for 1 hour. The samples in the following lanes were treated with FBS for 1 hour, at which time Actinomycin D was added (time 0) and samples were taken for RNA extraction at the following time points: 3, ActD 30 min; 4, ActD 60 min; 5, ActD 90 min; 6, ActD 120 min . GAPDH was used as a control for equal sample loading.

simultaneously with Actinomycin D in a manner similar to the above experiment. A strong signal was seen 60 minutes after addition of the inhibitors (figure 14B, lane 3). At succeeding times, the signal became fainter but was clearly visible after 2 hours (Figure 14B, lane 5). However, the signal disappeared within eight hours after the inhibitors were added (not shown).

1 2 3 4 5

32b1 ⟶

GAPDH ⟶
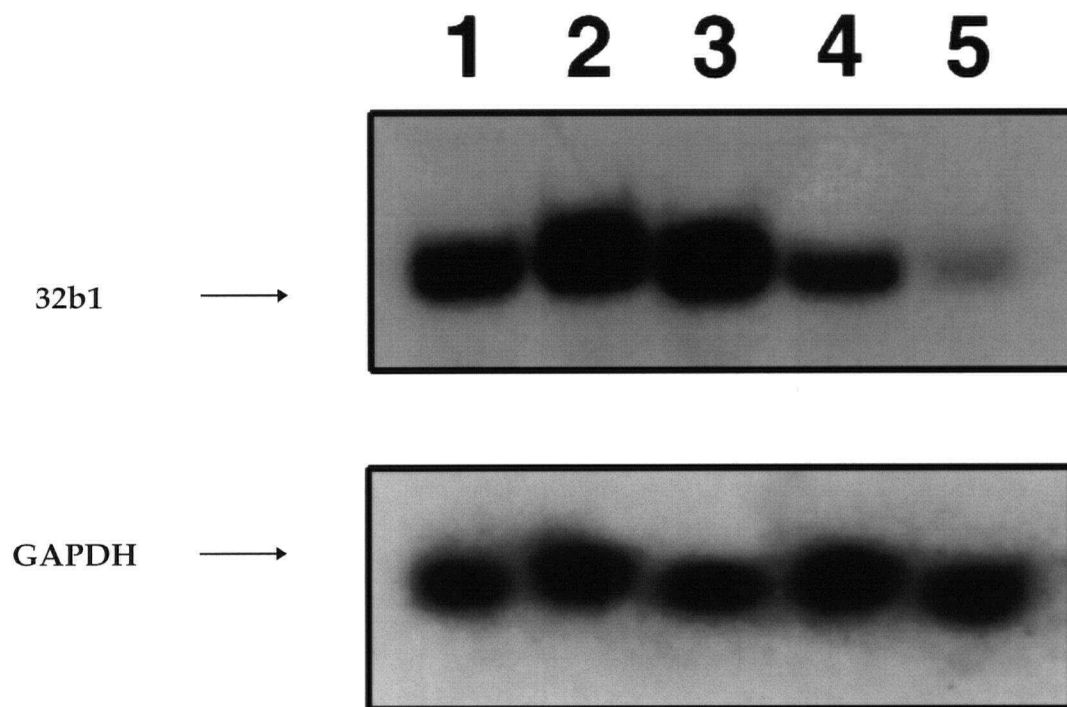
Figure 14B. A Time Course observing 32b1 mRNA hybridization patterns following treatment of Rat-2 fibroblasts with Actinomycin D and Cycloheximide. Rat-2 fibroblasts were rendered quiescent via density arrest and treated with 20% FBS for 1h. Cells were then treated with 1ng/ml actinomycin D (ActD) and 10ng/ml cycloheximide, simultaneously (section 2.7, 2.8 in Materials and Methods). 20 µg of each RNA sample was separated in each lane and probed with a 32b1 cDNA (described in section 2.9, Materials and Methods). Lane 1, quiescent Rat-2 cells induced 1 hour with 10% FBS; 2, ActD & Cycloheximide (Cyclo), 30 min; 3, ActD & Cyclo, 60 min; 4, ActD & Cyclo, 90 min; 5, ActD & Cyclo, 120 min. GAPDH was used as a control.

Discussion

For clarification purposes, I have divided this section into two halves. The first part discusses 32b1 as a cDNA product of its human gene. This will describe conclusions drawn from its coding sequence and its 3′ untranslated region that may predict its induction kinetics. There will also be discussion of information derived from its tissue distribution and lack of expression in various human cell lines seen through Northern hybridization experiments.

This project took an interesting turn when the 5' end of 32b1 could not be found in various libraries derived from human cell lines. The only cell line seen to express the mRNA product after treatment with serum and various growth factors was in another species, a Rat-2 fibroblast cell line. Various time course experiments were done in these Rat-2 cells to produce an induction profile on what is presumably an mRNA transcribed from a rat gene with very high sequence identity with 32b1. As seen in the results, to prevent any confusion, the rat mRNA that hybridizes to the 32b1 probe in Northern blots will be referred to as 32b1R mRNA.

The cross-species hybridization was carried out in Rat-2 fibroblasts because in other cloning projects, the detection of mRNA species under stringent conditions, that is, similar conditions to the Northern blot used with 32b1, led to discovery of new genes encoding cDNA sequences of higher than 90% similarity at the amino acid level (Calalb, M. et al., 1992, Stacker, S. et al., 1993). Further analysis of the 32b1R species was deemed interesting for two reasons. First, it was interesting because it was a transcript novel to this species; secondly, because it allowed comparison of functional differences of two highly similar genes, cyr61 and 32b1R in mouse and rat fibroblasts respectively.

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Any differences in results could later be further analysed through studying any sequence or environmental divergence of the genes and their expression. It was hypothesized that studies in the fiboblasts cell lines that expressed cyr61 and 32b1R would most likely be similar seeing that they were both derived from rodent cells. Although with different cell lines, previous clones have been seen to act in a similar manner after transfection into both rat and mouse fibroblasts, as seen with five cosmids involved in commitment of fibroblasts to adipogenesis (Colon-Teicher, L. et al., 1993). Regardless of the outcome, the results of the comparison may lead to clues to the function of 32b1.

1. What can the DNA sequence of the 32b1 cDNA tell us about the gene products?

The predicted amino acid sequence of the 1.3 kb fragment of 32b1 is 91% identical to cyr61 and contains the high number of cysteine residues in the colinear positions that are characteristic of this family. Unfortunately, because the putative consensus polyadenylation signal (AATAAA) was not found, a part of the 3′ end is still considered missing. In other genes, including cyr61, this putative signal was found within 20bp of the poly A tail (O'Brien et al., 1990; Ryseck et al., 1991). A 49 bp direct repeat was found in the 3′UTR of cyr61 but not in 32b1. This may be due to missing DNA from the 3′ end or simply to its absence in 32b1. The 3′UTR seen in the 32b1 clone is made up of 60% A and T residues and contains many in-frame stop codons. This AU-rich region contains several U-rich regions and three AUUUA pentamers at positions 1148, 1242 and 1259 (see Figure 15). While the pentamers, referred to as AU-Rich Elements (AUREs/AREs), have previously been found to facilitate degradation of

mRNA, nearby U-rich elements aid in mRNA destabilization and deadenylation, the first step in the degradation pathway (Ross, 1995; Chen et al, 1994; Shaw and Kamen, 1986). This is similar to cyr61 which also contains multiple copies of these pentamers (O'Brien et al., 1990). The first two pentamers in 32b1 mRNA are part of an octanucleotide consensus sequence (TTATTTAT) also shown by Shaw and Kamen (1986) to destabilize the GM-CSF mRNA. Thus, it can be predicted that the 32b1 mRNA should be very transient and short lived; my data in Rat-2 fibroblasts confirm this prediction. The portion of 32b1 3'UTR which may aid in conferring instability is shown underlined in Figure 15. As well, in the 3' UTR of 32b1, there were two copies of a 6mer, TTTGTA, and one 7mer, TTTATA, which were previously found to be responsible, together with a 5' element, for serum inducibility in immediate early genes (Freter et al., 1992). It is a feature commonly found in certain types of IEGs such as trancription factors, for example c-fos and c-myc, but not in the cytoplasmic signal transduction proteins, structural proteins or cell surface receptors (Freter et al., 1992). These results provide evidence suggesting that 32b1 gene is immediate early. The bolded italic regions in Fig. 15 represent these sequences.

2. What does the novel 5' sequence tell us?

The significance of the novel 32b1 sequence lies in the circumstances under which it was found. The partial cDNA, z50168, was discovered during a subtractive hybridization search for proteins downregulated in skeletal muscle tumors referred to as rhabdomyosarcomas (RMS) (Genini, M. et al., 1996). These small-cell tumors are insufficiently differentiated into muscle cells. Since z50168 (the 5' end of 32b1) was

```
 600  TTTAGGGACTAAATTGCCTACCTGGGTTTCCAGGGCACACCTAGACAAACAAGGGAGAAG   659
 201  PheArgAsp***                                                 203

 660  AGTGTCAGAATCAGAATCATGGAGAAAATGGGCGGGGGTGGTGTGGGTGATGGGACTCAT   719

 720  TGTAGAAAGGAAGCCTTGCTCATTCTTGAGGAGCATTAAGGTATTTCGAAACTGCCAAGG   779

 780  GTGCTGGTGCGGATGGACACTAATGCAGCCACGATTGGAGAATACTTGCTTCATAGTATT   839

 840  GGAGCACATGTTACTGCTTCATTTTGGAGCTTGTGGAGTTGATGACTTCTGTTTTCTGTT   899

 900  TGTAAATTATTTGCTAAGCATATTTTCTCTAGGCTTTTTTCCTTTTGGGGTTCTACAGTC   959

 960  GTAAAAGAGATAATAAGATTAGTTGGACAGTTTAAAGCTTTTATTCGTCCTTTGACAAAA  1019

1020  GTAAATGGGAGGGCATTCCATCCCTTCCTGAAGGGGGACACTCCATGGAGTGTCTGTGAG  1079

1080  ACGGCAGTCTATCTGCACTCTAAACTAAACTGCAAACAGAAATCAGGTGTTTTAAGACTG  1139

1140  AATGTTTTATTTATCAAAATGTAGCTTTTGGGGAGGGAGGGGAAATGTAATACTGGAATA  1199

1200  ATTTGTAAATGATTTTAATTTTATATCAGTGAAAGATTTTATTTATGGAATTAACCATT  1259

1260  TAATACCCGAATTCC                                              1274
```

Figure 15 Identified elements found in the 3′ UTR of 32b1. The underlined bases at positions 1148, 1242 and 1259 are thought to confer instability to the mRNA message (Shaw and Kamen, 1986). The bolded, italic sequences at positions 897, 1201 and 1219 were identified by Freter et al. (1992) as elements characteristic of immediate early genes.

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found in normal cells but was absent in the tumors, these results suggest that 32b1 plays a significant role in differentiation, perhaps as a tumor suppressor gene.

It was also important to look for clues to 32b1's function in the other cells from which it was isolated. The many partial 32b1 sequences derived from the WashU-Merck EST project revealed the gene to be expressed in adult female uterus (AA044451, Hillier,L. et al., 1995), normal lung tissue from a 72 year old male (T92167, Hiller, l. et al., 1995), and human fibroblasts (W51901, Hillier, L. et al., 1995). 32b1 was also isolated in different projects from the fetal heart (N86328, Liew, C, 1996) and from benign tissue of prostate cancer patients (Y09859, Pilarsky, C. et al., 1996). This was especially interesting because the reference, although not yet published, also refers to 32b1 as a partial cDNA sequence (226bp) which codes for a putative tumor suppressor gene for prostate cancer (Pilarsky, C. et al., 1996). This evidence strongly suggests that 32b1 plays an important regulatory function in humans, consistent with the evidence from other family members.

3. Why was the search for the 5′ end of 32b1 unsuccessful?

There are many possible explanations why the 5′ end could not be found in the various cDNA libraries looked at. The libraries searched were several years old and the missing fragment could have been simply lost over time. It was also conceivable that the library became contaminated with 32b1 and that it was not normally present in this source. Since 32b1 encodes a novel sequence, another possibility is that it may only be found as this 1.3 kb fragment in these particular cell lines.

The lack of detection seen in the Northern blots using RNA from leukocytic cell lines implies that 32b1 mRNA is an extremely rare transcript. Northern blot analysis requires approximately 1×10^{7} target transcripts in order to be detectable on the gel (Hengen, 1995). These results were supported by the lack of expression seen in blood leukocytes on the human tissue blot (Figure 9 seen below). It is interesting to note that PMA failed to induce 32b1R expression in Rat-2 fibroblasts as well as in the human blood leukocyte derived cell lines tested. Perhaps a different mitogen may induce 32b1 or it may simply not be expressed in these cell lines. Another possible reason for lack of induction in these cell lines may be because they are oncogenic in nature. They are less dependent on serum for cell growth, therefore they may continue to proliferate after serum starvation in which case they would be less responsive to addition of serum (Yu et al, 1993). It is also important to note that the PCR fragments cloned were all truncated to the same nucleotide. This gene could have a secondary structure that does not allow a reverse transcriptase to read through the mRNA. This would yield a truncated message such as the original 32b1 clone because most of the isolation methods attempted were based on reverse transcription.

Other researchers have found similar results where a cDNA could be isolated using PCR but not with Northern blot hybridization (Ashworth, 1993). They concluded that the isolation of a PCR product from a particular cDNA sample does not necessarily indicate that this gene is significantly expressed in that tissue. Rather the extreme sensitivity of PCR led to its isolation and Northern blotting should be used to confirm its meaningful expression (Ashworth, 1993).

4. What does the human tissue distribution of 32b1 tell us when compared with other CCN family members?

The human 32b1 mRNA was detected only in a purchased tissue distribution blot. Here, the probe 32b1 hybridized to two transcripts: a smaller 2.6kb band and a 4.4kb fragment. A similar larger RNA has been seen in tissue blots with other transcripts such as Gab1, where the larger transcript was thought to be representative of alternative splicing or to be derived from a related gene (Holgado-Madruga, 1996). As seen in Table 2, most of the other family members were strongly expressed in the lung but the purchased Northern tissue blot did not have lung so no comparison could be made. Cyr61, the mouse homologue displays a different tissue distribution than 32b1. There were only 4 common tissues probed with cyr61 (O'Brien et al, 1990): both messages were expressed in the ovary and testis; but while 32b1 was expressed in the small intestine and spleen, cyr61 was not. As mentioned above, no message was detected in the peripheral blood leukocytes. The expression found in the thymus, which contains a high number of immature T cells, correlated with this result by having a weaker expression relative to the other tissues. Northern blot analyses also echoed this result when the blood leukocyte cell lines, such as Jurkat T cells, U937 cells, and HL-60 cells, tested did not express the 32b1 transcript even after treatment with various agents. Information about the tissues that express 32b1 will eventually lead to clues about its general and tissue-specific functions in comparison to other CCN family members.

Table 2. Tissue distribution of 32b1 and various other CCN family members. The information compiled in this table was derived from a 32b1 probed Northern blot of Human tissue and from published literature. Low, lowest, moderate (mod), high, highest and undetectable (undetect) concentrations of mRNA expression seen were indicated with respect to the tissues tested for each family member. "-- --" represents data not available.

Tissues	32b1	cyr61	CEF-10	fisp-12	nov
spleen	mod	undetect	low	mod	low
thymus	low	-- --	-- --	undetect	-- --
prostate	mod	-- --	-- --	-- --	-- --
testis	mod	low	-- --	lowest	-- --
ovary	high	low	-- --	-- --	-- --
intestine	mod	undetect	low	undetect	-- --
colon	high	-- --	-- --	-- --	-- --
blood leukocyte	undetect	-- --	-- --	-- --	-- --
heart	-- --	mod	undetect	mod	undetect
kidney	-- --	low	low	mod	v. low
liver	-- --	undetect	undetect	undetect	undetect
adrenal gland	-- --	low	-- --	-- --	-- --
brain	-- --	low	low	mod	mod
uterus	-- --	mod	-- --	-- --	-- --
muscle	-- --	mod	undetect	-- --	undetect
pancreas	-- --	undetect	-- --	-- --	-- --
lung	-- --	highest	highest	highest	highest
bursa	-- --	-- --	low	-- --	-- --
stomach	-- --	-- --	low	-- --	-- --

5. Is 32b1 the human homologue of cyr61?

In order to determine whether 32b1 is the human homologue of cyr61, it is first important to clearly define the word "homology". The word has acquired numerous different meanings over the years that have caused much confusion in the scientific literature when used in comparisons between different species. In biological terms, homology has traditionally been defined as "having a common evolutionary origin" but in recent years, it has also been loosely used in the context of "possessing similarity or being matched" (Reeck et al., 1987). To prevent confusion, it has been suggested by various researchers that the term 'homology' used in this context be replaced by the terms "similarity","identity", "isology" or not used at all (Reeck, 1987; Dover, 1987; Aboitiz, 1987; Wegnez, 1987).

With respect to biological evolution, homology itself is a quality. A gene is either homologous or not. At present, it can only be hypothesized that the species compared have a common ancestor. The protein product of the homologous genes may not serve the same function or be similarly regulated depending on its environment. Examining sequence similarity or identity between the two genes can help determine the probability that the two genes are homologus. In the case of the genes coding for 32b1 and cyr61, with 91% sequence similarity, the real question is, what is the likelihood that these two genes became this similar from two divergent genes. The convergence of two sequences can happen in two different ways; first, two different proteins may be trying to fulfill the same function in similar cellular environments. The second way may be through genomic changes, that is, genes become crossed over, have point mutations, etc. to arrive at similar sequences. It is highly unlikely that through these two ways, a

gene would converge to have such high sequence similarity (Dover, 1987). Therefore, there is a high probability that 32b1 is the human homologue of cyr61.

The cross-species hybridization of the 32b1 probe to an mRNA fragment found in Rat-2 cells under highly stringent washing conditions implies that there is also a high sequence similarity between 32b1 and 32b1R. Based on the arguments seen above, it is also reasonable to assume that 32b1R is also the rat homologue of cyr61.

Despite high sequence similarity, these genes do not seem to be functional homologues. Comparison of the induction kinetic profiles of 32b1R with its mouse homologue cyr61 indicate that the mRNA products of both genes function at different times of the cell cycle. The mRNA product of 32b1R is very transient when induced by serum or EGF while the expression of cyr61 following serum stimulation continues for up to 8 hours. Cyr61 is also induced by phorbol esters while 32b1R does not respond to PMA stimulation. Perhaps the fact that 32b1 is lacking the 49 bp repeat found in the cyr61 3'UTR contributes to the differences seen in their induction patterns. These regulatory differences are also seen when other family members are compared (Kireeva et al., 1996, Xin et al., 1996). Cellular environment may play a role in the induction of mRNA expression of the two genes causing them to become differentially induced but from the results obtained here, cyr61 and 32b1R likely perform different functions in the cell lines in which they are expressed.

6. What can be learned about the induction kinetics of 32b1R after stimulation with various mitogens?

In comparing the induction kinetics of 32b1R with similar experiments done for other CCN family members, it must be noted that minor differences in conditions may cause a varied response in gene expression. Methods used to render the cells quiescent have an impact on the immediate early response of the fibroblasts to various growth factors and cytokines (Jahner et al, 1991a). As well, immortal cells and primary cells do not carry out completely normal cell functions. The age and type of cell line used in experiments may also give various results depending on the length of the cell cycle and the proteins expressed. Finally, it is vital to remember that the majority of the experiments are done using cDNA from a human gene to probe mRNA extracted from a Rat-2 fibroblast cell line. The results may not reflect how 32b1 is expressed in humans.

The primary reason for doing the three induction time course experiments was to establish a context in which the gene is induced and the length of time it is expressed. From our knowledge of events that follow treatment with these agents, one can infer the distinct signal transduction pathways that involve the transcription of 32b1R in Rat-2 fibroblasts.

The mRNA of 32b1R was expressed after serum and EGF, but not PMA, was added to quiescent cells. These results indicate that there may be a serum response element (SRE) element present in the promoter of 32b1R, as seen with cyr61 (Latinkic et al, 1991). This element binds to the serum response factor (SRF) and plays an important role in the serum induction and transcriptional repression of c-fos (Latinkic et al, 1991). The induction kinetics of the 32b1R mRNA and c-fos are similar in their transient

appearance but in contrast, cyr61 transcription is mantained for 8 hours following serum induction (Latinkic et al, 1991). Other factors, such as cell culture environment or the presence of a regulatory factor which inhibits or induces mRNA transcription, must play a role in serum induced expression to exhibit the different kinetics.

One growth factor present in serum is EGF which is needed for growth by almost all cells in culture. EGF activates 32b1R in a manner which is more transient than expression seen with serum which implies that serum contains additional regulatory proteins that also play a role in induction. EGF is an activator of a receptor tyrosine kinase (RTK) which couples to both the phosphatidylinositol pathway, leading to activation of the Protein Kinase C (PKC) and an increase in the intracellular (Ca^{2+}) concentration (Boonstra, 1995), as well as to the Ras pathway which sends messages to the nucleus via the MAP kinases (Karnitz, 1995). To test the role of the PKC and calcium concentration in the induction of 32b1R and for comparison to other family members, quiescent Rat-2 cells were treated with a phorbol ester PMA. The main biological response to treatment with PMA is the induction of the phospholipid dependent Protein Kinase C (PKC) pathway by substituting for DAG (Darnell et al, 1990). The effect of PMA addition on the expression of 32b1R was very important because CEF-10 and cyr61, the chicken and mouse homologue displayed different patterns of induction. Unexpectedly, 32b1R was uninduced by phorbol esters. The validity of the experiment was further tested by probing the same Northern blot with c-fos, a gene known to be phorbol ester inducible. Its pattern of induction was the same as previously published (Huang et al, 1995; Bi et al, 1994; Borner et al, 1995). Because the phosphatidylinositol pathway failed to induce 32b1 mRNA transcription, it is most

likely that EGF induces 32b1R through the Ras pathway or through another pathway that has not been previously described.

7. What is the effect of inhibition of protein synthesis and translation on the stability of the immediate early gene 32b1R?

Actinomycin D was used to shut off transcription to qualitatively estimate the half life of 32b1. Although a higher frequency of time points would have given a more accurate estimation, the half-life of 32b1 was approximated to be less than 30 minutes. As well, cycloheximide was used in conjunction with actinomycin D in order to test the effect of translation on the stability of the mRNA transcript. The superinducing effect of cycloheximide may be due to two independent factors, an increase in stability of the mRNA message and a prolonged transcription of the genes. The ability of cycloheximide to stabilize the 32b1 mRNA message suggests one of two things: either a newly synthesized or labile nuclease is required for degradation or inhibition of the mRNA transcript is linked to translation (Lau and Nathans, 1991). In the latter circumstance, perhaps there is a site that must be translated in order to initiate the mRNA degradation process. Treatment with cycloheximide increased the stability of the 32b1 message at least 3-fold. Previous studies with c-myc revealed that mammalian cells treated with cycloheximide extend mRNA expression due to a retardation of deadenylation ability but the actual degradation after this primary step is not affected (Ross, 1995). From the experiments done with 32b1 and assuming the smaller band seen just prior to disappearance of the hybridizing band is due to deadenylation, the results do not concur with those from c-myc. Although the deadenylation step does

take a longer period to complete, the time required for the degradation step is also considerably lengthened. This supports a possibility that either the smaller band seen is not due to deadenylation or there are other factors influencing the stability of the 32b1 mRNA message besides translation. These results confirm that translation plays an important role in increasing the stability of the message.

8. Is 32b1R an immediate early gene?

Immediate early genes (IEGs) derive their name from their analogy with primary transcriptional response of DNA viruses (Lau and Nathans, 1987). A gene is referred to as being " immediate early " when it meets the three defining criteria previously discussed in the Literature Review (Lau and Nathans, 1991). Based on these requirements, 32b1R is most likely an immediate early gene meeting two of the three characteristics. Besides being induced immediately after stimulation with various mitogens without de novo protein synthesis, the 32b1R mRNA is a rare transcript in control cells and becomes superinduced in the presence of transcription inhibitors such as cycloheximide; these are all general traits of IE genes (Simmons et al, 1989). The 32b1R mRNA species induced by different agents is expressed in a very transient manner and has a very short half-life of less than 30 minutes, estimated using Act D, consistent with the behavior of group I members of the immediate early gene group (Lau and Nathans, 1991; Lau and Nathans, 1987). The final experiment required in order to prove without a doubt that 32b1R is an immediate early gene would be a nuclear run-on assay. The results from this experiment would indicate if the gene is transcriptionally activated as a result of mitogen stimulation or simply stabilized.

9. What is the likelihood that 32b1 is RBF-1?

Although further studies must be done in order to fully understand 32b1's role in the cell, there are many results that support the probability that 32b1 is not RBF-1. The RBF-1 and RBF-2 factors are thought to be required for Ha-Ras responsiveness in Jurkat T lymphocytes, with or without PMA induction, and in macrophages. From electrophoretic mobility shift assays (EMSAs) of the mutated RBE binding sites done with Jurkat nuclear extracts, it was determined which areas were important for RBF binding in response to Ras induction (Bell, B. et al., unpublished). The major contradictory result found for 32b1 was that it appeared to be a very rare transcript in this same Jurkat T lymphocyte cell line, and in most blood leukocytes, seen through lack of detection in Northern blots. Perhaps 32b1 was isolated in the Southwestern blot screening because it contained many modules for binding and oligomerization (Bork, 1993). Results seem to suggest that 32b1 and its family members play a role in the mediation of cell proliferation and wound repair.

10. A model for 32b1

Experimental results with 32b1 were unexpected because the 5' end could never be found in the Jurkat T cell line from which it was originally isolated. The cDNA was repeatedly isolated from blood leukocyte derived cell lines using PCR but never detected in Northern blot analysis. This implies that the 32b1 gene is not significantly expressed here. The numerous times that 32b1 was isolated during PCR in both Jurkat T cells and U937 cells treated with PMA for 3.5 days suggests that it is not likely to be a

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coincidence. In gathering clues from the accumulated results, it seems that in these cell lines, 32b1 could only be found in this 1.3 kb length as a rare transcript without the 5′ end seen in its mouse homologue, cyr61. The 32b1 transcript is encoded by a gene with homology with the CCN family which members are important in mediating cell differentiation. The FASTA results indicate that 32b1 may be a tumor suppressor gene, from the findings of two separate research groups where 32b1 was found in normal cells but not in tumors (Genini et al., 1996, Pilarsky et al., 1996). This also supports a regulatory function for 32b1. It has been previously found in nov, another CCN family member, that there is a regulatory function in the amino terminus of the protein. Full length nov negatively regulates cell growth but an amino truncated version can transform CEF cells (Joliot et al., 1992). An interesting hypothesis may be that since the Jurkat T cell line is oncogenic and 32b1 is a tumor suppressor gene, perhaps 32b1 is similar to nov in that its regulatory portion is found in the amino terminus while the carboxy terminus, without this control, contributes to oncogenicity. The 5' end of 32b1 could be absent in these leukocyte cell lines because this truncated version of 32b1 contributes to the uncontrolled growth in these cells albeit at low levels. This would explain the absence of the 5' end.

A model for 32b1 function may be that it functions in a similar way to cyr61 because of their high sequence similarity and any differences seen here may be due to cellular environment. In this case, 32b1 may act as an extracellular matrix molecule as well as a tumor suppressor gene. 32b1 would be lacking its regulatory domain in the amino terminus and may actually be signaling cell proliferation in an uncontrolled manner as a signaling molecule bound to the extracellular matrix causing cancerous

growth. Under normal circumstances, the 5' end of 32b1 would regulate the signaling function of 32b1 and promote cell proliferation in a controlled manner, perhaps through the binding of an insulin-like growth factor with domain I of its protein which has sequence similarity with IBPs.

11. Where do we go next?

The induction kinetics profile of 32b1R hybridizing with a human cDNA probe has left some interesting hypotheses to be tested. At this point, 32b1 and 32b1R should be separated into two distinct sets of experiments and eventually converged to allow further speculation on the homologous background of the two genes.

In studying 32b1, the human cDNA clone, the first step would be to find a human cell line that expresses the mRNA transcript. Because some of the CCN families were found in fibroblasts, a human fibroblast cell line may be a reasonable place to start. Before wasting time on looking in a library for a very rare species, induce quiescent cells with serum and extract RNA in a time course experiment. A Northern blot of the various time points using 32b1 as a probe will allow estimation of when (or if) 32b1 is expressed. From here, RACE can be used on that RNA sample to isolate the 5' end of 32b1, or alternatively a library can be made derived from RNA extracted at the time point where 32b1 is expressed at its highest level. This library can then be searched using Touchdown PCR or conventional screening methods. Once the full length clone is found, then its induction kinetics can be studied in that particular cell line. It can also be used to probe previously tested Rat-2 Northern time courses or to see if a different induction pattern is seen. It can be further tested by doing serum induction

experiments in the 3T3 mouse fibroblast cell line to see if it reacts similarly to cyr61. That is, is the difference in induction kinetics and tissue expression a function of the cellular environment? If dissimilar, further studies could be done to find any significant differences in the two gene products that may play a role in serum induction kinetics. Some interesting experiments that would answer some questions that have arisen in comparison with other family members would be a Northern blot testing the tissue distibution of 32b1 mRNA in human lung relative to other tissues. As seen with many other CCN members, is it most highly expressed in the lung?

Another extremely interesting experiment would be to repeat a similar experiment to the one described for nov where its 5' truncated transcript was tested to see if it induced transformation in cells. This would be interesting in the leukocyte cell lines where the truncated cDNA clone was only found missing the 5' end.

With respect to 32b1R, it would also be important to return to the Rat-2 fibroblast cell line and find the rat homologue of 32b1. Since we know where and when it is expressed, it would seem like a simple task to return to the RNA extractions expressing 32b1R in Northern blots and use the techniques discussed in this thesis to find the full-length clone. Another important experiment would be to fully define 32b1R as an immediate early gene by doing a nuclear run-on assay to confirm that transcription is activated during mitogen stimulation. For other comparisons which may give clues to the function and regulation of 32b1R, further time course experiments using other growth factors such as fibroblast growth factor (FGF), Platelet derived growth factor (PDGF) and Transforming growth factor beta (TGF-β) could be carried out. DNA

sequencing of both cDNA clones will allow further comparison and would be seen as the discovery of two novel members of the CCN family.

Conclusion

The fact that these "CCN" family members are found in a wide range of species implies their importance in regulating cell proliferation. Many of the genes were discovered in fibroblasts, cells involved in this function and were seen to be induced by growth factors, such as TGF-β, PDGF and EGF that have been previously discovered to play a significant role in wound repair. Although there have been many results supporting this general function for CCN family members, the specific members when compared in similar experiments display completely different regulation as well as different roles in cell differentiation. Cyr61 has been hypothesized as an extracellular matrix signalling molecule, CTGF is a growth factor, nov is a negative regulator for growth and 32b1 as a tumor suppressor. This is a relatively new group of immediate early genes on which further study must be carried out, especially to determine the significance of the 38 aligned cysteines in their amino acid structure and their specific roles in vivo. During this term of research on 32b1, two novel CCN family members were discovered. 32b1, discovered from human Jurkat T lymphocytes, and another 32b1 homologue in Rat-2 fibroblasts (32b1R). Although the complete 32b1 gene was never found, much information was found with respect to its induction patterns and RNA stability which may help in further research in the future.

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