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Transcriptional Regulation of the Tissue Inhibitors of Metalloproteinases

By

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Abstract

The Tissue Inhibitors of Metalloproteinases-1 (*Timp-1*) gene is important in modulating the net proteolytic balance within the ECM. Typically present at low levels, transcriptional control enables the rapid increase in *Timp-1* levels following growth factor and cytokine stimulation in many situations where ECM remodelling is required. The key regulatory element involved in both basal and induced promoter activity is a non-consensus AP-1 binding site (TGAGTAA) located at –63 bases upstream of the most 5'- transcription start site. This binding site, which differs by a single base from the consensus AP-1 site (TGAGTCA) is conserved in all the *Timp-1* genes identified from rodents, humans, and horse (Richardson and Dodge, 1998). I have shown that the base change imparts to the region the ability to interact with a single-stranded DNA-binding protein, termed here ssT1. The ssT1 factor also interacts with an upstream region of the promoter, -115/-100, and is important for regulating the basal activity of the promoter from both these sites. This data make the ssT1 an important regulator of overall *Timp-1* transcription levels.

In addition to these studies on *Timp-1*, I performed the initial promoter analysis of the recently identified *Timp-4* promoter. Using RACE-PCR, three putative transcription start points were identified. A deletion analysis of the upstream promoter region has shown several regions important in trancriptional regulation. First is a region in close proximity to the putative transcription start points sites, which has a positive regulatory function. In addition, at least two elements are located in regions far upstream which were able to enhance expression from another promoter. Overall, these studies have contributed to the characterization of transcriptional regulatory mechanisms governing *Timp* expression. These data are highly relevant towards our understanding of pathological situations that arise from ECM dysfunctions in which TIMPs are involved, such as arthritis, fibrosis, and cancer invasion and metastasis.

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Abbreviations

ADAM - a disintegrin and metalloproteinase domain
AMV - avian myeloblastosis virus
AP1 - activating protein 1
BSA - bovine serum albumin
cAMP - cyclic adenosine monophosphate
CAT - chloramphenical acetyl transferase
CBFA - core binding factor 1
CEprMss - coding element-purine-rich motif, single-stranded
CMC - cytomegalovirus
CRE - cyclic AMP response element
DIG - digoxygenin
DMF - dimethyl formamide
DMEM - Dulbecco's modified eagle's medium
DNA - deoxyribonucleic acid
dNTP - deoxynucleotide tri-phosphates
DTT - dithiothreitol
ECM - extracellular matrix
EDTA - ethylene-diaminetetraacetic acid
EGF - epidermal growth factor
EGTA - ethyleneglycol bis-N,N,N',N'-tetra acetic acid
ELISA - enzyme-linked immunosorbant assay
EMSA - electromobility shift assay
EPA - erythroid potentiating activity
FAK - focal adhesion kinase
FBS - fetal bovine serum
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
GFP - green fluorescent protein
GITC - guanidinium isothiocyanate
GM-CSF - granolocyte macrophage - colony stimulating factor
HCl - hydrochloric acid
HGF - hepatocyte growth factor
IL - interleukin
ILK - integrin linked kinase
LB - Luria Bertani
LBP - leader binding protein
MAPK - mitogen activated protein kinase
MF-3 - muscle factor 3
MMLV-RT
MMP - matrix metalloproteinases
MT-MMP - membrane-type matrix metalloproteinases
NFκB - nuclear factor κB
OSM - oncostatin M
PAGE - polyacrylamide gel electrophoresis
PAI - plasminogen activator inhibitor

PBS - phosphate buffered saline
PDGF - platelet derived growth factor
PEA3 - polyoma element activator 3
PMA - phorbal myristate acetate
RA - retinoic acid
RPM - revolutions per minute
SCID - severe combined immunodeficient
SDS - sodium dodecyl sulphate
SH - Src homology
SSC - sodium citrate-sodium chloride
ssT1 - single-stranded Timp-1 promoter binding protein
TACE - TNFα-converting enzyme
TAE - tris-acetic acid-EDTA buffer
TE - Tris-EDTA
TGF - transforming growth factor
TIE - TGFβ-inhibitory element
TIMP - Tissue Inhibitor of Metalloproteinases (protein)
Timp-1 - Tissue Inhibitor of Metalloproteinases-1 (murine gene)
TIMP1 - Tissue Inhibitor of Metalloproteinases-1 (human gene)
TNF - tumour necrosis factor
tPA - tissue plasminogen activator
TPA also known as PMA) - phorbol myristate acetate
uPA - urokinase plasminogen activator
uPAR - urokinase plasminogen activator receptor
WT - wild-type

Chapter 1 Introduction

1.1 Overview

The extracellular matrix is a dynamic structure which is in a constant state of both synthesis and degradation. Whether the matrix increases or decreases in bulk depends upon which of the opposed forces are dominant. The matrix metalloproteinases are the principal family of ECM degrading proteinases, and are capable of degrading all of the ECM components. This makes the ECM family important in cases where rapid ECM remodelling is required, as well as very hazardous, when the MMPs are abnormally expressed, resulting in a number of diseases. Because of this destructive potential, the MMPs are strictly regulated at three levels, transcriptional control, activation of latent proenzymes, and finally, through interaction with a specific family of inhibitors, called the tissue inhibitors of metalloproteinases (TIMPs).

There are four members of the TIMP gene family (TIMP-1 through -4), and each function by interacting with the MMP active site, therefore, removing the enzymatic potential of the protease. The TIMPs therefore represent the last mechanism in place to prevent the unwanted or unnecessary destruction of the ECM. Because the MMP/TIMP interaction occurs with 1:1 stoichiometry, the levels of each respective protein is important for the overall activity of the MMPs. An analysis of the regulatory mechanisms leading to the expression levels of both active MMPs, and TIMPs is therefore warranted.

TIMP-1, the prototypic MMP inhibitor, is inducible by many different types of stimuli, and has been shown to be upregulated in a number of pathologies. The principal mechanism of regulation has been shown to be transcriptional, and TIMP-1 has many different regions which contain cis-acting regulatory elements. This thesis aims to

analyze the transcriptional mechanisms in which the murine Timp-1 gene is regulated. In addition, I also performed the first experiments to identify the potential Timp-4 promoter, and map out key transcriptional regulatory elements.

1.2 The Extracellular Matrix

The extracellular matrix (ECM) is a latticework which provides support for cells and tissues. It provides the appropriate cellular microenvironment for diverse cellular functions, such as adhesion, migration, and proliferation. The ECM is composed of a meshwork of many different types of molecules: proteins, proteoglycans, and glycoproteins. The principal component of the ECM is collagen, which is well suited for its supportive role by providing tensile strength to the ECM. Additional contributions are provided by other molecules such as elastin and proteoglycan, which contribute to the properties of elasticity and bulk respectively (Mosher et al., 1992). Once thought to perform a role mainly in cellular support, it is now known that the ECM is also involved in a diverse array of different functions, including signalling, adhesion, motility, and cell growth.

The basement membrane is a specialized form of the ECM which forms a physiological, semi-permeable barrier between organs and organ systems (Yurchenco and O'Rear, 1994), and is composed mainly of collagen type IV, heparan sulphate proteoglycan, laminin, entactin, and osteonectin (Fox et al., 1991; Yurchenko et al., 1992). The basement membrane is important in the function of many tissues, and is especially evident in the circulatory system, which depends upon the basement membrane for development as well as support.

Cell-cell communication and signalling has more recently been established as a function of the ECM, and occurs through several different mechanisms. The ECM is capable of immobilization of ligands, after which they can either be sequestered from, or presented to their appropriate cellular receptors, as examples of both positive and negative control of signalling (Keifer et al., 1991; Raines et al., 1992). The ECM could also bind factors and modify their activity, or affect their interaction with receptors (Damsky and Werb, 1992; Klagsbrun and Baird, 1991). Additionally, specific components of the ECM itself are important agents in signalling through interaction with both integrin and non-integrin receptors (Schwartz et al., 1995).

The integrins are a family of heterodimeric transmembrane receptors which are important in cell-cell adhesion and attachment of cells to the ECM, largely through the formation of the focal adhesion complex (Schwartz et al., 1995). The focal adhesion complex plays a critical role in allowing the ECM to communicate with the cytoskeleton and to help direct cell-cell interactions and motility in addition to cellular signalling (Guan et al., 1991; Hanks and Polte, 1997). This can be seen in the cytoskeletal reorganization that occurs upon cell-ECM attachment (Schaller et al., 1992), as well as the recruitment of specific intracellular kinases such as ILK and FAK to the focal adhesion complex, followed by the activation of signals that regulate gene expression.

Because of the diverse functions of the ECM, and the possibility for disorders to develop when it is not controlled, the regulation of the ECM is of paramount importance. This regulation occurs by the delicate balance between synthesis and degradation of the individual components. A variety of different cell types are able to express and secrete

the ECM proteins, and are regulated by various intra and extracellular cues, some even originating within the extracellular matrix itself.

Alternatively, the levels of the various components of the ECM are reduced or cleared through proteolytic degradation. This function is performed primarily by three families of proteases; serine proteases, cysteine proteases, and the matrix metalloproteinases.

1.3 Degradation of the ECM: the Proteases

1.3.1 Serine Proteases

While involved in the proteolytic degradation of a number of ECM components, the principal function of the serine protease family is in the activation of the MMPs (Chen, 1992). Two key members, urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), interact with and proteolytically cleave plasminogen, to produce the proteolytically active plasmin. In the case of uPA, the formation of activated plasmin is enhanced by an interaction between uPA and a receptor, uPAR (Ossowski et al., 1991). After its activation, plasmin then is able to degrade proteins within the ECM, or activate other proteases, resulting in a net increase in ECM catabolism (Blasi and Stoppelli, 1998). The enzymes are kept in control by a group of inhibitors (the plasminogen activator inhibitor group (PAI)), which are able to interact with the serine protease at the plasma membrane, leading to internalization of the complex (Conese et al., 1995). In this manner, the inhibitors are able to control both uPA and tPA proteolytic potential, as well as the activation of other proteases, like the MMPs.

1.3.2 Cysteine Proteases

There are 2 families of cysteine proteases, which are classified based on their dependence upon a thiol nucleophilic attack upon cysteine residues for their proteolytic activity. One group is related to IL-1β converting enzyme, and the other is the papain family (Chapman et al., 1997). The papains contain many members, notably cathepsin B, H (Wang et al., 1998), L, and S, of which most are only weakly active at neutral pH, therefore demonstrating their primary functions within the acidic environment of cellular vesicles (Chapman et al., 1997). Because of this common intracellular localization, the cathepsins are usually involved with intracellular processes (Chen, 1992; Czaplewski et al., 1999). However, there are reported examples where bone tissue cells, specifically osteoclasts, express cathepsins (Bromme et al., 1996), where they may be involved with the process of bone resorption (Inui et al., 1999). It has also been demonstrated that some cathepsins posses collagenolytic and elastolytic activities (Bromme et al., 1996), and might therefore have a function in tissue remodelling (Gelb et al., 1996). A recent study has also linked cathepsin activity to the activity of the MMPs. It was shown that cathepsin B is able to fragment both TIMP-1 and -2 (Kostoulas et al., 1999), thereby affecting the extracellular proteolytic balance. Similar to the other proteases described, the cysteine proteases must be regulated to prevent inappropriate proteolytic activity. A primary mechanism of regulation is through interaction with a specific family of inhibitors, called the cystatins (Barrett, 1987), which bind reversibly, but tightly to cysteine proteases to inactivate them (Ni et al., 1998).

1.3.3 Matrix Metalloproteinases

The matrix metalloproteinase (MMP) family of ECM degrading enzymes are zinc-dependant proteins (discussed in more detail in section 1.4.1) which collectively have a broad substrate-degrading capacity. Because of their capacity to degrade all components of the ECM, the MMPs are under tight regulatory control. They are typically expressed at low levels, however, when ECM remodelling is required, most MMPs are responsive to multiple cytokines, growth factors, and physical stress (Nagase and Woessner, 1999), and are rapidly produced (section 1.4.1.3). To prevent massive and unwanted degradation of the ECM, the MMPs are expressed as proenzymes which must be activated (section 1.4.1.4). The final mechanism of regulating the MMPs is by the interaction with a specific family of inhibitors, the TIMPs (section 1.5), which interact with the MMPs and remove their proteolytic potential.

An important point when examining the function of each of the proteinase families, is that each has a specific family of inhibitors. This highlights the physiological danger of having active proteases. In all cases, inhibitors represent the last defensive mechanism controlling the active proteinases.

1.4 Metzincins

The proteinase family known as the metzincins is composed of a large number of proteins, each which interacts with and is dependent upon zinc as a catalytic co-factor, and also require both zinc and calcium for stability of their tertiary structure. There are 2 principal criteria for inclusion within the metzincin family. First is a highly conserved active site with the sequence (HEXXHXXGXXH/D) which interacts with zinc through a

conserved series of zinc-binding ligands (Hooper, 1994). The tightest regions of conservation are the zinc-binding residues, in which the first two are always His, and the third residue is either His or Asp (Bode et al., 1993; Rawlings, 1997). In addition to the conserved zinc-binding regions of the active site, the family also contains a conserved Met residue located within a turn (the met-turn) underlying the proteinase active site (Bode et al., 1993). Although the metzincins consists of a large proteinase family, the focus of discussion here will be on the reprolysin sub-family (specifically on the adamalysins) and the matrixins, or matrix metalloproteinases.

1.4.1 The Matrix Metalloproteinases

1.4.1.1 Family Members

The matrix metalloproteinases are a family of zinc and calcium dependent extracellular enzymes (Corcoran et al., 1995; Woessner, 1991) which have a diverse number of ECM and protein substrates. Specifically, the MMPs collectively are able to degrade all protein components of the extracellular matrix, making them the most important ECM degrading protease family. This rapidly growing enzyme family contains approximately 20 members, organized into four distinct subgroups, based on substrate specificity, function, and structure (Pendas et al., 1997). The groups are as follows: interstitial collagenases, stromelysins, gelatinases, and membrane-type metalloproteinases (table I).

Table 1. MMP family members and substrates.

subclass	Name	MMP #	substrates
Collagenases	Interstitial collagenase	1	Coll III,I, II, VII, X, gelatin, proteoglycans
	Collagenase 2	8	Coll I, II, III, gelatin, fibronectin, proteoglycans, serpins
	Collagenase 3	13	Coll II, I,III,X, gelatin
	Collagenase 4	18	Coll III,I, II, VII, X, gelatin, proteoglycans
Gelatinases	Gelatinase A	2	type I collagen, all denatured collagen,elastin, laminin, fibronectin and aggrecan
	Gelatinase B	9	all denatured collagen,elastin, laminin, fibronectin and aggrecan
Stromelysins	Stromelysin 1	3	Coll III,IV,IX,X, IGF-BP3, IL-1β, MMP-1,-8,-13,-9
	Stromelysin 2	10	Coll III,IV,V,fibronectin, laminin, aggrecan, proteoglycans
	Stromelysin 3	11	α2-antiplasmin and plasminogen activator inhibitor 2
	Matrilysin	7	collagen I, III, IV, V, fibronectin, laminin, entactin/nidogen, aggrecan, cartilage link protein, elastin and tenascin-C tumor necrosis factor a precursor, u-plasminogen activator precursor (pro-uPA)
MT-MMPs	MT1-MMP	14	gelatinase A, fibronectin, vitronectin, B chain of laminin and dermatan sulfate proteoglycan, gelatin, casein and elastin, interstitial collagens, Coll I, III
	MT2-MMP	15	laminin
	MT3-MMP	16	Coll III, digested fibronectin, gelatin, casein,
	MT4-MMP	17	Not determined
	MT5-MMP		Not determined
Others	Macrophage elastase	12	elastin,entactin, laminin, proteoglycans, Coll IV, fibrinogen, MBP, TNFα, plasminogen
	?	19	Not determined
	enamelysin	20	Not determined
	X-MMP (xenopus)	21	Not determined
	C-MMP (chicken)	22	Not determined
	?	23	Not determined

Reference: Matsumoto et al., 1997; Nagase and Woessner, 1999; Pei, 1999.

The collagenases are composed of three members, macrophage/fibroblast (MMP-1), a neutrophil form (MMP-8), and the most recently identified, collagenase-3 (MMP-13). The principal substrates of these enzymes are interstitial collagen (I, II, III), making the collagenases very important in the general degradation of the ECM which has collagen as its primary component (Nagase and Woessner, 1999). Cleavage of these collagen substrates results in a characteristic N-terminal ¾ and C-terminal ¼ product fragments (Gomis-Ruth et al., 1996).

The gelatinases are able to degrade collagens type IV, V, X, elastins, and gelatin, which is a breakdown product of collagen cleavage (Mackay et al., 1990). Similar to the degradation of collagen by the collagenases, the breakdown of type IV collagen produces characteristic ¼ and ¾ breakdown products (Collier et al., 1988; Wilhelm et al., 1989). Having collagen IV as a principal substrate makes the gelatinases important in processes involving basement membrane proteolysis, like cancer invasion (Sugiura et al., 1998). Unlike the other MMP groups, the gelatinases contain a fibronectin-homology domain which enables their interaction with gelatin.

The stromelysins are a group of MMPs which are able to degrade fibronectin, proteoglycans, laminin, and some collagens. There are 2 stromelysins, stromelysin –1 and –3. The physiological function of stromelysin 1 is not clear, and stromelysin-1 knock-out mice had no obvious phenotype (Birkedal-Hansen, 1995). However, the recent use of mammary epithelial cells with inducible stromelysin-1 expression (tet-on) demonstrated a function in tumour promotion (Sternlicht et al., 1999). When the inducible cells were injected into SCID mice fat pads, mesenchymal-like tumours were formed, however, without overexpression of stromelysin-1, the cells formed epithelial

glandular structures. Furthermore, overexpression of stromelysin 1 in transgenic mice resulted in mammary abnormalities, including malignancies (Sternlicht et al., 1999).

Stromelysin-3 is of particular interest because of the observation that it is expressed in stromal cells around breast tumours, and is correlated with clinical aggressiveness of the disease (Wolf et al., 1993). Additional data regarding the tumour-promoting function of stromelysin 3 was seen in mice which had targeted knock-out of the gene (Masson et al., 1998). While the mice were fertile, with no obvious abnormalities, they showed a decreased tumorigenesis from exposure to the chemical agent, 7,12-dimethylbanzanthracine (DMBA). Presently, the mechanism in which either stromelysin-1 or –3 promote tumour progression is unknown.

The MT-MMPs are the most recently identified MMP subfamily, and consist of five members, designated MT-MMP 1 through 5 (Pei, 1999; Takino et al., 1995; Will and Hinzmann, 1995). All family members contain the characteristic RXKR site, which is a furin recognition sequence (Imai et al., 1996). This area of the protein has particular importance in the process of proenzyme activation, because furin, found in the Golgi apparatus, is able to activate MT1-MMP intracellularly. Another feature defining the MT-MMP subfamily is a C-terminal hydrophobic trans-membrane domain (Polette and Birembaut, 1998). This location within the plasma membrane differentiates the MT-MMPs from the other members of the family, which are all soluble. There are exceptions to this transmembrane localization rule, as the recently identified MT-MMP-5 is shed from the membrane by proteolytic cleavage, giving it the unique property of being either membrane associated or soluble (Pei, 1999). Also, MT4-MMP has been shown to be GPI-anchored as opposed to containing a transmembrane domain (Itoh et al., 1999).

Like other MMPs, the MT-MMPs have a variety of ECM substrates, however, the analysis of the family is still in its infancy, and the majority of research has been done on the MT1-MMP. An important substrate of MT1-MMP is progelatinase, whereby an interaction results in the plasma membrane-dependent activation of progelatinase A (Kinoshita et al., 1996). However, additional substrates include fibronectin, laminin, vitronectin, dermatan sulphate proteoglycan, and collagen type I, II, and III, but not types IV, and V (Ohuchi et al., 1997; Pei and Weiss, 1996). Recent substrate analysis has shown that MT3-MMP is able to degrade collagen type I, III and fibronectin (Matsumoto et al., 1997; Shofuda et al., 1997).

The importance of MT1-MMP in connective tissue biology was demonstrated by examining mice in which the gene is disrupted by gene targeting (Holmbeck et al., 1999). The mice were viable at birth, however, displayed runting and wasting quickly thereafter. An analysis of the developing mice demonstrated skeletal dysplasia (incomplete ossification), and impaired collagen turnover mediated by both fibroblasts and osteoblasts (Holmbeck et al., 1999), which might contribute to the abnormal physiology of the knock-out mice.

1.4.1.2 Domain Structure

A comparison of the peptide sequence of all family members reveals several common characteristics among the MMPs (Borkakoti, 1998). They all contain a pre-domain, also called a signal peptide. There is a pro-domain which is important for the maintenance of latency in the enzymes. A catalytic domain contains the conserved sequence

HexxHxxGxxH, which is a zinc binding motif. And finally, they contain a C-terminal hemopexin-like domain implicated in substrate recognition (see figure 1).

The prodomain, containing the conserved PRCG(V/N)PD motif, consists of three alpha helices separated by flexible spacer regions (Becker et al., 1995). The most important feature of the prodomain is a highly conserved cysteine residue which interacts with a zinc ion located within the active site of the MMP. The resulting MMP configuration is inactive as a protease. Furthermore, it enables a mechanism for activation through disruption of this interaction within the prodomain, either physically, by proteolysis of the enzyme to remove the pro-domain, or chemically through chelation of the zinc ion. This method of MMP regulation has been described as the cysteine switch model for activation (Birkedal-Hansen et al., 1993; Springman et al., 1990). While this was demonstrated structurally from the X-ray structure of prostromelysin (Becker et al., 1995), the sequences corresponding to the cysteine switch within the N-terminal domain are conserved, suggesting similar activation mechanisms for all MMPs.

The hallmark of the catalytic domain is its dependence upon zinc as a cofactor for both its proteolytic activity, and for structural integrity. The tertiary structure consists of a 5-stranded β-sheet lying along 2 helices. A third helix forms the deep active site cleft, which houses the zinc-binding motif (HExxHxxGxxH)(Bode et al., 1992; Hooper, 1994). The active site of MMPs also contains a critical β-turn, dependent upon a methionine residue, which is necessary for the structural integrity of the proteins (Bode et al., 1993). The MMPs are unique among all metalloproteinases due to the presence of a second

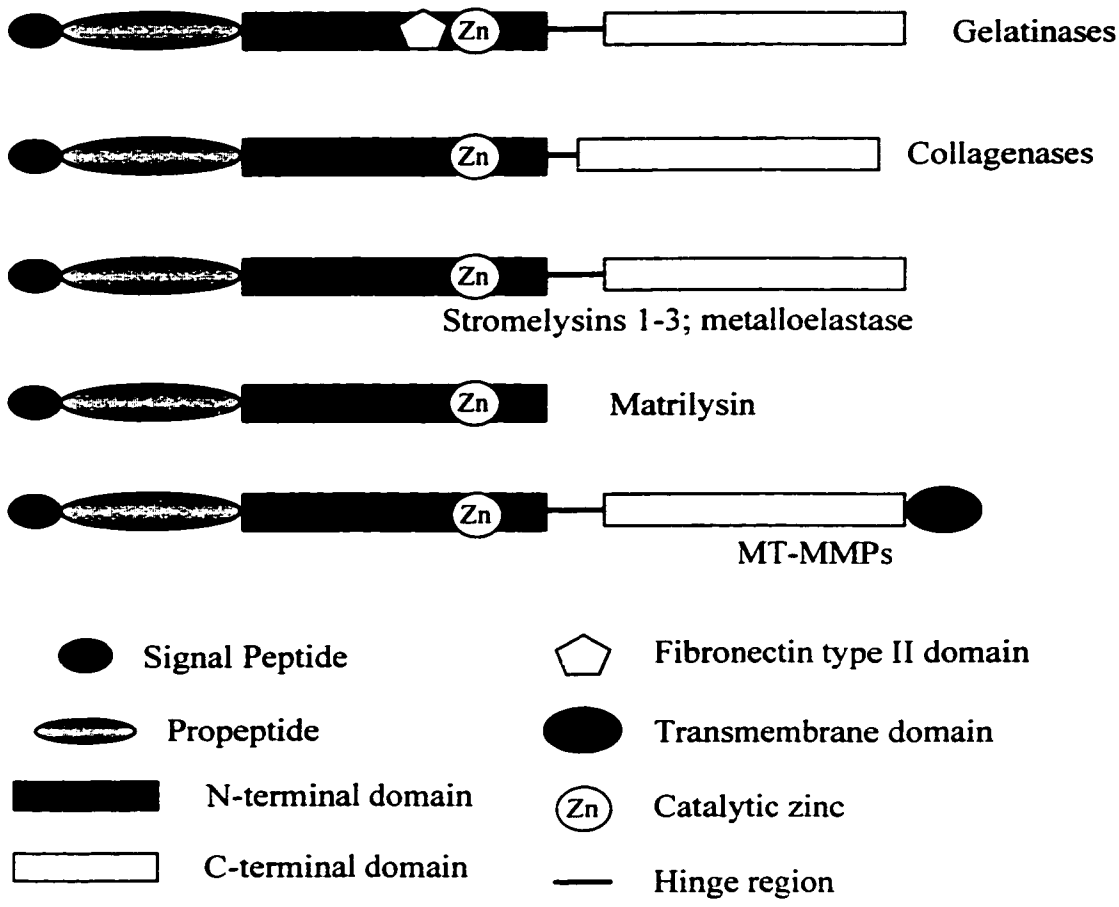


Figure 1. Domain structure of the MMPs (based on Nagase & Woessner, 1999).

structural zinc ion, required for their tertiary structure (Borkakoti, 1998). In summary, the active site domain is a combination of the active site cleft, and the catalytic zinc, both of which are required for stability of tertiary structure. It is this zinc requirement which makes the MMP enzymes sensitive to ion chelators such as EDTA.

The C-terminal hemopexin domain has representation in all family members except for matrilysin (Murphy and Knauper, 1997). The X-ray crystal structure of the hemopexin domain from several MMPs reveals a β-propeller structure with 4-fold symmetry, in which each blade is composed of anti-parallel four stranded β-sheets (Murphy and Knauper, 1997). The function of the hemopexin domain has largely been hypothesized following observations of C-terminal deletions, which in many cases causes an impaired interaction with native collagens (Allan et al., 1991; Murphy et al., 1992). That led to the idea that the hemopexin domain is important for substrate recognition and interaction. Additionally, it appears that the hemopexin domain and the C-terminal regions of the protein are important for interaction with specific MMP inhibitors, the TIMPs. While the inhibitors interact with the active site of the MMPs, the rate of interaction is enhanced through an initial C-terminal docking process upon the MMP (Howard and Banda, 1991; O'Connell et al., 1994). A stromelysin which has the C-terminus truncated binds to TIMPs with a much lower affinity (K_i=5.95x10^{-9}M) compared with the full length (K_i=8.3x10^{-10}M)(Baragi et al., 1994). Therefore, while the active site is the primary region of interaction with the inhibitor, there are contributions from other areas of the MMPs as well.

1.4.1.3 MMP Gene Expression

The degradative potential of the MMPs dictates that they must be strictly regulated. This occurs by several different mechanisms. The primary method for controlling MMP expression and unwanted ECM degradation is by producing either low or negligible levels of proteases, which is commonly seen in normal cells. However, mechanisms must be in place to ensure that the MMPs can be rapidly expressed during periods of desired matrix remodelling. The large diversity of the MMPs and their variable tissue distribution makes a summary of transcriptional regulation difficult. But there are some common patterns, as well as the fact that many of the MMPs are coordinately regulated, implying that common mechanisms might be operating.

A summary of responsiveness of many of the MMPs to various stimuli is depicted in table II. A striking feature of MMP induction if that not only are many MMPs responsive to multiple stimuli, but also that different signalling pathways and promoter elements are involved. An important observation, however is that many of the inducible

MMPs, depicted in table II, respond similarly to the same cytokines or growth factors. For example, IL-1 and PMA both consistently upregulate the expression of many MMPs (Bond et al., 1998; Borghaei et al., 1999; Pendas et al., 1997; Vincenti et al., 1998; White et al., 1997; Zhang et al., 1998). This would represent a mechanism in which a number of MMPs can be rapidly mobilized to meet sudden tissue requirements of ECM degradation. In addition to this response, however, there appear to also be signalling mechanisms which are specific to a smaller number of MMPs, thereby

Table 2. Growth factors and cytokines which regulate MMP expression.

MMP	Factor	+/-	Cell Line	Cis-Element	Reference
MMP-1	V-Src	+		PEA3, STAT	Vincenti et al., 1998
	PMA	+		PEA3, STAT	Vincenti et al., 1998; White et al., 1997
	TGF-beta	-	KMST human fibroblasts		Uria et al., 1998
		-	lung fibroblasts		Eikelberg et al., 1999
	EGF	+		Ets, AP1	Watabe et al., 1998
	IL-1	+	synovial fibroblasts	AP-1, NFkB	Vincenti et al., 1998b
	P53	-		AP1, others	Sun et al., 1999
	C-2 ceramide	+	fibroblasts	AP1	Reunanen et al., 1998
	GM-CSF/IL-1	+-synergy	monocytes	prostaglandin pathway	Zhang et al., 1998
	Okadaic acid	+	fibroblasts	JNK and p38 path	Westermarck et al., 1998
	alpha5beta1 integrin	+	synovial fibroblasts	Rac1 and ros	Werb et al., 1989
	oncostatin M	+	human astrocytes	MAPK and STAT pathways	Korzus et al., 1997
MMP-2	RA + Bt2cAMP	+ - synergy	HT1080	CRE-like	Hasan et al., 1999
	RA	-	adenocarcinoma cells		Nakajima et al., 1989
	LIF/OSM	no change	osteoblasts		Varghese et al., 1999
MMP-3	IL-1	+	synovial fibroblasts	AP1, NFkB, others	Borghaei et al., 1999;Quinones et al., 1994
	TNF-alpha	+	synovial fibroblasts	AP1, NFkB, others	Borghaei et al., 1999
	PMA	+	Synovial fibroblasts	AP1, Ets	White et al., 1997
	RA	-	Synovial fibroblasts; embryo fibroblasts		Saus et al., 1988; Nickolson et al., 1990
	dexamethasone	-	Synovial fibroblasts		Saus et al., 1988; Frisch and Ruley, 1987
	TGF-beta	-	Rat fibroblasts		Kerr et al., 1990
MMP-9	EGF	+	fibroblasts	AP1, Ets	Watabe et al., 1998
	PDGF or bFGF	+	fibroblasts		Watanabe et al., 1998
	and IL-1 or TNFa	+ syn	dermal fibroblasts, monocytes	AP1, NFkB, others	Bond et al., 1998; Zhang et al., 1998
	RA	-	Mammary adenocarcinoma		Nakajima et al., 1989
	Ha-ras	+	OVCAR cells	PEA3, AP1, NFkB, GT box	Gum et al., 1996
	TNF-alpha	+	monocytes		Zhang et al., 1998
MMP-13	GM-CSF	+	monocytes		Zhang et al., 1998
	TGF-beta	+	KMST fibroblasts	AP1	Uria et al., 1998
	cbfa overexpress	+	osteoblasts	Cbfa motif	Jimenez et al., 1999
	PTH	+	in vivo (mice)	AP1, cbfa1 (core binding fact)	Porte et al., 1999
	TPA	+	Hela and COS cells	AP1	Pendas et al., 1997
	LIF/OSM	+	osteoblasts		Varghese et al., 1999

allowing a more focussed proteolytic response. Alternatively, certain factors, such as retinoic acid (RA) and transforming growth factor β (TGFβ) have an inhibitory effect upon many MMPs (Hendrix et al., 1990; Nakajima et al., 1989; Uria et al., 1998), which would provide a safeguard to turn off a massive proteolytic response.

1.4.1.4 MMP Activation

Following the transcription of MMPs, a second mechanism for regulation is the expression of latent enzymes which require proteolytic cleavage for activity. For most MMPs, this activation event takes place extracellularly. However, there are several exceptions, as stromelysin 3 and MT1-MMP are both activated intracellularly by furin.

Activation of the enzymes involves the removal of the pro-peptide sequence by proteolysis, mercurial agents (Itoh et al., 1995; Ogata et al., 1995), heat, or otherwise disrupting the interaction between the cysteine within the MMP pro-domain and the zinc ion (e.g. SH-reactive agents). As described previously, a key cysteine residue within the prodomain (the cysteine switch) interacts with the active site zinc, which therefore requires cleavage for the MMP to become proteolytically active. The actual agents which cleave of the prodomain, however, are quite diverse, and often specific to the MMP itself.

With a few exceptions, all MMPs are typically activated through an initial cleavage by plasmin, resulting in a product which is a substrate for further cleavage by stromelysin, or through autoproteolysis (Matrisian et al., 1985; Whitham et al., 1986; Wilhelm et al., 1987) (figure 2). This mechanism highlights the importance of other protease families, namely the serine proteases, in the activation of the MMPs through the

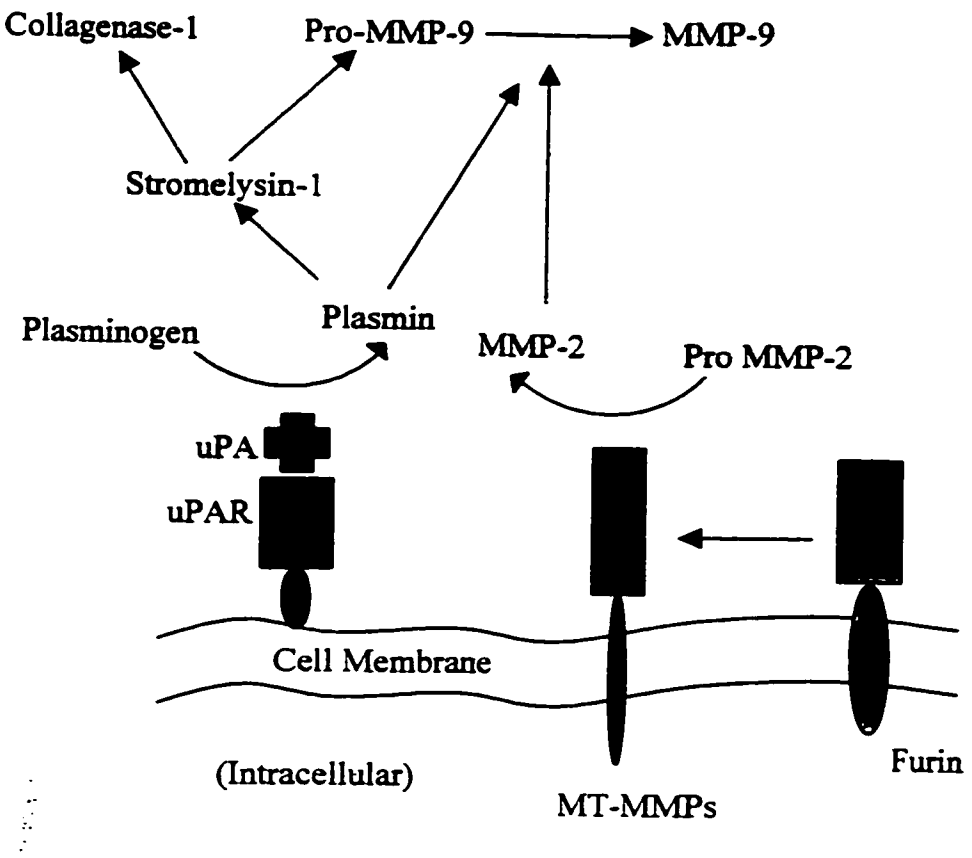


Figure 2. MMP activation cascade at the cell surface (after Yong et al., 1998). Plasmin is produced by the cleavage of plasminogen by urokinase plasminogen activator (uPA). The uPA receptor is anchored to the membrane by its receptor, uPAR. Plasmin activates stromelysin-1 and MMP-9. Stromelysin is able to activate other MMPs, amplifying the initial activation cascade. The furin proteinases activate the MT-MMPs, which in turn, can activate MMP-2.

proteolysis of plasminogen to form active plasmin. Should the function of the serine proteases be impaired (for example by their inhibitors, the PAIs), plasmin will not be produced, and the major MMP activator mechanism will not be in place. Direct physiological evidence of the role of plasmin in the activation of several MMPs (MMP-3, -9, -12, and –13) was seen in u-PA-deficient macrophages, which when supplemented with plasminogen were unable to efficiently activate the aforementioned MMP, while wild type macrophages were (Carmeliet et al., 1997).

The primary mechanism of MMP-2 activation is different from the general mechanism. While MMP-2 is activated by a number of the usual agents such as organomercurials, matrilysin, and collagenase (Crabbe et al., 1994; Sang et al., 1996; Stetler-Stevenson et al., 1989) and even self activation, in the presence of heparin (Crabbe et al., 1993), it is not efficiently activated by plasmin. Recent data regarding MMP-2 activation indicates that it takes place as a membrane-dependant event (Strongin et al., 1993; Ward et al., 1991). It is thought that TIMP-2, which interacts with proMMP-2, is required to transport the MMP-2 to the cell surface (see figure 2). This was demonstrated by observing iodinated TIMP-2/MMP-2 complexes, which bind to HT1080 cells, while MMP-2 alone did not (Emmert-Buck et al., 1995). The plasma membrane localization of MMP-2, and its activation was not inhibited by Timp-1, which suggested a specific membrane-associated MMP-2 protease. Truncated MMP-2 proteins indicated that this cell-surface activation of Gelatinase A requires the gelatinase A C-terminal domain (Strongin et al., 1993). Important observations that MT-MMP-1 associates with TIMP-2 led to the present model where TIMP-2 acts as a bridge, by interacting with MT-MMP-1 at its N-terminal domain (Butler et al., 1998) and to progelatinase A at its C-

terminal domain, enabling the activation of the progelatinase A under the correct circumstances. This suggests that the levels of TIMP-2 are critical in the activation of progelatinase A. If TIMP-2 levels are too low, progelatinase A will not get anchored to the membranes where it is activated. Alternatively, if TIMP-2 is too high, the MT1-MMP is inhibited, and unable to activate the progelatinase A.

Aside from MT1-MMP, there are other proteases which are thought to be involved in the activation of pro-gelatinase A. These include other members of the MT-MMP family, specifically MT1-, MT2-, MT3-, and MT5-MMP. Thrombin has also been shown to activate pro-gelatinase A, in a process which was not inhibited by either TIMP-2 or 1,10 phenanthroline, therefore it appears to be a process independent of MT1-MMP (Nguyen et al., 1999). Furthermore, in addition to TIMP-2, the $\alpha V\beta 3$ integrin can also anchor proMMP-2 to the cell surface for activation, however there appear to be cell-specific differences in this process (Brooks et al., 1996).

MMP-9, on the other hand, appears to be activated by a number of proteins, including other MMPs. A principal mechanism might be through plasmin, which is ineffective at directly activating MMP-9 (Okada et al., 1992), but will activate stromelysin-1 (MMP-3), which in turn activates MMP-9 (figure 2) (Ogata et al., 1995; Ramos-DeSimone et al., 1999). There is also evidence that other MMPs, including MMP-2 can activate MMP-9 (Fridman et al., 1995). The proform of MMP-9 is able to interact with TIMP-1, which results in a complex that is resistant to activation by plasmin-activated stromelysin-1, which therefore defines an additional level of regulation for MMP-9 (Ramos-DeSimone et al., 1999).

1.4.2 The ADAMs

The recently identified ADAMs (a disintegrin and metalloproteinase domain) family of proteins are a large family of metzincins with very divergent functions. Presently 23 members have been identified, and the proteins are grouped according to function. Structurally, most ADAMs adhere to a similar domain organization, with a pro-domain, an MMP-like protease domain, and a disintegrin domain. The ADAMs therefore are sensitive to chelation agents which disrupt the metal-dependent active site, such as EDTA or o-phenanthroline (Black and White, 1998). Additional domains present in some, but not all of the ADAMs is a cysteine rich, EGF-like repeat, and a transmembrane domain terminating in a cytoplasmic tail. It is primarily the transmembrane and intracellular regions which separate the ADAMs from the closely related but soluble snake venom metalloproteinases.

Although such structural similarities exist, the ADAMs all have different and specific functions. The prototypic ADAMs, fertilin-α and -β, are necessary for the fusion of sperm and egg during fertilization (Black and White, 1998; Cho et al., 1998; Myles et al., 1994; Wolfsberg et al., 1993). It was the observation that the shedding of TNF-α from its plasma membrane associated state is inhibitable by synthetic hydroxamate MMP inhibitors which finally linked the ADAMs to membrane associated proteolysis (Gearing et al., 1994; McGeehan et al., 1994). This observation led to the cloning of the TNF-α converting enzyme (TACE) (Black et al., 1997; Moss et al., 1997), which is a member of the ADAM family. In addition to releasing TNF-α, TACE is also important in the generation of EGF ligands, notably TGF–α. On a similar note to TACE, the *Drosophila*

ADAM, kuzbanian (ADAM 10) processes Notch, the receptor for the signalling molecule, delta (Pan and Rubin, 1997).

1.5 The Tissue Inhibitors of Metalloproteinases (TIMPs)

1.5.1 TIMP Structure and Function

The third event important for the regulation of the MMPs is through an interaction with a specific family of inhibitors, the TIMPs. The TIMPs function by directly interacting with the zinc-binding active site of all of the MMPs (Birkedal-Hansen et al., 1993). This interaction is essentially non-reversible, and occurs with a 1:1 molar stoichiometry, and renders the MMP unable to degrade its substrates.

Four TIMPs have been characterized so far (TIMP-1, -2, -3 and –4) (De Clerck et al., 1989; Docherty et al., 1985; Greene et al., 1996; Leco et al., 1997; Leco et al., 1994), all of which share certain structural similarities. All family members have 12 conserved cysteines, which organize the protein structure to enable the formation disulphide bonds resulting in a series of 6 loops. This structure divides the protein into two functional domains, the N-terminal and C-terminal domain. It appears that this bipartite structure of the TIMP separates two different functional regions of the protein. It is the N-terminal domain of the TIMP protein which contains the MMP inhibitory function, evidenced by a truncated TIMP-1 which contained only the N-terminal domain folded into the first three loops and still retained its ability to inhibit MMPs (Murphy et al., 1991). Accordingly, the N-terminal domain is relatively well conserved among all four TIMPs, specifically at the VIRAK sequence, which is present in them all. However, aside from these regions of similarity, there is divergence in structure and function among the family members. The

recently identified TIMP-4 has 37% identity with TIMP-1 and 51% identity with TIMP-2 and –3 (Greene et al., 1996). As I have discussed earlier, TIMPs can form complexes with pro-MMPs. Thus both TIMP-1 and TIMP-3 interact with proMMP-9 (Butler et al., 1999), and TIMP-2, -3, and -4 interact with pro-MMP-2 (Bigg et al., 1997; Butler et al., 1999; Goldberg et al., 1992). Again, the use of truncated TIMP mutants has localized this function to the C-terminus of the TIMP protein. As described previously, this interaction has particular importance in the activation of MMP-2, and possibly MMP-9.

The structures of both TIMP-1 and -2 have been recently resolved using nuclear magnetic imaging, and X-ray crystallography (Fernandez-Catalan et al., 1998; Gomis-Ruth et al., 1997; Tuuttilla et al., 1998). The C-terminal domain of recombinant TIMP-2 is composed of a parallel stranded β-hairpin and a β-loop-β motif (Tuuttilla et al., 1998). The N-terminal domain is a 5-stranded β-sheet which is folded to resemble a barrel, reminiscent of the oligonucleotide/oligosacchiride binding (OB) fold family of DNA binding proteins (Williamson et al., 1994).

There are important regions within TIMP molecules which enable them to bind to the active sites of MMPs. One such region is the AB-loop, which is located between the A and B strand of the N-terminal β-barrel (Fernandez-Catalan et al., 1998; Tuuttilla et al., 1998). The AB-loop undergoes a tilting, which enables it to make direct contact with the protease, while the other regions of the TIMP molecule change only slightly to adapt to the enzyme interaction (Tuuttilla et al., 1998). An important difference in structure among the TIMP molecules is the length of the AB-loop (Butler et al., 1999). While TIMP-1 and -3 have short AB loops, both TIMP-2 and -4 have longer AB loops. The AB loop of TIMP-2 enables an interaction with a surface pocket of MT1-MMP, created by a

structure called the MT-loop (Fernandez-Catalan et al., 1998). The importance of the AB loop of TIMP-2 in the MT1-MMP association was demonstrated by mutating Tyr36 within the loop, which disrupted the MT1-MMP interaction, but did not compromise its interaction with other MMPs (Butler et al., 1999). The shortened AB loop of TIMP-1 might provide a plausible explanation as to its inability to inhibit the MT1-MMP protease (Butler et al., 1999). In addition to the N-terminal region, the TIMPs possess unique C-terminal tails, which are thought to play additional roles in specificity. The charged tail of TIMP-2 is thought to be important for pro-MMP-2 binding (Willenbrock et al., 1993). However, little is known about the C-terminal tail regions of the other TIMPs.

The crystal structures of the TIMP-1/MMP-3 and TIMP-2/MMP-3 complexes have recently been resolved (Gomis-Ruth et al., 1997; Muskett et al., 1998). The TIMP-1 molecule is shaped like an elongated wedge, occupying the active site cleft of MMP-3 (Gomis-Ruth et al., 1997). The first cysteine (Cys-1) is important in the interaction, and chelates the central zinc residue within the MMP active site. The Threonine-2 side chain of TIMP-1 then extends into the large, specificity pocket of MMP-3. In addition, the Cys-1/Cys-70 disulphide bond creates a surface ridge which enables Cys 1 to Val 4 and Met 66 to Val 70 to contact the MMP active site. The importance of Cys 1 in MMP inhibition is demonstrated by observing an engineered Timp-2, with an alanine appended to the amino-terminus, which was inactive as a protease inhibitor, however, retained the ability to complex with pro-MMP-2 (Wingfield et al., 1999). These residues are highly conserved among TIMPs, and therefore it is thought that this mechanism can be generalized to the other TIMP family members (Gomis-Ruth et al., 1997).

The C-terminal domain of TIMPs has two specific functions. First, an interaction occurs with the C-terminal, hemopexin domain of the MMP. This docking process provides an initial contact point between TIMPs and MMPs, and facilitates the inhibitory interaction between the TIMP N-terminal domain with the MMP active site (Taylor et al., 1996). Alternatively, the C-terminal domain of TIMPs also enables an interaction with progelatinases under certain circumstances. Each of TIMP-2, -3, and -4 are able to form complexes with pro-MMP-2 (Bigg et al., 1997, Will et al., 1996), while TIMP-1 and -3 interact with pro-MMP-9. This interaction occurs with the C-terminal, hemopexin-like domain of the MMPs (Overall et al., 1999), and functions in regulating the activation of the pro-MMPs. The activation of pro-MMP-9 is impaired by an interaction with TIMP-1 (Itoh and Nagase, 1995). The N-terminal domain of TIMP-2 complexed with MT-MMP creates a docking complex for the recruitment of pro-MMP-2, a pivotal step in MMP-2 activation (Butler et al., 1998).

The members of the TIMP family display different levels of glycosylation (see table 3). TIMP-1 is a highly glycosylated protein with a molecular weight of 28.5 kDa (Gomez et al., 1997). TIMP-3 is also glycosylated, with a MW of 24-27 kDa. Neither TIMP-2 nor –4 are glycosylated, and migrate with 21 and 22.5 kDa respectively. Another difference between the family members is in the localization of the protein. While TIMP-1, -2, and –4 are freely soluble, TIMP-3 is insoluble, and confined to the ECM (Blenis and Hawkes, 1984; Leco et al., 1994; Staskus et al., 1991). The tissue distribution of the TIMPs suggests that there are important differences in the regulation

Table 3. Characteristics of Members of the TIMP family

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Molecular weight	28.5	21	24-27	24
Chromosome (human)	Xp11.23-11.4	17q2.2-2.5	22q12.1-13.2	3p25
Number of Exons	6	5	5	5
RNA	0.9	3.5, 1.0	4.5, 2.8, 2.4	1.1
glycosylated	yes	no	yes	No
soluble	yes	yes	No - ECM associated	Yes
inducible	yes	constitutive	yes	Slightly
Tissue localization	Bones, lung, ovary,	Lung, brain testes	Lung, brain kidney,muscle,	Brain, heart
Inhibit MT-MMP	no	yes	yes	yes
Inhibit ADAMs	no	no	Yes – ADAM-17	no

of the TIMPs. While TIMP-2 is widely distributed, there is more specificity found in the expression of TIMP-1 (bones, lung, ovary) and –3 (lung, brain, kidney, muscle) (Flenniken and Williams, 1990; Nomura et al., 1989). TIMP-4 shows even more restricted expression, and is found mostly in heart, brain tissue, mammary epithelial cells, and vascular smooth muscle cells (Dollery et al., 1999; Fata et al., 1999; Greene et al., 1996; Leco et al., 1997). However, the recently cloned rat Timp-4 has indicated more diverse expression pattern, with Timp-4 detected in the skin, retina, smooth muscle, and pancreas tissues (Wu and Moses, 1998). This difference in spatio-temporal expression of the TIMPs might be a consequence of their different inducibilities, which will be discussed later.

While all TIMPs are similar in their ability to inhibit most MMPs (Liu et al., 1997; Ward et al., 1991), there is a marked difference in the interactions with the MT-MMP group. TIMP-1 is unable to inhibit the MT-MMPs, while the other members of the TIMP family are able to do so (Matsumoto et al., 1997). On a similar note, is the inhibition of the recently identified ADAMs family of proteases, in which only certain TIMPs are effective against certain ADAMs. For example, only TIMP-3 is able to inhibit ADAM-17 (Amour et al., 1998), implying unique target recognition sequences within the TIMP proteins.

1.5.2 TIMPs in Cell Growth and Development

1.5.2.1 TIMPs as Regulators of Cell Growth

While it is easy to consider the primary function of the TIMPs as inhibitors of the MMP, each member also has additional properties independent of this role. The prototypic family member, TIMP-1 was initially identified based on having a growth factor effect on leukaemic cell lines (Hayakawa et al., 1992) which gave it its initial name of erythroid potentiating activity (EPA)(Avalos et al., 1988). This phenomenon is independent of its TIMP function because non-specific MMP inhibitors do not have a similar effect. Note, TIMP-2 also has EPA, and acts as a growth factor in certain metanephric mesenchymal cells (Barasch et al., 1999; Hayakawa et al., 1994; Hayakawa et al., 1992). A further role in gene regulation by TIMP-1 has been seen using a TIMP-1 – EGFP fluorescent protein (Ritter et al., 1999). It was seen that the TIMP-1-EGFP conjugate attached to the plasma membrane of MCF-7 cells, then after 72 hours was internalized and localized to the nucleus. This indicates a possible role in either replication or transcription. A similar accumulation of TIMP-1 within the nucleus was also seen by a different group (Zhao et al., 1998). In this case, TIMP-1 was seen in the nucleus in a cell-cycle associated manner, reaching maximal nuclear levels in S-phase. A similar nuclear migration was not seen for either TIMP-2 or –3. However, there is evidence that, like TIMP-1, TIMP-3 regulation and expression is also linked to the cell cycle, where TIMP-3 mRNA was increased in WI-38 fibroblast cells in G1 stage of the cycle (Wick et al., 1994). The functional significance of the cell cycle association to both gene expression and cellular localization, however, is unknown, however, a function in

both gene expression, cell division, or the inhibition of a nuclear MMP can be hypothesized.

1.5.2.2 TIMPs and Apoptosis

Many recent reports have suggested that each of the TIMPs have a function in the induction, or suppression of apoptosis in a variety of cell lines. Overexpression of TIMP-3 by adenoviral delivery into rat smooth muscle cells caused them to undergo apoptosis as defined by the characteristic morphological features (Baker et al., 1998). This was confirmed by the addition of recombinant TIMP-3 to the same smooth muscle cells (Baker et al., 1998), and by the overexpression of TIMP-3 in SK-Mel-5 and A2058 melanoma cells (Ahonen et al., 1998). Furthermore, the conditioned media derived from TIMP-3 overexpressing DLD cells (colon carcinoma) resulted in apoptosis in fibroblast recipients (Smith et al., 1997). That the apoptotic effect was dramatically reduced by including TNF-α receptor antibodies into the conditioned media suggests that the apoptosis is triggered somehow through the TNFα signalling pathway (Smith et al., 1997). This is a TIMP-3 specific response, as none of TIMP-1, TIMP-2, or the synthetic MMP inhibitor BB94 resulted in this effect.

While it can be seen that TIMP-3 has a pro-apoptotic effect, the opposite response is associated with TIMP-1. In trials with Burkitts lymphoma cell lines, it was seen that TIMP-1 expression correlates with resistance to apoptosis (Guedez et al., 1998). Furthermore, the addition of recombinant TIMP-1 was able to block apoptosis in the same cells – a response which is resistant to alkylation, and therefore independent of the MMP inhibiting function. One possibility is that the expression of apoptosis genes is affected, as TIMP-1 increased expression of Bcl-X_L while decreasing NFκB levels

(Guedez et al., 1998). This goes back to the idea of TIMP-1 acting as a growth factor. In addition to TIMP-1, TIMP-2 also appears to have an anti-apoptotic effect. When overexpressed in B16F10 murine melanoma cells, the clones were more resistant to apoptosis compared to parental lines (Valente et al., 1998).

1.5.2.3 TIMP-3 and Sorsby's Fundus Dystrophy

The additional specific functions of TIMPs is highlighted by the discovery of TIMP-3 point mutations in a retinal degenerative syndrome called Sorsby's Fundus Dystrophy. Positional cloning identified the initial mutation associated with SFD to a chromosomal location around the TIMP-3 gene (Weber et al., 1994). Since then, a series of 6 mutations within the TIMP-3 gene have been isolated which all lead to the pathological phenotype. All mutations result in a single amino acid change to a cysteine residue, and all are located on the C-terminus of the protein. It is therefore thought that the SFD mutations affect the proper folding of the TIMP-3 protein. However, data from our lab has indicated that the MMP inhibitory function, localized to the N-terminus, is not affected by the mutations. Rather, the TIMP-3 proteins with the SFD mutants appear to have altered adhesion characteristics, and have the propensity to form higher molecular weight aggregates with TIMP activity (Langton et al., 1998; Yeow, 1999).

1.5.3 TIMP Induction

All four TIMPs may be similar in their exon/intron organization, yet they are unique in their response to stimuli and in their cellular localization. Of the four identified TIMPs, TIMP-1 and –3 are induced by many different types of stimuli (Table 4) and show tissue specific expression patterns. On the other hand, TIMP-2 is largely uninducible, and constitutively expressed throughout the different tissues. The most recently identified family member, TIMP-4, has not been thoroughly examined in detail. However, it does not appear to be very inducible, and shows an extremely restricted pattern of expression. Of the inducible TIMP genes, TIMP-1 has been the most extensively studied, therefore accounting for the apparently larger number of inducing stimuli in the summary table. However, an important point is that in many cases, TIMP-1 and –3 are coordinately regulated, with some important exceptions, which will be discussed later.

In addition to serum and PMA, a number of physiological growth factors and cytokines are also able to induce TIMP-1 and –3. One of the most studied of the inducing stimuli is TGFβ, which is important in ECM regulation for several reasons. First, during murine development, there are similarities in the expression patterns of both TGFβ and Timp-1 (Flenniken and Williams, 1990; Pelton et al., 1990), indicating that TGFβ may be an important regulator of TIMP-1 during development. Second, is that TGFβ is an important factor in the regulation of the overall composition of the ECM based on its ability to downregulate several of the MMPs, namely MMP-1, -3, and –13,

Table 4. growth factors and cytokines which regulate TIMP expression

Gene	stimulus	response	cell type
Timp-1	serum	+	fibroblast; mouse, human
	phorbol ester	+	fibroblast; mouse, human
		+	macrophages, human
	bFGF	+	fibroblasts, human
	EGF	+	fibroblasts; human
	TGF-beta	+	various; mouse, human
	IL-1	+	fibroblast, monocytes; human
		no effect	fibroblasts, chondrocytes, human
	IL-6	+	various; mouse, human
	Onc-M	+	fibroblasts,synovial cell; human
	retinoic acid	+	fibroblasts; human
		-	calvarial cells
	LPS	+	macrophages, human
	angiotensin II	+	astrocytes; rat
	virus-NDV	+	mouse L-cells
	virus-HTLV-1,-2	+	T-cells; human
	progesterone	+	uterine, cervical fibroblasts;rabbit
	dexamethasone	-	various
	retinol	+	endothelial cells; human
	concanavalin-A	+	monocytes; human
		-	fibroblasts; human
	TNF-alpha	+	monocytes, human
TIMP2	various	constitutive	various; human, mouse
	retinoic acid	+	endothelial cells; human
	phorbol ester	-	HT1080; human
		constitutive	various; human
	TGF-beta	-	various; human
	progesterone	+	uterine cervical fibroblasts; rabbit
	LPS	-	macrophages; human
TIMP3	serum	+	various; human, mouse
	phorbol ester	+	various; human, mouse, chicken
	TGF-beta	+	various; human, mouse
	dexamethasone	+	fibroblasts; mouse
	Onc-M	-	synovial cells; human
		+	chondrocytes; human
	PDGF	+	aortic smooth muscle; rabbit
	LIF	no change	osteoblasts

* from Edwards et al., 1999; Zhang et al., 1993; Eikelberg et al., 1999, Li et al., 1998, Varghese et al., 1999

while upregulating both TIMP-1 and –3 (Edwards et al., 1987; Eickelberg et al., 1999; Matrisian et al., 1986; Overall et al., 1989; Overall et al., 1991; Rydziel et al., 1997). For TIMP-3, a TGFβ induction has been seen in several different cell lines, and has been disrupted by tyrosine kinase inhibitors, giving insight into the signalling pathways utilized by the TGFβ stimuli (Huang et al., 1998; Su et al., 1998). Because of this antagonistic response of MMPs and TIMP-1 stimulation, the net effect would be the net deposition of ECM due to decreased proteolytic enzyme, and increased inhibitor. In fact, this has been demonstrated by the increase of both collagenous and non-collagenous components of the ECM in TGFβ stimulated osteoblasts (Hock et al., 1990), and an increase in bone formation following the local administration of TGFβ in mice (Rosen et al., 1994).

Despite the fact that TGFβ has a strong transcriptional activating effect on both TIMP-1 and –3, efforts at identifying the signalling pathways or the promoter elements have been unsuccessful. In the case of the MMPs, the TGF-β-inhibitory element (TIE) directs the inhibitory response. A site responsible for the activating response within the Timps has not yet been identified. Unpublished data from our lab, however indicates that a TGF-β responsive element lies within the upstream promoter region of Timp-1 (-2700/-223).

Aside from TGFβ, there are also many other growth factors and cytokines which differentially affect expression of the MMPs and their inhibitors. This type of response can also be seen from the cytokine IL-10, where the IL-10 stimulation of primary human prostate cancer cells resulted in an upregulation of TIMP-1, and a repression of both MMP-2 and –9 (Stearns et al., 1999). In the model system used, it was hypothesized that

this differential effect might be involved in the increased angiogenesis often associated with prostate tumours.

While the inducibility of TIMP-1 and –3 can often be generalized, there are still clearly circumstances in which they are differentially regulated. A recent study analyzed the effect of proinflammatory cytokines, and demonstrated a strong upregulation of TIMP-1 following IL-1β/TNFα stimulation within brain endothelial cells. However, this same combination blocked the expression of TIMP-3 (Bugno et al., 1999). Clearly, the pattern of TIMP gene expression depends upon both the stimulus used, as well as the cell/tissue being studied.

1.6 Remodelling of the Extracellular Matrix

1.6.1 Physiological ECM Remodelling

There are many normal processes which require alterations to components of the ECM. The process of wound healing is associated with several important steps in which MMPs and overall ECM regulation are affected: inflammation, angiogenesis, tissue formation, and matrix deposition (Madlener et al., 1998). Accordingly, during the process of wound healing, changes in the expression levels of the MMPs and their inhibitors are often seen, such that there is often an upregulation of MMPs and TIMPs, implying a tight control of ECM regulation (Madlener et al., 1998). There is also the case of trophoblast invasion into the uterine wall during early development, where the trophoblast must express and release active metalloproteinases in order to break down the ECM of the maternal tissue. Accordingly, increased levels of many MMPs have been associated with the invasive trophoblast, including collagenase, stromelysin, and

gelatinases A and B (Alexander et al., 1996; Leco et al., 1996). The major TIMP expressed in the maternal decidua is TIMP-3, whose levels surround the region of the invading trophoblast correlate closely with those of MMP-9, which suggests a function in restricting the degree of invasion of the trophoblast (Leco et al., 1996).

1.6.1.1 Angiogenesis

The process of angiogenesis is defined as the formation of new blood vessels, which reemerge from preexisting vessels (Stetler-Stevenson, 1999). The process requires that the basal lamina of the existing blood vessels be degraded, followed by a migration of endothelial cells into the tissue stroma. Because of the invasiveness of this process, it is suspected that MMPs might play an important role in it. Many of the experiments in the field have utilized endothelial cells as a model system, as dividing endothelial cells is a hallmark of the angiogenic process. It was shown that bovine aortic cells overexpressing TIMP-1 were 89% less invasive as measured in gelatin coated Boyden chambers (Fernandez et al., 1999). Further evidence from the in vitro model showed that both TIMP-1 and –2 were able to inhibit angiogenesis within the chick yolk sac (Takigawa et al., 1990). The physiological significance of this observation would be that the endothelial cells need functional MMPs for invasion through the basement membrane.

The critical nature of ECM regulation during angiogenesis can also be seen by disrupting the delicate balance between the MMPs and their inhibitors. Stimulating human bone marrow endothelial cells with interleukin 10 results in an increased expression of TIMP-1, and a concomitant decrease in both MMP-2 and –9 (Stearns et al.,

1999). This stimulation also blocked the cells from forming microvessels in 3-dimensional collagen gels, which demonstrates the importance of ECM regulation and proteolysis in endothelial vessel formation.

1.6.2 Pathological ECM Remodelling

Just as there are many normal situations which require alterations in the ECM, there are also many pathological situations which arise resulting in the dysregulated destruction or accumulation of ECM. MMPs have been associated with accelerated breakdown of ECM and connective tissue, which can lead to arthritis (Murphy and Hembry, 1992; Wernicke et al., 1996), tumour metastasis, multiple sclerosis, and Alzheimer's disease (Yong et al., 1998).

1.6.2.1 Fibrosis

Because liver fibrosis is the result of an increased deposition of ECM components, principally collagens type I, III, and IV, the loss of ECM regulation is an important factor in the development of the disease (Schuppan, 1990). It has been demonstrated that there is an increase in MMP-2, MMP-9, and TIMP-1, and –2 during the development of hepatic fibrosis (Iredale et al., 1996; Kossakowska et al., 1998; Takahara et al., 1995). The results demonstrate that there is a loss of the regulatory ECM controls within the diseased liver. Immunolocalization has shown that it is the hepatic stellate cells within the liver that are primarily responsible for the expression of both MMPs and the TIMPs (Arthur et al., 1992; Theret et al., 1997). Because the hepatic stellate cells both proliferate and increase matrix production in the diseased liver, they are

a primary target for intervention in the disease. The recovery of the liver, as it returns to normal health is termed resolution of the fibrotic condition. It was shown that during the resolution of carbon tetrachloride-induced fibrosis of the rat, there is an increase in collagenolytic activity (Iredale et al., 1998). Paradoxically, there is no net increase in MMP-13 levels during liver recovery, which is the only collagenase in the rat. However, it was shown that the TIMP-1 and –2 levels are decreased as the liver recovers from fibrosis (Iredale et al., 1998), which led to the hypothesis that the same amount of MMP-13 is still present, but more active. Another interesting result from the liver recovery model was that as the TIMP-1 levels were dropping, the hepatic stellate cells underwent apoptosis (Iredale et al., 1998). This observation might indicate that TIMP-1 promotes the survival of the hepatic stellate cells. Collectively, these studies all highlight the importance of the levels of the MMPs and their inhibitors in the pathogenesis and recovery from a fibrotic state. It is these levels which regulate the amount of active proteolysis occurring in the liver.

1.6.2.2 Cancer

The most dangerous step in the development of a tumour is its metastatic spread to multiple sites within the body. However, in order to spread, the cells must pass through a number of physiological barriers, such as basement membranes, stromal matrices, and cell-cell junctions (Coussens and Werb, 1996). Because of their degradative potential against many of those barriers, there has been considerable interest in the role of MMPs and TIMPs in the progression of cancer (Johnson et al., 1998; Liotta et al., 1991).

Many studies have examined the expression and activity of MMPs, as well as their inhibitors, in malignant tissue as compared to normal tissue. The function of the MMP-TIMP system in facilitating tumourigenesis was elegantly demonstrated by using antisense RNA technology to block TIMP-1 levels in Swiss 3T3 cells (Khokha et al., 1989). The result was cells which were not only more invasive in amnion invasion assays, but also more metastatic following injection into athymic mice. Other approaches have studied the expression of MMPs and TIMPs in malignant tissue as compared with adjacent normal tissue.

In most tumour tissue examined, a pattern can be seen where there are increases in both MMPs, such as collagenase, gelatinase-A, and gelatinase B, as well as an increase in some of the TIMPs, usually TIMP-1 (Kossakowska et al., 1991; Kossakowska et al., 1993; Murray et al., 1998; Urbanski et al., 1993). There are several possible explanations for this paradoxical upregulation of both proteases and inhibitors found in tumours. The TIMP-1 protein has been demonstrated to possess a growth promoting effect (section 1.5.2.1), and might be contributing to the growth of the tumour. Alternatively, the increased TIMP expression might be a defensive mechanism of tissues adjacent to the tumour, and function to restrain the growing tumour.

Many studies have demonstrated the importance of the gelatinases in tumour invasion and metastasis (Kanayama et al., 1998; Sugiura et al., 1998). The source of gelatinases appears to be largely stromal in nature, therefore deriving from the host. The importance of gelatinase B in invasion was demonstrated further by injecting the highly invasive melanoma cells, B16-BL6, into MMP-9 deficient mice (Itoh et al., 1999). The result was a decrease of 45% in the number of metastatic colonies found compared to

wild-type control mice. The expression of gelatinases is thought to be an important part of tumour metastasis because the gelatinases are able to degrade type IV collagen, a constituent of basement membranes, and an important structure for confining tumours.

1.7 Transcriptional Regulation of the MMPs and TIMPs

1.7.1 Promoter Organization of the MMPs

When comparing the promoters of the MMPs, the overwhelming feature is, with a few exceptions, the presence of at least one AP1 binding site in the promoter regions of the MMPs (Figure 3). This immediately suggests a common mechanism whereby a cell can coordinately regulate the activities of the ECM degrading enzymes. With the exceptions of MMP-2 and –11 (Benbow and Brinckerhoff, 1997), there is an AP-1 binding site located at approximately –70 base pairs upstream of the MMP transcription start site. Also noteworthy in the promoters of the MMPs is a prevalence of PEA3 binding motifs, although there is a great variability in both the number and placement of these sites. While there are clearly additional contributions from other regions of the promoter, and other cis-acting elements, it is the action of, and interaction between the AP-1 and PEA3 sites which is the most important feature of MMP transcriptional regulation.

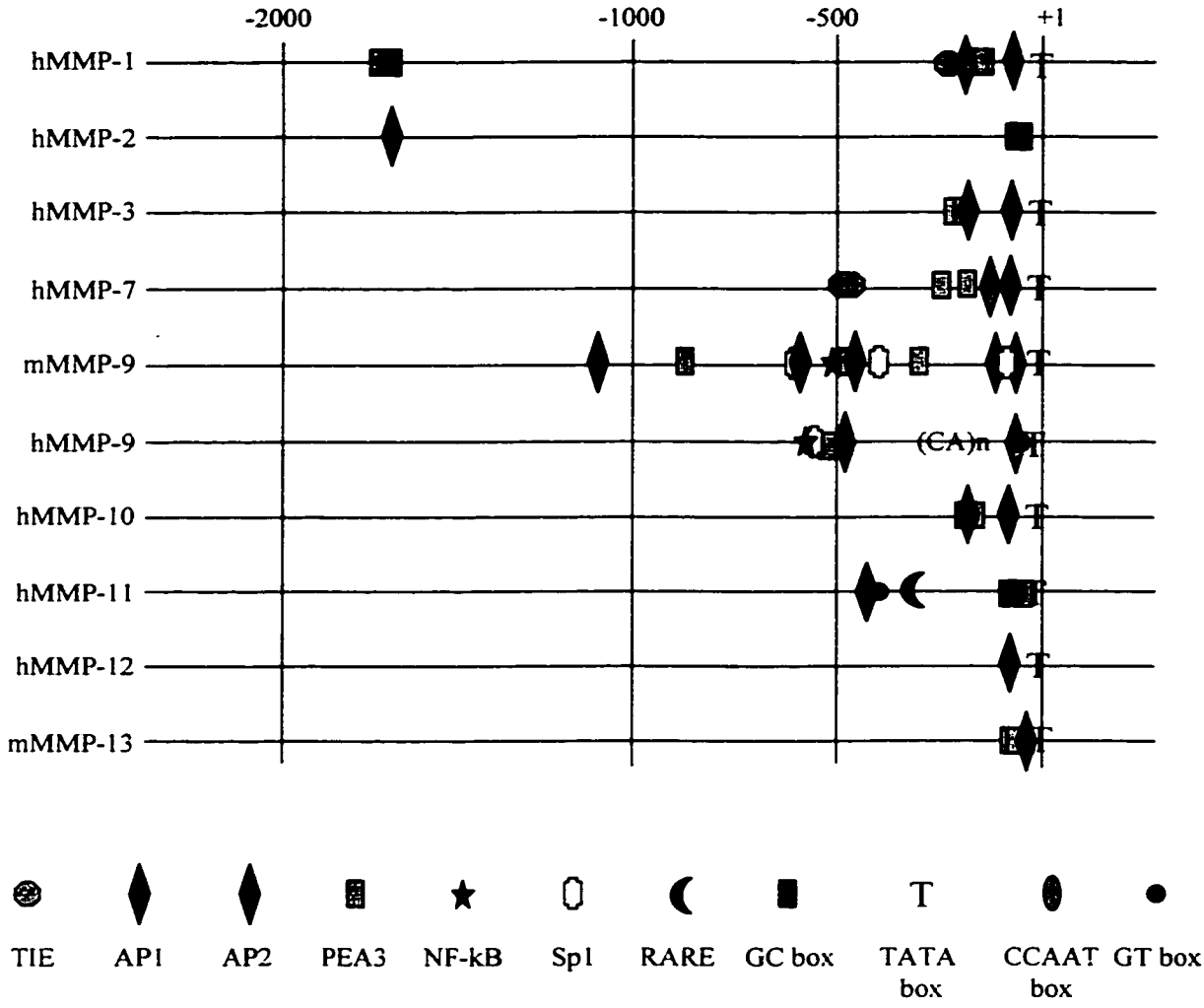


Figure 3. Promoter map of Matrix Metalloproteinases. Displayed are a series of maps to several MMP promoters. The transcription start site is designated +1, and the specific transcription factors corresponding to the legend, are placed approximately to scale. Of particular interest is the clustering of AP1 binding sites near the transcription start point. Additionally, there are often PEA3 elements adjacent to AP1 binding sites. From Benbow and Brinckerhof, 1997, Buttice et al, 1996, Munaut et al 1999, Gaire et al., 1994, Anglard et al., 1995..

1.7.1.1 The AP1 Transcription Factor Superfamily

The activating protein-1 (AP-1) transcription factor is especially relevant to the MMPs because it is the predominant means of upregulating the inducible MMPs. As well, the mechanism of AP-1 interaction with the AP-1 binding site (TRE) was first identified and studied on the collagenase-1 promoter (Angel et al., 1987). The AP-1 factor represents three different groups of proteins, each which form functional homo- or heterodimers through a bZIP (basic region leucine zipper) region. The AP-1 protein members belong to either the Fos (v-Fos, c-Fos, FosB, Fra1, Fra2), Jun (v-Jun, c-Jun, JunB, JunD) or ATF (ATF2, ATF3, B-ATF) protein groups (Angel and Karin, 1991). The ATF transcription factors can either homodimerize, or heterodimerize with Jun proteins, and are then able to interact with the cAMP-response element (CRE). Alternatively, Jun/Jun homodimers or Jun/Fos heterodimers interact with the AP-1 binding site (also called (TPA)-responsive element, TRE; TGACTCA). The possibility for many different dimerization combinations is thought to enable both binding specificity, and target response of the AP-1 transcription factor (Karin et al., 1997). The c-fos gene is induced very rapidly by a large number of stimuli, therefore placing c-fos in the grouping of immediate early genes. Alternatively, c-jun is expressed at low basal levels, although it is still induced by many stimuli (Karin et al., 1997).

The use of gene knockout models in mice has led to a better understanding of the functions of the individual AP-1 components. While c-fos is clearly induced by a number of growth factors, and is thought to play a critical role in cell growth and differentiation, the targeted elimination of the c-fos gene had only minor effects on the mice, which were growth retarded, and had bone deficiencies (Johnson et al., 1992).

Similarly, disruption of fosB also had only minor behavioral phenotypes in knockout mice (Brown et al., 1996). Alternatively, targeted deletion of c-jun has a more drastic effect on mouse development, and was lethal to the mice by mid-gestation (Johnson et al., 1993). The drastic phenotype in c-jun knockout mice might be explained because c-jun is prevalent in all AP-1 complexes. These results do not diminish the contribution of c-fos, but likely, demonstrate redundancy among the different members of the c-fos group.

1.7.1.2 The Ets Transcription Factor Superfamily

The Ets transcription factors family contains numerous members (c-ets-1, v-ets, erg, fli-1, FEV, PEA3, sp-1/Pu-1, erf) which are involved in development, signal transduction, and cell transformation (Laudet et al., 1999). All Ets proteins contain a conserved 85 aa sequence called the ETS domain, which targets the binding sequence GGA(A/T) (Wasylyk et al., 1993). A characterization of the ETS domain does not reveal any identity with other characterized DNA binding motifs, however, the 3-dimensional structure shows a winged-helix-turn-helix structure, which might be a feature necessary for its DNA interaction (Laudet et al., 1999). While the Ets family members are able to interact with DNA at their specific binding sites (Wasylyk et al., 1991), an important mechanism of regulating or directing Ets function is accomplished by protein-protein interactions. Specifically relevant to the regulation of MMPs is the interaction between Ets and AP-1. First described on the polyoma enhancer, it has now been shown that a number of genes have an Ets-AP-1 composite element important for gene regulation (Wasylyk et al., 1998; Wasylyk et al., 1990). A similar cooperation between Ets and AP-

1 factors has been seen in activation of the stromelysin-1 gene (Wasylyk et al., 1991), as well as the serine protease, uPA (Nerlov et al., 1991).

1.7.2 Transcriptional Regulation of the MMPs

It has been demonstrated in many of the MMP promoters that the AP-1 site located at –70 is important to both the overall basal expression of the gene, as well as induction by PMA and other cytokines and growth factors (Benbow and Brinckerhoff, 1997). This can be demonstrated by mutating the consensus AP-1 binding site (usually TGAGTCA, but often with slight variations) to render it incapable of interacting with the AP-1 factor. Additionally, making 5' deletions of the promoter regions until the AP-1 binding site is removed enables the study of the importance of different promoter elements. Both approaches have demonstrated that the proximal AP-1 binding site (-70) is important in regulating basal expression from the promoters of MMP-1, MMP-3, and MMP-9 (Auble and Brinckerhoff, 1991; Buttice et al., 1991; White and Brinckerhoff, 1995; White and Brinckerhoff, 1995). There is also evidence that the AP-1 sites of MMP-1, -3 and –9 are involved in cytokine and growth factor stimulation of these genes. Both MMP-1 and –3 are inducible by PMA, which is a strong protein kinase C agonist. However, reporter constructs containing 5' deletions of either MMP-1 or MMP-3 such that the AP-1 site is deleted, show a decreased response to PMA (Buttice et al., 1991; Gutman and Wasylyk, 1990; White and Brinckerhoff, 1995), demonstrating the importance of the AP-1 binding site in conveying the PMA response.

Most MMPs are induced by the cytokines EGF, PDGF, TNFα, and IL-1 (Benbow and Brinckerhoff, 1997). While studies have shown that the proximal AP-1 binding site

is often required for the full effect of the stimuli, the disruption of the element does not completely obliterate cytokine responsiveness of the promoter examined, therefore other regions within the promoter are important for the response as well. This is exemplified by the MMP-9 promoter, which clearly requires additional elements in addition to the –70 AP-1 site for full cytokine responsiveness (Sato et al., 1993; Sato and Seiki, 1993).

The AP-1 transcription factor complex is joined by the Ets family of transcription factors as critical regulators of MMP expression. The importance of c-Ets in MMP expression was seen in stably transfected endothelial cells whicn either expressed low or high Ets-1 (Oda et al., 1999). The cells which expressed high levels of c-Ets also expressed high levels of MMP-1, -3, and –9, and were more invasive in matrigel invasion assays when compared to the cells which expressed low levels of c-Ets. In another example, the overexpression of different Ets family members in stromal fibroblasts resulted in a different response on MMP-1 expression depending on the Ets family member (Westermarck et al., 1997). C-Ets-1 overexpression resulted in a 10 fold increase in MMP-1 whereas other Ets proteins, ERGB/Fli-1 and PU.1 had no effect on the basal activity of the MMP-1.

What is becoming clear in the transcriptional regulation of the MMPs, is that there is an interplay between the AP-1 and PEA3 transcription complexes in the activation of the MMPs. In many cases (though not necessarily at the –70 AP-1 site) there are AP-1 and PEA3 binding sites in close proximity. Both Ets and AP-1 are required for the full response of insulin in activating MMP-1 (Chapman et al., 1999), as well as in the regulation of MMP-3 (Wasylyk et al., 1991) and MMP-9 (Gum et al., 1996).

It has been demonstrated that there is a direct physical association between AP-1 and PEA3 factors, and that interactions can occur between c-Ets and Jun/Jun, or c-Ets and Jun/Fos (Bassuk and Leiden, 1995; Carrere et al., 1998; Wasylyk et al., 1990). Collagenase 1 is upregulated by the Ets protein, Erg, however, mutagenesis of the collagenase 1 AP-1 binding site abolished the response (Buttice et al., 1996). Furthermore, gel shift analysis showed that Erg can complex with Fos/Jun, and that this association is necessary for it to interact with the adjacent Ets-binding site. Interestingly, it appears that different members of the Ets family differentially augment AP-1 factors. For example, Ets-1 and Ergb/Fli-1 are both able to increase the response of components of the AP-1 factor, but in different manners. Ets-1 potentiated either JunB or c-Jun while ErgB/Fli-1 augmented only c-Jun (Westermarck et al., 1997). Alternatively, PU.1 abolished the JunB and c-Jun response, and deletion analysis showed that this disruption in AP-1 signalling is dependent on an intact AP-1 site (Westermarck et al., 1997). These studies collectively demonstrate the importance of c-Ets family members in the regulation of the MMP genes, and also that each specific family member also affects the overall response to the gene.

In addition to the aforementioned signalling pathways and stimulating agents, there are other cytokines which affect the MMPs. However, this appears to be a bit more individualistic, and serves to highlight the uniqueness of each of the family members. Studies on MMP-9 have shown that the gene is induced by scatter factor (hepatocyte growth factor, HGF), which appears to utilize the MAPK signalling pathway, as the stimulatory response is blocked by the MEK inhibitor PD098059 (Hasan and Nakajima, 1999). Additional studies have demonstrated an upregulation of MMP-9 by activated ras

(Gum et al., 1996), which is dependent upon a functional AP-1 binding site. Further importance to the MMP-9 AP-1 binding site is demonstrated by overexpressing v-src in human HT1080 fibroblasts, which results in the upregulation of MMP-9 mRNA. A functional AP-1 binding site is required for this upregulation, along with a GT-box downstream of the AP-1 (Sato et al., 1993). These data demonstrate that there are multiple signalling agents, using different signalling pathways which converge on the AP-1 binding site, but there are also contributions outside of AP-1 signalling which are important.

While there are examples of many MMPs being repressed by retinoic acid (see below), MMP-2 is enhanced by it, and it has been demonstrated that RA will synergize with cAMP, an effect which is dependent on a Cre-like sequence located at –245 of its promoter (Hasan and Nakajima, 1999). Another interesting mechanism in which MMP-1 is activated is through the contact between $\alpha_2\beta_1$ integrin and type I collagen (Pilcher et al., 1999). The mechanism appears to operate through the EGF signalling pathway, as EGF neutralizing antibodies block the response.

Additional promoter mapping studies have highlighted the importance of MMP transcription during osteoblast function. The collagenase, MMP-13, which is specifically localized to osteoblasts, is activated by the transcription factor Core Binding Factor I (CbfaI) (Jimemez et al., 1999; Porte et al., 1999). Direct in vivo evidence of a functional role for Cbfa1 is seen in Cbfa1 targeted knock-out mice, which lack mature osteoblasts (Jimemez et al., 1999).

There have been many examples of positive transcriptional regulation of the MMPs. However, there will also be situations which require the inhibition of expression.

In fact, promoter analysis has identified several examples of negative transcriptional regulation. Several MMPs have a TGFβ-inhibitory element (TIE; figure 3), and an analysis of the region has been performed in MMP-1 and -3. Deletion of the TIE disrupts the inhibitory effect of TGFβ stimulation (Kerr et al., 1990). In addition to the inhibitory response dependent upon the presence of the TIE, it is also hypothesized that TGFβ might be altering the composition of the AP-1 factor, and thereby repressing transcription in that manner. TGFβ has been demonstrated to induce JunB, which could then replace other members of AP-1 and repress the AP-1 response. Similarly, retinoic acid (RA) also has an inhibitory effect, which is often dependent upon the presence of an intact AP-1 (Pan et al., 1995). Another inhibitory mechanism by RA stimulation might be to alter the composition of the AP-1 factor, thereby affecting transcription (Uria et al., 1998).

There also appear to be other mechanisms by which the MMPs can be inhibited. A recent report has shown that the tumour suppressor gene p53 is able to inhibit the collagenase promoter in reporter assays (Sun et al., 1999). However, 4 of 6 p53 mutants lost this inhibitory potential. In contrast, MMP-2 is upregulated by wild-type p53 expression, but not by expression of the p53 mutants (Bian and Sun, 1997). These observations not only link ECM to the cell cycle control, but also have relevance to the ECM remodelling which is associated with cancer invasion and tumour suppression, as p53 mutations are common to invasive tumours.

While it has been demonstrated that there are many common features to the MMP promoters which enable a coordinate expression in times of required tissue remodelling, many of the MMPs also have specific tissue distribution. Two studies have examined different regions of the MMP-9 promoter with respect to tissue distribution. The first

looked at long MMP-9 promoter regions linked to lacZ in transgenic mice (Manaut et al., 1999), and compared expression patterns. The authors were able to look at the patterns of expression from constructs with the 5' MMP-9 promoter endpoint at –2700, or further upstream from –7200, with 3' endpoints either within the first exon, or including the first intron. The expression pattern showed that the –2700 LacZ mice did not show correct expression compared to the endogenous gene screened by in situ hybridization, even if the intron was included, although *in vitro*, in stimulated cultured cells, the fragment did resemble the endogenous response to various stimuli. Alternatively, the –7200 fragment did show similar expression patterns compared to the endogenous regardless of the presence of the first intron. Another study (Mohan et al., 1998) had a similar approach, but used much shorter constructs. A –522/+19 MMP-9-LacZ construct showed appropriate expression in vivo. However, a series of deletions, many of which also showed correct expression in vitro, were not expressed in the transgenic mice. These studies make two important points. The first is that in vivo studies are able to provide a more informative analysis of different promoter regions, and are vital for proper distribution studies. Also, there are many promoter elements which are involved in regulating the distribution of a specific MMP, and one cannot only focus on specific regions, like the proximal AP-1 site as the most important.

1.7.3 Gene Structure of the TIMPs

There are several indications that the members of the TIMP gene family arose through duplication of a single ancestral gene. There is a similarity in their intron/exon structure, where TIMP-2, -3 and –4 are all composed of 5 exons (Edwards et al., in press;

Olson et al., 1998). TIMP-1 differs slightly in that it is composed of 6 exons, however, Exon 1 is untranslated, and the first 2 exons in TIMP-1 contain the same information as the first exon of the other family members. Therefore, all TIMP genes have 5 protein coding exons.

All of the TIMP genes are located on different chromosomes: TIMP-1 on X (Willard et al., 1989), TIMP-2 on 17, TIMP-3 on 22, and TIMP-4 on 25 (Olson et al., 1998). One common feature of the genomic TIMP location is their placement within the synapsin gene family. TIMP-1 is located in an inverse orientation within intron 5 of the gene synapsin 1 (Derry and Barnard, 1992). The recently identified *Drosophila* synapsin gene also contained a TIMP-1 gene within its 5th intron, indicating that the chromosomal location of the two genes has been evolutionarily conserved from invertebrates to humans (Klaggs et al., 1996; Pohar et al., 1999). It was also recently demonstrated that the TIMP-3 gene is located within intron 5 of the synapsin 3 gene, which shows that at least TIMP-1 and –3 were duplicated (Pohar et al., 1999). The authors are presently studying TIMP-2 and –4 to determine whether the pattern holds for the remaining family members as well. Despite the evolutionary conservation in localization between the TIMPs and the synapsins, no functional or regulatory link between the two genes has been identified.

1.7.4 TIMP Promoter Organization

While there are indeed many conserved similarities between the TIMP genes, the TIMPs have very individual characteristics as well, namely in their inducibility and tissue distribution. Accordingly, there is a great divergence in the promoter regions of the family members. At this time, very little is known about the promoter of TIMP-4,

however, a lot of work has been performed in identifying important regions and elements within the promoters of TIMPs 1 through 3.

The promoter of Timp-1 differs from the other family members in that there is an absence of a TATA box, multiple transcription start sites, and several SP1 binding sites clustered within the first intron, and the upstream promoter regions (see figure 4). These are all highly indicative of a housekeeping gene, however, there are also 2 AP-1 sites, and several PEA3 binding motifs, which are thought to contribute highly to the inducible nature of the gene. The human TIMP-1 gene shares many of the same features as the murine gene including the AP-1 and PEA3 sites. However, there are some notable exceptions, as there is a canonical AP-1 binding site within the first intron of the human TIMP-1 located at +150 which is not present in the murine promoter (Clark et al., 1987). TIMP-2 and –3 each initiate transcription from a single transcription start site, and both contain a TATA box (with the exception of human TIMP-3). TIMP-2 is quite devoid of transcription factor binding sites, containing several SP1 sites, which are not commonly associated with induction, and an AP-1 site and two PEA3 sites located quite far upstream for the transcription start site (between –700 and –900). Deletion studies have not shown any particular regions of the promoter which are important regulatory elements, including the AP-1 site at –288 (De Clerck et al., 1994). Similar to TIMP-2, TIMP-3 does not have any cis-acting elements in close proximity to the transcription start point, however, there are a number of AP-1 sites which are important in the regulation and induction of the gene (Wick et al., 1994).

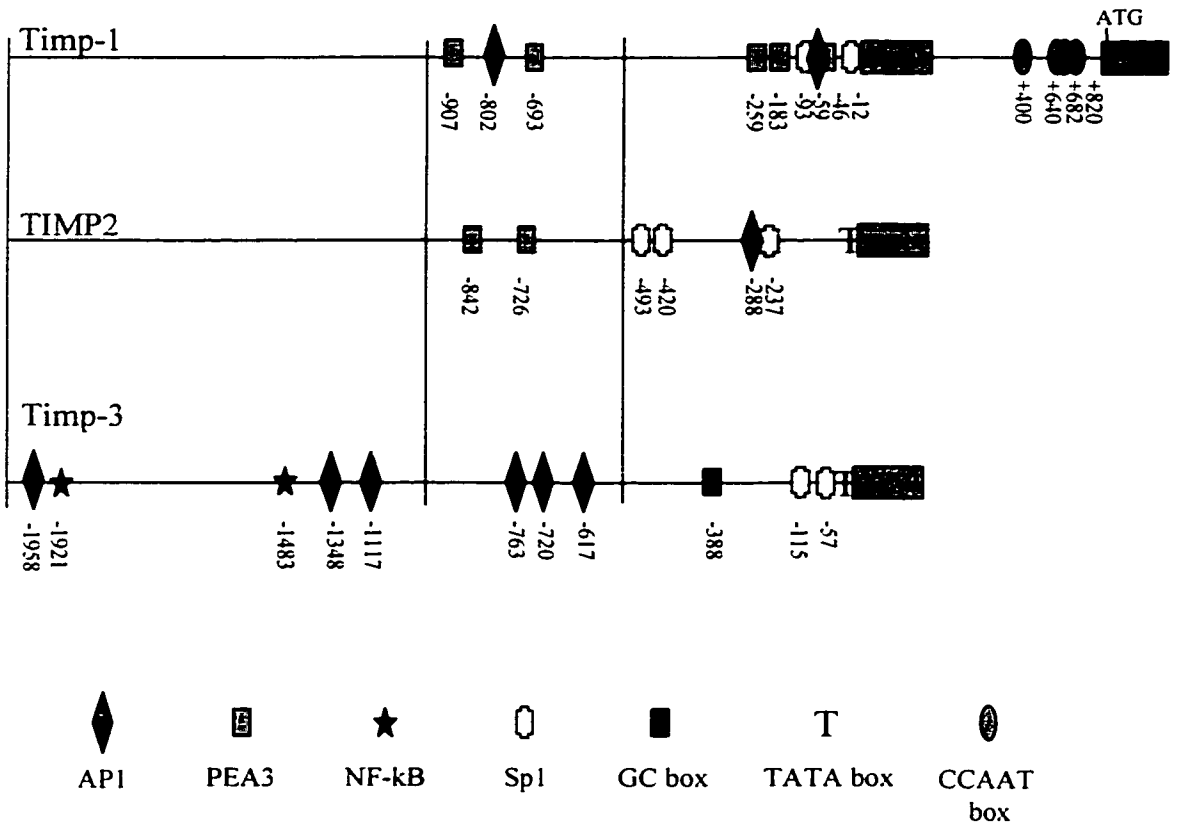


Figure 4. Promoter Map of the Tissue Inhibitors of Metalloproteinases. Depicted are the cis-acting transcriptional regulatory regions of the Timps - specifically, the elements presently identified. Not only are there differences in the organization and type of elements regulating the transcription from the gene family, but Timp-1 contains enhancer elements within the first intron.

1.7.5 The Murine TIMP-1 Promoter and Cis-Acting Elements

1.7.5.1 The Promoter Proximal Region (-223/+47)

Both deletion and mutagenesis analyses have been used to identify key regulatory regions of the *Timp-1* promoter. The promoter can be broken up into several functional units, defined arbitrarily by restriction enzyme sites within the gene. The promoter proximal region lies between a HindIII and BamHI sight, from –223 to +190 respectively (where +1 designates the transcription start site). This fragment contains many key elements involved in *Timp-1* regulation. Of critical importance is an AP1 binding site in close proximity to a PEA3 element. There are several interesting points about the *Timp-1* AP1 site. First is that the *Timp-1* AP-1 binding site (TGAGTAA) differs from the consensus AP-1 sequence (TGAGTCA) by a single base. This is not unique to the murine *Timp-1* gene, but has been conserved in the TIMP-1 genes identified in human, horse, and mouse (Clark et al., 1997; Edwards et al., 1992; Richardson and Dodge, 1998). While initial studies suggested that the *Timp-1* AP-1 sequence in isolation is unable to interact with AP-1 (Risse et al., 1989), later studies from our lab (Edwards et al., 1992) demonstrated not only that AP1 interacts with the sequence, but that it has a very strong positive effect, and is largely responsible for the serum activation of the gene. The failure of the TGAGTAA isolated sequence (Risse et al., 1989) to interact with AP-1 suggests that additional flanking sequences are required for the interaction.

Investigations from within our lab have examined whether there are differences between the consensus AP-1 binding site compared to that found in the Timp-1 promoter. The Timp-1 site interacts with both c-Fos and c-Jun, because EMSA reactions reveal a complex which is not only competed by a cold sequence corresponding to the collagenase

AP-1 site, but also, is supershifted by c-Fos and c-Jun antibodies (Sharma, 1993). It was also revealed that the Timp-1 AP-1 binding site interacts with additional proteins, as demonstrated by a higher mobility band on EMSA. Experiments in this thesis define some of the characteristics of these high mobility bands.

In addition to the observation from our lab, another group has also seen multiple protein-DNA interactions with the Timp-1 AP-1 binding site in activated hepatic stellate cells (Bahr et al., 1999). EMSA analysis showed two complexes formed at the AP-1 binding site, defined by a fast (high mobility) or slow (low mobility) migration in the native gels. The low mobility shift formed shortly after induced-activation of the hepatic stellate cells, and appears to be composed of AP-1 proteins (Bahr et al., 1999). However, the high mobility shift was of an unknown composition, but possibly contained different Fos/Jun family members, or alternatively, AP –1 proteins heterodimerized with other transcription factors. This data suggests that there might be differences in the composition of the AP-1 factor for different sequences of the AP-1 binding site.

Directly adjacent to the TIMP-1 AP-1 binding site is a PEA3 element, which is protected following DNAse I footprinting analysis. This site is a binding site for the c-Ets family of transactivator proteins (Macleod et al., 1992; Xin et al., 1992). Similarly to the case described for many MMPs, there is a synergistic response between AP-1 and PEA3 in the regulation of *Timp-1* (Logan et al., 1996). Therefore, a synergism between AP-1 and Ets factors appears to be a common mechanism in the control of the ECM regulatory genes. This might be a mechanism through which a coordinate regulation of the MMPs, and TIMPs can occur, as there are many examples where TIMP-1 and MMP-

1 and MMP-9 are coordinately upregulated in different pathological situations, such as in cancer metastasis.

In the case of *Timp-1*, a synergistic effect between AP1 and PEA3 was demonstrated by two different methods (Logan et al., 1996). First, using –70/+190 CAT constructs, it was shown that transfection with c-ets-1 alone had no effect on reporter expression. However, cotransfection with c-ets-1 plus low levels of AP1 resulted in high CAT activity, much higher than the same amount of AP-1 alone. Similar results were obtained when the –70/-40 region, which contains both the AP1 and PEA3 binding sites, were put into a reporter construct with a heterologous promoter (tkCAT), where the combination of AP1 and c-Ets-1 resulted in enhanced reporter activity (Logan et al., 1996).

What makes this region within the promoter proximal region even more complicated is the linking of Oncostatin M (OSM) induction of TIMP-1 to a STAT binding site located between the AP-1 and PEA3 elements within the proximal promoter (Bugno et al., 1995; Richards et al., 1997). This was demonstrated by the presence of two bands in an EMSA of a region including the AP1 binding site to just upstream of the PEA3 site. One complex, a constitutive band, was competed by cold AP1. Another complex, induced by OSM was competed by cold STAT (Bugno et al., 1995), as well as by induction from a –95/+47 CAT reporter construct following stimulation by OSM (Richards et al., 1997). While it has been observed that OncM signals through the Timp-1 AP-1 binding site (Botelho et al., 1998), there has been no evidence described that there is synergy between OncM and either AP-1 or PEA3.

1.7.5.2 Upstream Promoter (-2700/-223)

Though the importance of the promoter proximal region in *Timp-1* gene regulation is indisputable, there are clearly other regions which contribute towards *Timp-1* expression. Comparing a large fragment of the promoter, -2700/+47 to just the promoter proximal (-223/+47) region of the promoter results in an increase in basal activity, as well as a slight increase in serum inducibility (Edwards et al., 1992). There are several regulatory elements in this large fragment of the promoter, including an AP-1 site, and two PEA3 elements. When a series of nested deletions of this site were made (from –2700/-223 to –347/-223) and subcloned into a tkCAT driven vector, CAT reporter assays resulted in high CAT activity from the –1030/-223tkCAT construct, which contains all the elements defined above, and a decreasing CAT activity as the elements were deleted away (Edwards et al., 1992).

Additional evidence that there are important regulatory elements within the upstream region of the promoter comes from growth factor responsiveness. Specifically, the Timp-1 gene undergoes a process of synergism at the mRNA level when it is stimulated with the combination of TGFβ and PMA together (Edwards et al., 1987). This phenomenon, which is demonstrated by the endogenous gene, is not replicated by the promoter proximal (-223/+47) region of the promoter. However, inclusion of the entire upstream region of the promoter (-2700/+47) did show the superinductive effect between TGFβ and PMA *in vitro* (Edwards, unpublished data). Taken together, these data emphasize that while the promoter proximal region is the key regulatory region, there are contributions from many other areas of the promoter which are required to produce the spectrum of responses seen for the *Timp-1* gene.

1.7.5.3 Intron 1 Regulation of the Timp-1 Gene.

Some of the early work on the Timp-1 promoter was performed on the region upstream of the first protein coding exon. However, shortly thereafter, an untranslated first exon was identified, which meant that the preliminary gene regulation work was performed upon the first intron (figure 4). It was shown by using the intron fragment (+85/+760) bound to a reporter that this region resulted in increased basal reporter expression (Coulombe et al., 1988). A detailed analysis of the sequence revealed the presence of several CCAAT boxes, to which the increased activity was attributed.

While the importance of these CCAAT boxes for basal expression of Timp-1 has been demonstrated, there are also additional regions which appear to be important. This can be demonstrated by comparing constructs which contain part of the intron (-223/+190) to constructs which terminate in the center of the first exon (-223/+47) in reporter assays, where in *Timp-1*, the first intron extends up to +90. In these experiments, it was shown that the inclusion of part of the intron resulted in a greater basal activity. Several groups have indicated that there might be a second AP-1 binding site present in the *Timp-1* promoter, and it is thought to be located between the regions, +47/+190 (Logan et al., 1996). In key experiments demonstrating this, CAT reporter constructs driven with either –70/+190 or –50/+190 were co-transfected with c-Fos and c-Jun expression plasmids. As expected, reporter expression from the –70/+190 construct was upregulated by overexpression of c-Fos-c-Jun, as this region still retains the promoter proximal AP-1 binding site. However, surprisingly, expression from the –50/+190 reporter construct, in which the AP-1 site is deleted, was also increased (Logan et al.,

1996). These experiments were performed on the mouse *Timp-1* promoter, however, data from the human intron reveals the presence of a canonical AP-1 site which was not conserved in the mouse.

Following the cloning of the human TIMP-1 gene and promoter region (Clark et al., 1997), a series of deletions were made examining the important regions of the intron. These deletions showed important elements located in the 5'-end of the first exon, and also demonstrated that inclusion of the exon-1/intron-1 boundary is important to TIMP1 expression (I. Clark, personal communication).

While there are several regions within the intron that act in a positive fashion, there is also evidence for a silencer element within the intron, which comes from a pair of intron constructs driving the CAT reporter. One construct spans the large promoter fragment of –2700/+190, and the other contains most of the intron, at –2700/+770. The interesting observation is that the truncated construct has quite high CAT activity; however, inclusion of the intron had a strong suppressive effect (Logan, S. Personal communication).

1.7.6 TIMP-2 and TIMP-3 promoters

Unlike TIMP-1 and –3, which are inducible by a large number of stimuli, TIMP-2 shows more signs of being a housekeeping gene. It has a TATA box, and 5 SP1 sequences, and is expressed in most tissues (De Clerck et al., 1994). Additionally, the promoter is not responsive to a number is stimuli, including TPA, TGFβ, IL-1 and TNFα, despite containing an AP1 binding motif (De Clerck et al., 1994).

The promoter of TIMP-3 differs from that of TIMP-1 primarily in organization. The TIMP-3 promoter contains 5 exons, therefore does not have the untranslated exon seen in TIMP-1. However, similarly to TIMP-1, TIMP-3 lacks a TATA box, and contains many potential transcription factor binding sites. Such sites include the factors SP1, LBP, GM-CSF, AP2, and c-myb [Zeng, 1998 #172; Clark, 1997 #17; Edwards, 1999 #78].

1.8 Objectives

What is becoming more apparent in a growing number of diseases, is that the pathological remodelling of the ECM is often associated with a loss of regulation of either MMPs, TIMPs, or both. Earlier work from our lab has shown that the principal method of *Timp-1* regulation is transcriptional based. Furthermore, many regions have been identified which are critical in the regulation of *Timp-1* expression, as described. However, there are clearly many regulatory elements within the promoter which have not been characterized.

The recently identified *Timp-4* gene has not been examined in detail yet. While the *Timp-4* gene itself shows a very restricted pattern of expression, it has recently been shown to be upregulated in some disease processes, such as glioblastoma invasion (Groft, unpublished data) and during arterial injury (Dollery et al., 1999). These results highlight the need for further research into the regulation of this gene.

The specific aims of this research were :

1) **The role of intron-1 in the regulation of *Timp-1*.** Transgenic mice containing the promoter region lacking the first intron were used for this approach. Furthermore, a search for positive regulatory regions between +47 and +190 was undertaken, specifically to identify the location of a putative AP-1 binding site.

2) **Identification of the location of regions within the proximal promoter that are important for Timp-1 basal expression.** Previous results have highlighted the importance of a region, -223/-95, upstream from the AP-1/PEA3 elements in the positive regulation of basal *Timp-1* transcription. This region, in addition to the irregular AP-1 binding site itself were examined in detail.

3) **Preliminary analysis and identification of the Timp-4 promoter.** While the gene itself has been cloned in both humans and mice, the promoter has not yet been identified in either species. Therefore, an initial screen was performed using RACE-PCR, and based on the identification of several transcription start points, deletion analysis was used to locate regions important for transcriptional regulation.

This research is necessary in order to fully understand ECM regulation that occurs during both normal and pathological situations. While changing expression levels of both the MMPs and their inhibitors are associated with many normal and pathological states, there is still no understanding of the mechanisms leading to these changes. A fundamental knowledge of the regulation of gene expression necessitates the examination of the promoters involved. This thesis is aimed at studying the mechanisms of transcription of both *Timp-1* and *Timp-4*. The protein levels of these inhibitors are important in the overall regulation of the ECM. Knowledge of their transcriptional

regulation is essential for understanding how ECM balance is swayed to favour proteolysis or synthesis.

Chapter 2. Materials and Methods

2.1 Cell Culture

2.1.1 Maintenance of Cell Lines

Murine fibroblast C3H10T1/2 cells were cultured in Dulbecco's Modified Eagles medium/Ham's F12 (DMEM/F12) containing 10% fetal bovine serum (FBS, Life Technologies, Inc). Whereas antibiotics were added for experimental procedures (1% antibiotic/antimycotic, Life Technologies, Inc), routine maintenance of cells was done free of antibiotics.

2.1.2 Transient Transfections: Calcium Phosphate Precipitation

Transient transfections were performed by two different methods. Initially, the calcium chloride precipitation protocol of Chen and Okoyama (1987) was used. However, recently, the commercially available FuGENE 6 transfection reagent was utilized (Roche). For both protocols, the cells were plated at a density of $5x10^5$ cells per 10cm dish. It was previously recommended to use $1x10^6$ cells per dish, however, I found that there was an increased transfection efficiency at the slightly lower density for C3H10T1/2 cells. Following the plating, the cells were grown overnight in media containing 10% FBS. It is important in the calcium chloride precipitation protocol that the cells do not achieve confluency, as the uptake of the DNA is dependent upon the presence of dividing cells. The cells were transfected the following day, using either the calcium phosphate precipitation method or FuGENE (discussed separately).

Transfection of the cells using the calcium phosphate precipitation protocol, 20 μg plasmid was brought to 900μl final volume in TE, followed by the addition of 100μl $CaCl_2$ (2.5M). The mixture was then added dropwise to 1 ml of 2xBES-buffered saline

(50mM BES, 280 mM NaCl, 1.5mM Na_2HPO_4, pH to 6.96) with constant mixing on a vortex mixer. The mixture was allowed to stand for exactly 20 minutes without any agitation - this is critical to the formation of DNA-salt precipitate that is of the most efficient size for uptake into the cells. Then, 1 ml of the precipitate solution was added dropwise to each of 2 plates of cells. The cells were incubated for 18 hours at 37°C in an atmosphere of 97% (V/V) air, 3%(V/V) CO_2, which was also important for optimal uptake of the precipitate. The following day, the cells were allowed to recover by replacing the media with fresh 10% FBS media, and incubated at 5% CO_2 (V/V). After an 8 hr recovery period, the cells were serum starved for 24 hours prior to serum stimulation. Unstimulated cells were given a change of serum free media after the recovery. Twenty-four hours after stimulation, cell extracts were collected by one of two methods. Three cycles of freeze-thaw were performed by moving the extracts between the -70°C freezer and the +37°C water bath. It is important to let the samples reach -70°C by leaving them at that temperature for at least 30 minutes. After the final freeze-thaw, the supernatant was collected by centrifuging the sample at full speed (13,200 RPM) for 5 minutes, then transferring it to a new tube. Alternatively, the cell extracts were collected using the commercially available Reporter Lysis Buffer (Promega). To lyse the cells, 500 µL of the detergent-containing buffer was dropped onto the cells, and the plate was agitated so that the entire surface is covered. The cells are then incubated for 30 minutes before they were scraped into a tube using a rubber policeman. Similar to the freeze thaw protocol, the supernatant was then collected by centrifugation of the sample, and transferred to a new tube.

2.1.2.2 Transient transfections: FuGENE

The FuGENE transfection protocol relies on the mechanism of lipid fusion for internalization of the plasmid DNA. FuGENE 6 (Roche) is a non-liposomal lipid blend which is mixed with DNA, then added to the cells. For transfection of C3H10T1/2 cells, I followed the protocol recommended for adherent cells. Because of the increased transfection efficiency of the FuGENE protocol, smaller culture dishes (35mm) were used (cells were plated at $1x10^5$ for 6 well dishes, or $5x10^5$ for 90mm plates. Similar to the calcium phosphate precipitation protocol, transfection was most efficient when the cells were in the log growth phase. To prepare the FuGENE 6, 4uL was added to 100uL of serum free media in a 5 mL snap-top tube (Falcon). Care was taken to avoid touching the FuGENE 6 reagent to the tube, which inactivates it. This mixture was incubated at room temperature for 5 minutes, then added dropwise to a second tube, which contained 1ug of plasmid DNA. The solution was carefully mixed, then incubated for 15 minutes at room temperature. Finally, the solution was added dropwise to a plate of cells.

Prior to switching between the two transfection protocols, I performed a side-by side comparison of the two, both in 100 mm culture dishes. Three different constructs were each transfected by FuGENE 6 as well as by the calcium phosphate precipitation protocol (figure 5). With all constructs examined, the use of the FuGENE reagent resulted in approximately 2 fold greater overall CAT activity compared to the calcium phosphate precipitation method. Additionally, I have since found the FuGENE method to be more consistent (data not shown).

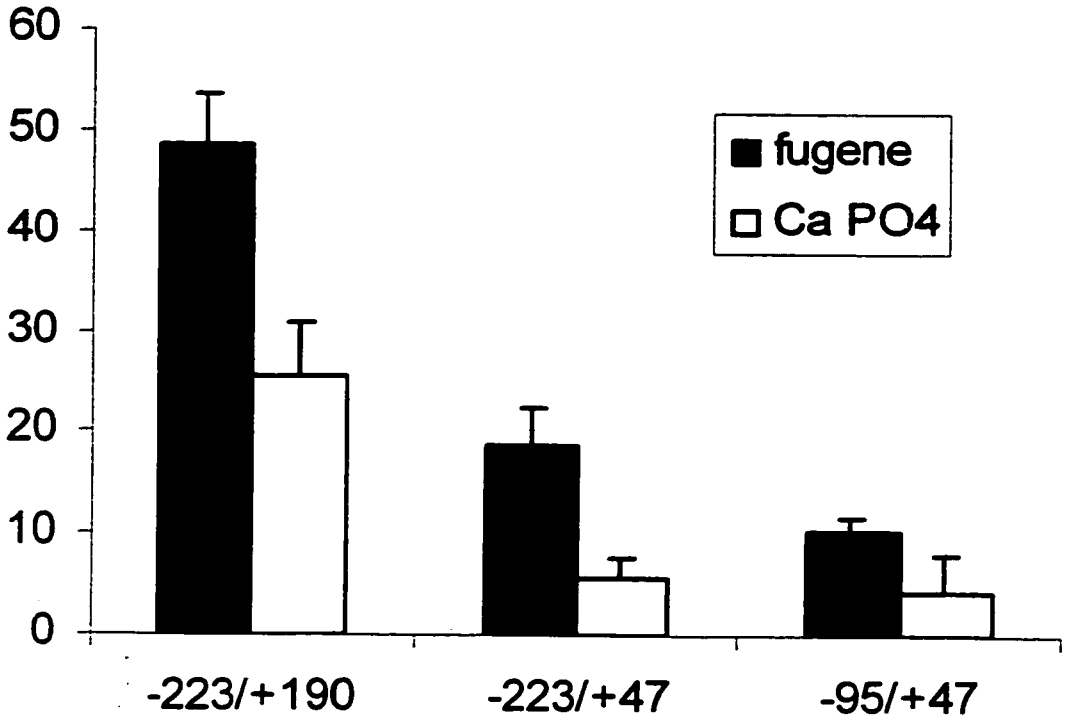


Figure 5. Comparison of the FuGENE 6 and the Calcium Phosphate Precipitation Transfection Protocol. A cis-by side comparison was performed between the two protocols, using the same number of cells. For the Calcium phosphate precipitation, 10ug of DNA was used per plate, compared to 2ug for the FuGENE 6. While the transfection varied by method, the post-transfection treatment, as well as harvesting and CAT assays were identical in methodology (see methods and materials).

2.1.2.3 Transfection Standardization

All transfections were standardized with the Hirts assay for DNA input as described (Hirt, 1967). When collecting the cell extract for reporter analysis, the remaining components (DNA, RNA, cell membranes, etc) were left in a pellet. These pellets were lysed in 0.5ml Hirt's solution (0.6% SDS, 10mM Tris-HCl (pH 7.5), 10mM EDTA) for 10 minutes at room temperature. This was followed by the addition of 60µl of 5M NaCl and 20µl proteinase K (10mg/ml), which was then incubated for 3 hours at 37°C. After the protease digestion, the mixture was centrifuged for 5 minutes at 12,000 RPM, and the supernatant retained and extracted with 1:1 phenol:chloroform. The samples were then prepared for slot-blot transfer by mixing 50 µl supernatant with 16.7 µl 1M HCL for 5 minutes to denature, followed by the addition of 117 µl neutralization solution (0.9M NaOH, 2.25M NaCl) for 15 minutes. The volume was increased to 510 µl with buffer (1.4M Tris-HCl (pH 7.0), 1.5M NaCl) and then loaded onto a slot-blot manifold for transfer onto duralon-UV membranes (Stratagene, La Jolla CA). Following transfer, the samples were fixed by UV crosslinking, hybridized to specific probes (pBLCAT3, or pGL2-basic) and exposed onto X-ray film (Kodak). Intensity of signal (measured densitometrically) was then used to standardize the reporter expression to amount of input plasmid.

2.2 Histology

2.2.1 Animal Handling and Treatment

Transgenic mice were produced in the host strain of BALB/c. The protocol for the production and husbandry of transgenic mice was approved by the University of

Calgary Faculty of Medicine Animal Care Committee. All animals were treated in accordance with guidelines established by the Canadian Council on Animal Care.

2.2.2 Tissue Collection, and Processing

Homozygous transgenic mice (no older than 6 months old) were mated, and mating was monitored by the appearance of a mucous plug. The pregnant mice were sacrificed at specific times following mating (12.5 days, 14.5 days, etc) by cervical dislocation. The uterus was exposed by making a mid-line incision into the abdomen, and was removed into a 75cm^2 petri dish containing ice-cold PBS. The embryos were dissected out of the uterus, and placed into sample trays filled with embedding media chilled to 4°C (OCT, Sigma). In order to snap-freeze the tissue block, the sample was placed into liquid nitrogen for 10 seconds, then stored at –70°C until processing. The samples were processed within 6 months to prevent dehydration of the tissue blocks in the freezer.

2.2.3 Sections and Staining

The frozen tissue blocks were warmed up to –30°C by placing them in the cryostat microtome chamber. 10μm tissue sections were cut, and mounted onto gelatin coated microscope slides. The slides could then be stored at –70°C indefinitely prior to staining.

Staining of the embryo sections for β-gal activity was performed in four steps. The slides were first soaked in 0.1M PBS (pH 7.4) for one minute in coplin jars. Next, they were transferred to a fixative (0.2% glutaraldehyde, 0.1M phosphate buffer (pH=7.4), 2mM $MgCl_2$, 5mM EGTA) for 15 minutes. The glutaraldehyde was washed

out with 2x15 minute changes of wash buffer (0.1M PBS [7.4], 2mM $MgCl_2$, 0.01% (w/v) sodium desoxycholate, 0.02% (w/v) Nonidet P-40). Finally, the samples were stained in an X-gal-containing staining buffer (wash buffer + 1mg/ml X-gal (in DMF), 5mM $K_4Fe(CN)_6 3H_2O$, 5mM $K_3Fe(CN)_6$, 1mM spermidine hydrochloride). The slides were incubated in the staining buffer overnight (18-36 hours) at 37°C, with gentle shaking. It is important to ensure the staining buffer is kept dark during the staining period as the X-gal is light sensitive. After staining, the slides were rinsed in water, and counterstained with eosin before coverslip mounting in permount mounting medium. The sections were then examined by light microscopy.

2.3 Recombinant DNA techniques

2.3.1 Polymerase Chain Reaction (PCR)

Many of the constructs used in this thesis were generated by amplifying specific promoter fragments using PCR. For each PCR reaction, 10ng template DNA (or 2μL of reverse transcription product) was mixed with PCR buffer (10mM Tris-Cl [8.3], 50mM KCl, 5mM $MgCl_2$, 0.2mg/mL BSA). To this mixture, dNTPs were added to a final concentration of 600μM and sense and antisense primers were added (to a final concentration of 400pM), along with water for a final volume of 50μL. A hot start was used, where the samples were placed into the PCR block (Hybaid, or DNA Engine) at 94°C, and 1 unit of *Taq* polymerase added at that temperature. Samples were run on a standard thermocycle program: 1) denature, 94°C, 4 minutes. 2) thermocycling, 94°C, 1 minute, 55°C, 1 minute, 72°C, 1 minute. 3) extension, 72°C, 4 minutes. Following PCR

amplification, the samples were analyzed by gel electrophoresis, and gel purified on a 1-2% agarose gel.

2.3.2 RACE PCR

The 5'-RACE-PCR was performed using the 5'/3' RACE kit (Roche) according to the manufacturers protocol. The multistep procedure used 3 day murine heart RNA as the template material. Three antisense primers were produced based on the first exon of Timp-4 (see figure 33), SP1 (CAGTCCGTCCAGAGACACTC), SP2 (GATACTGCTTGTGACTGTTG), and SP3 (CGTATCACTAGAGCCGAGTG). The three Timp-4-specific primers provided three distinct levels of specificity to the PCR protocol. The first step was the first strand synthesis of the mRNA extracted from day 3 mouse heart. The AMV reverse transcriptase was used because its optimal incubation temperature is higher than MMLV-RT, and therefore fewer regions of RNA secondary structure would be encountered by the enzyme. A total of 2μg of total RNA was reverse transcribed using the SP1 primer as per manufacturers directions (2μg mRNA, 1x cDNA synthesis buffer (Roche), SP1 primer (1.25μM), and dNTP [0.5μM]). The mixture was incubated at 55°C for 1 hour.

The purity of the DNA is important, therefore, the RT product was cleaned using the high pure PCR product purification kit (Roche) as per manufacturers directions. The product was then poly A tailed using terminal transferase (1x reaction buffer (Roche), 19μL of purified cDNA, 200μM dATP, 10 U terminal transferase – final volume 25uL). The reaction was incubated at 37°C for 20 minutes, then the enzyme was denatured at

70°C for 10 minutes. This tailed cDNA was then used for amplification by PCR using the Timp-4-specific primer (SP2) as the 3' primer, and an oligo dT-anchor primer as the 5' ($T[T]_{18}T[A/C/G]$). The PCR reaction was performed using a high fidelity PCR kit (Expand® high fidelity PCR system, Roche) as per manufacturers protocol. Following the first round of PCR, the product was examined on a 1% agarose gel. If the product was not a clear single band, a second round of PCR was performed using the first PCR product as a template. In addition, for greater specificity, the SP3 primer was used.

After the second round of PCR, the resulting product was gel purified, and subcloned into an AT-PCR product vector (Pharmingen). The resulting clones were then sent for commercial sequencing (Cambridge Bioscience).

2.3.3 Cloning techniques

2.3.3.1 Restriction digests

A total of 5μg of plasmid was routinely used for restriction digest for the purpose of cloning. The DNA was mixed with the buffer recommended by the restriction enzyme manufacturer, along with water to a final volume of 29μl, such that the buffer was diluted to 1x. A volume of 1μl (10 units) of the appropriate restriction enzyme was added to the mixture, and allowed to incubate at 37°C overnight. The restriction digests were terminated by the addition of 6x DNA gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 100mM EDTA). The samples were then analyzed by PAGE, and compared to a 100bp DNA ladder (Roche) for a confirmation that the digests went to completion, and that the product was the appropriate size. Fragments were then photographed, or gel purified (see next section).

2.3.3.2 Gel purification

DNA was electrophoresed on 1% agarose gels containing 0.3 μg/mL ethidium bromide until sufficient resolution of the bands occurred. The bands were then eluted by making an incision in the gel immediately in front of the band of interest, and placing a small sheet of DEAE cellulose membrane moistened with 1xTAE in the space. The gel was then placed back into the electrophoresis tank and a current applied until the band migrates onto the membrane. Next, the membrane was removed, and dipped into TE to rinse off any agarose. Then, the membrane was placed into a tube containing 300 μl of high salt elution buffer (50 mM Tris-HCl [8.0], 1 M NaCl, 10mM EDTA [8.0]) and incubated at 65°C for 30 minutes. At this point, the membrane was checked under UV light to ensure that the DNA was eluted. The eluate was then purified by phenol-chloroform extraction, then ethanol precipitated. The concentration of the DNA recovered was determined by measuring the optical density (260nm).

As an alternative to the above protocol, DNA can be eluted by cutting out the band with a scalpel, and using the QIAX2-gel purification kit according to manufacturers directions (Roche).

2.3.3.3 Klenow Polymerase Fill in Reaction

Cloning of inserts into blunt-ended vectors was performed by filling in the 5'-overhangs of the cDNA or enzyme-digested vector, using klenow polymerase enzyme (Gibco-BRL). The DNA (up to 200μg) was added to a mixture of 1x react 2 buffer (Roche), 16μM dNTPs, and 1 unit of klenow polymerase (Roche). The mixture was

incubated for 30 minutes on ice, and the enzyme removed by phenol-chloroform extraction.

2.3.3.4. Ligations

All ligations were performed using the Rapid DNA Ligation Kit (Roche) according to manufacturers directions. All DNA fragments used for ligations were first gel purified, and phenol chloroform extracted (or purified by ion exchange). Typically, a vector:insert ratio of 1:3 was used, and the final amount of DNA used for the kit did not exceed 100 ng. The DNA was brought up to 20µl volume in DNA ligase buffer (Roche), and 10 units of T4 DNA-ligase was added. The reaction proceeded at room temperature for 5 minutes, then the ligated sample was used directly to transform competent bacteria, or stored at –20°C until needed.

2.3.3.5 Preparation of Competent Bacteria

The recipient strain (XL-1 blue, or DH1) were grown in 500mL of LB media to log phase (OD_{600}=0.2), then chilled on ice for 15 minutes. The cells were kept at 4°C for the rest of the procedure. The cells were collected by centrifugation at 2000g for 15 minutes (4°C), then resuspended in 200mL of cold, sterile $CaCl_2$ (100mM). The suspension was chilled on ice for 20 minutes, centrifuged, the pellet resuspended in 5mL of sterile 100 mM $CaCl_2$, and stored on ice for 24 hours. The next day, sterile glycerol was added dropswise to the cell suspension (10% vol/vol). The glycerol was added dropwise, as heat released by the dissolving glycerol can reduce the efficiency of the competent cells. The cell mixture was then aliquoted into 1.5mL sterile tubes (200µL or

400μL aliquots) and snap-frozen in liquid nitrogen. Cells may be stored at –70°C for up to 1 year.

2.3.3.6 Transformation of Competent bacteria

The primary method used for transformation of plasmid DNA into bacteria was by heat shock. The bacteria used were either competent XL1-blue, DH1 (section 2.3.4.4), or commercially available supercompetent XL1-blue bacteria (Stratagene). Either 1ng of plasmid DNA, or an entire ligation reaction were mixed with 100μL of competent cells (or 30 μL of commercial supercompetent cells), and chilled on ice for 20 minutes. The mixture was then placed in a 42°C water bath for exactly 90 seconds, and then returned to ice for 5 minutes. 300μL of LB was added to the cells and incubated at 37°C for 30 minutes. Different volumes of the bacteria were then spread onto ampicillin-containing LB-agar plates (10μg/mL). For the commercial competent bacteria, 20 μL and 60μL provide a good range of positive colonies. For self-prepared bacteria, 50μL and 200μL were plated. The bacterial plates were then incubated overnight at 37°C.

2.3.3.7 Preparation of DNA probes

Two different protocols were used to label DNA probes for screening bacterial colonies, nick translation and random priming. For nick translation, 100 ng of DNA was used as a template. The DNA was added to a nick translation buffer mix (50mM Tris-HCL. PH 7.6, 10mM $MgCl_2$, and 400 μm each of dATP, dGTP, and dTTP), followed by the addition of 20μCi of α^{32}P-dCTP (800 Ci/mmol), and 1 μL of DNA polymerase 1 enzyme mix. The enzyme mix was made from 0.01 μg/ml DNAseI, 1U/μl E. coli DNA

polymerase I, within an enzyme diluent (50mM Tris-HCl, pH 7.6, 10mM $MgCl_2$, 50% glycerol, and 100µg/ml BSA). The nick translation reaction was then incubated for 2 hours at 15°C. The reaction was stopped by the addition of 100µl of 25mM EDTA/1% SDS, and the unincorporated nucleotides removed using a G-50 spun column. The probe was then boiled for 10 minutes (in a 1.5 ml centrifuge tube) prior to adding to the hybridization mixture.

Another method used to prepare probes was random priming, using the Prime-It II Random Primer Labeling Kit as per manufacturer directions (Stratagene). Briefly, 25 ng of DNA was brought up to a volume of 23µL, then 10µl of random oligonucleotide primers was added ((27 ODU/mL). The sample was boiled for 5 minutes, then placed at 37°C to anneal. Next, the 10x buffer was added (5µl), then α-^{32}P-dATP (3000 Ci/mmol), and 1µl of klenow polymerase (5U/µl). This reaction was then incubated for 10 minutes at 37°C, and stopped with 0.5M EDTA (pH 8.0). Unincorporated nucleotides were removed using a G-50 spun column, then the probe was boiled (10 minutes) prior to adding to the hybridization mixture.

2.3.3.8 Filter hybridizations and colony screening

The bacterial colonies were transferred to Hybond membrane circles by carefully placing the orientation-marked filter on the agar surface. The first sheet was soaked with 10%SDS, and the filters were carefully removed from the bacterial plates, and placed colony side up on the soaked whatman paper for 5 minutes. The filters were then transferred colony side up onto denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 minutes, then 2 separate renaturation steps on whatman paper soaked with 1.5M NaCl,

0.5M Tris, (pH 8.0). The membranes were then UV crosslinked in a Stratalinker on autocrosslink cycle (Stratagene), then placed in hybridization buffer (50% formamide, 5X Denhardt's solution, 5X SSC, 1% SDS, 0.1 mg/µl salmon sperm DNA) for 2 hours in a 90mm culture dish.

After the 2 hour prehybridization was complete, the hybridization solution was aspirated off, and the hybridization buffer containing boiled probe was added. It was important that each membrane is in contact with the probe. To ensure this happens, the membrane may need to be added to the probe one at a time. The filters are then incubated overnight at 42°C to hybridize to the probe.

The following day, the filters were removed from the hybridization solution, and washed twice in a low stringency wash (2X SSC, 0.1% SDS) for 15 minutes each, then twice at higher stringency (0.2% SSC, 0.1% SDS) for 15 min each. All washes were performed at 42°C, with gentle rocking. The filters were then allowed to air dry, and were exposed onto Kodak X-ray film. Positive colonies were lined up against the colonies on the original plate by using the orientation markings.

2.3.3.9 Plasmid Preparation

Positive colonies identified by colony lifting were screened by plasmid minipreparation. The individual colonies were cultured in 2 ml of Luria-Bertani (LB) medium overnight. The next morning, 1 ml of the culture was transferred to a tube (the remaining culture stored at 4°C), and centrifuged at 6000 RPM for 5 minutes (room temperature). The supernatant was discarded, and the pellet resuspended in 100 µl of an isotonic buffer (50 mM glucose, 25 mM Tris base pH 8.0, 10 mM EDTA), and stored on

ice. The bacteria were then subject to alkali lysis by adding 200µl of lysis buffer (0.2M NaOH, 1% SDS) and mixing by inverting the tube. After about 2 minutes, neutralization buffer (5 M KOAc pH 4.8) was added, and the tubes inverted, and stored on ice for 5 minutes. The mixture was then centrifuged for 5 minutes (13,200 RPM), and the supernatant transferred to a new tube. The nucleic acids were precipitated out of solution by the addition of 2 volumes of absolute ethanol, followed by a 10 minute incubation at 4°C. The precipitate was then collected by centrifugation, and the pellet washed in 70% ethanol, absolute ethanol, and then lyophilized dry. The pellet was then resuspended in 50 µl of TE buffer, and a 10µl aliquot tested by restriction digest for confirmation of the insert.

The above procedures enabled the identification of bacterial colonies containing plasmids with the correct insert. Large scale isolation of DNA was obtained by performing a plasmid maxi prep. Colonies identified as positive were used to inoculate a larger volume of LB (100 ml). The next day, plasmid DNA was obtained from the bacteria using a Maxi-Plasmid Kit (Quiagen) as per manufacturer recommendations. The kit operates by using alkali lysis and centrifugation (similar to the mini-preparation protocol described above), and then the purification of the plasmid DNA using an ion exchange column. Another method that was used for purifying the DNA was by centrifuging it through a cesium chloride density gradient (Sambrook et al., 1989).

2.3.3.10 Sequence Analysis of Plasmid DNA

The identity of plasmids was confirmed either by restriction analysis (when appropriate sites were available) or by sequence analysis. Sequencing was performed manually using the sequenase sequencing kit (Roche) as per manufacturer directions. Alternatively, plasmids were sent away to Cambridge Bioscience for sequencing.

2.4. RNA Isolation and Analysis

2.4.1 RNA Isolation

The isolation of RNA from tissues and cells was performed by protocols described previously (Chomczynski and Sacchi, 1987). Tissue samples were homogenized in 1mL of GITC solution (4M guanidinium isothiocyanate, 25mM sodium citrate [7.0], 0.5% sarkosyl, 0.1M β-mercaptoethanol), 1mL phenol (water saturated) and 0.1mL 2M sodium acetate. The solution was then well mixed (10 seconds on a vortex mixer) and placed on ice for 15 minutes. The RNA was collected out by centrifugation (10,000g, 20 minutes, 4°C), and retention of the aqueous phase. Addition of 1 volume of isopropanol, and incubation at –20°C for 1 hour, followed by centrifugation (10,000g, 20 minutes, 4°C) enabled the precipitation of the RNA. The isopropanol was decanted off the pellet, which was then dissolved in 0.3mL of GITC solution and transferred to a 1.5mL centrifuge tube. A second phenol extraction step (1:1 H_2O saturated phenol:chloroform) at this point improved the purity of the RNA. One volume of isopropanol was then added to the aqueous phase of the phenol chloroform extraction, and chilled at –20°C for 1 hour. After centrifugation (maximum speed, 10 minutes, 4°C), the pellet was washed with chilled 70% ethanol, then absolute ethanol, and dried

(lyophilized). The RNA was then resuspended in high quality H_2O. For large RNA pellets, the sample were heated at 65°C to aid dissolving. The concentration and purity of the RNA were determined by measuring the optical density (OD_{260}; OD_{280}).

2.4.2 Semi-Quantitative RT-PCR

A comparison of Timp-4 activity was measured using the primer-dropping PCR protocol (Wong et al., 1994). Standard PCR was performed (see section 2.3.1), however, an internal control was added to the PCR mix. The GAPDH gene codes for a metabolic enzyme which is present at approximately equal levels in all cells and tissues. Therefore, I amplified both TIMP-4 as well as GAPDH in the same PCR reaction. Because GAPDH is expressed at a high level, fewer amplification cycles were required before the reaction begins to plateau. The TIMP-4 primers were still in a linear phase of amplification at 35 cycles, while GAPDH was optimally amplified at 26 cycles. Therefore, the GAPDH primers were added at the denaturing step (94°C) of cycle 10 for TIMP-4. This allows both genes to be amplified to optimal levels, yet prevents competition for reaction substrates (such as enzyme, or dNTPs). After the PCR cycling was complete, samples were examined on a 1.5% agarose gel, and visualized by ethidium bromide staining.

2.5 Constructs

2.5.1 PCR constructs

Inserts for a deletion series of the mTIMP-1 promoter were generated polymerase chain reaction (PCR) using standard protocols. The constructs, corresponding to -223/+47, -195/+47, -180,+47, -165/+47, -140/+47, -125/+47 and -95/+47 were generated,

gel purified, and restricted with XhoI and SacI to create sticky ends based on restriction tags placed on the PCR primers. The inserts were then subcloned into the luciferase reporter plasmid, pGL2-basic (Promega) with XhoI and SacI sticky ends. The primers used for PCR are depicted in table 5.

2.5.2 EMSA constructs

Oligos for bandshifts and southwestern blotting were synthesized by UCDNA synthesis lab (University of Calgary, Canada) (table 6). If annealing of oligos was required to produce double-stranded probes, equimolar amounts of sense and antisense strands were diluted to 1ug/uL in 10mM tris-HCl/100 mM NaCl, and heated to 95°C. To facilitate annealing, the oligos were allowed to cool slowly to 4°C. Probes were then diluted to a 10ng/μl working volume in 10 mM NaCl.

2.6 Reporter assays

2.6.1 Luciferase assays

Luciferase reporter assays were performed using the luciferase assay system according to manufacturers instruction (Promega, Madison, WI). Briefly, 20 μL of cell lysate was mixed with 100uL of luciferase assay reagent, then light emission was immediately measured on a luminometer (TD-20, Promega, Madison WI). Luciferase transfections were standardized to CAT activity by cotransfection with a cytomegalovirus promoter driven CAT construct (CMV-CAT).

Table 5. PCR primers for construct synthesis. The table shows the sequences of all oligonucleotides used in the design of deletion and mutant constructs. The constructs are all written in 5' to 3' orientation. The Sac primers are all sense, and the Xho 47 primer is antisense for production of deletion constructs. Directionality is mentioned for all other primers.

Name	Sequence
Timp-1 Sac 223	GGGAGCTCAAGCTTTAGGCGCTCTCTCT
Timp-1 Sac 195	GGGAGCTCTCAAGGCCGCCAGGAAGGAC
Timp-1 Sac 180	GGGAGCTCAGGACTGTGCATGACGTGGA
Timp-1 Sac 165	GGGAGCTCGTGGAGCTGGGGGAAGGGGG
Timp-1 Sac 150	GGGAGCTCGGGGGCGCAGTGGGCTGGCT
Timp-1 Sac 125	GGGAGCTCAGGAGGGAGTATCTTTGGGT
Timp-1 Sac 95	GGGAGCTCGCCCGCCCCTAGGCTTTGAC
Timp-1 Xho 47	GGCTCGAGATCTGCGATGATGGCGGCA
coll. AP1 top	GTGGGTGGATGAGTCATGCGTCCAGGAAG
coll. AP1 bot	CTTCCTGGACGCATGACTCATCCACCCAC
mutAP1 top	CAGCGGTGGGTGGAGGAGTGATGCGTCCAGGAAG
mutAP1 bot	CTTCCTGGACGCATCACTCCTCCACCCACCGCTG
mutPEA3 top	TGAGTAATGCGTCCTTGAAGCCTGGAGGCA
mutPEA3 bot	TGCCTCCAGGCTTCAAGGACGCATTACTCA
Hind -115mut1	CCAAGCTTGAGGAGTGGTTTATCCTGGGGCCCG
Hind -115	CCAAGCTTATCTTTGGGTTTATCCTGGG
Timp-1 Bam +47	AAGGATCCCTGCGATGATGGCGGCAGGG
Timp-4 -28anti	GGCTCGAGGTCTGCCTGTCGTAACAAAC
Timp-4 -87	CCGTCGACGGCTGCACGAAGCTTTCTGG
Timp-4 -161	CCGTCGACGGGTGGCCTGGTGCCAAAGG
Timp-4 -200	CCGTCGACCTCTGCAACTTCCCAACTGG
Timp-4 -720	CCGTCGACGCACTGGCTGTAAGAAAGGG

Table 6. Electromobility Shift Assay Primers. The sequence of all EMSA primers is listed. In all cases, top refers to the sense strand, and bot refers to the antisense strand.

Name	Sequence
TRE 1	GATCCGGCTGACTCATCA
TRE 2	AGCTTGATGAGTCAGCCG
-125/-95 bot	GATCCCCAGGATAAACCCAAAGATACTCCCTCCTA
-125/-95 top	AGCTTAGGAGGGAGTATCTTTGGGTTTATCCTGGG
-123/-110 top	AGCTTGAGGGAGTATCTTTG
-123/-110 bot	GATCCAAAGATACTCCCTCA
-115/-100 top	AGCTTATCTTTGGGTTTATCCG
-115/-100 bot	GATCCGGATAAACCCAAAGATA
-108/-92 top	AGCTTGGTTTATCCTGGGGCCCG
-108/-92 bot	GATCCGGGCCCCAGGATAAACCA
-115/-100 top mut1	AAAGCTTGATGAGTCGTTTATCCG
-115/-100 top mut2	AAAGCTTATCTTTGGTCAGCCGCG
-115/-100 top MUT -115/-114	AAAGCTTCGCTTTGGGTTTATC
-115/-100 top MUT -113/-111	AAAGCTTATTGGTGGGTTTATC
-115/-100 top MUT -110/-108	AAAGCTTATCTTGATGTTTATC
-115/-100 top MUT -107/-105	AAAGCTTATCTTTGGACCTATC
-115/-100 top MUT -104/-102	AAAGCTTATCTTTGGGTTCGCG
MTIMP-AP1 top	AGCTTGGATGAGTAATGCG
mTIMP-AP1 bot	GATCCGCATTACTCATCCA
RT7-D1 top	AATTGGCCTTAGGG
RT7-D1 bot	CCCTAAGGCC
NF*k*B top	AGCTTAGGGGACTTTCCCAG
NF*k*B bot	GATCCTGGGAAAGTCCCCTA

2.6.2 CAT assays

CAT assays were performed using standard methodology previously described (Edwards et al., 1992). First, 20ug of protein extract was incubated for 10 minutes at 37°C with ^{14}C-chloramphenicol (0.0002 μCi) in Tris-HCl pH 7.5 in a final volume of 100 μL. Next, 20 μL of 4mM acetyl coenzyme A was added and incubated for 1h at 37°C. The reaction was terminated by extraction with 900 μL of ethyl acetate. The sample was then lyophilized, resuspended in 20 μL ethyl acetate, and spotted onto a thin layer chromatography plate. Chromatography was performed in an atmosphere containing 95% chloroform/5% methanol. Finally, the plates were exposed onto X-ray film (Kodak).

Greater sensitivity and reliability was obtained using a CAT ELISA kit (Roche) as per manufacturers directions. To a final volume of 200uL, 10ug of cytoplasmic extract was diluted into sample buffer, with a final volume of 200μL. The entire volume was then loaded onto the supplied plates, which were pre-coated with anti-CAT antibodies, and incubated for 2 hours at 37°C. The plates were washed 5 times with 250μl of wash buffer (PBS-TWEEN) for 30 seconds each wash. The primary antibody anti-CAT-DIG, was then diluted in sample buffer, added to the ELISA wells in a volume of 200 μl, and incubated for 1 hour (37°C). The plate was then washed 5 times again, and the secondary antibody, anti-DIG-POD, was diluted in sample buffer, and 200μl was added to the wells. The plate was washed a final time, and 200μl of substrate (room temperature) was added to each well. The plate was covered, and incubated at room temperature for 15 minutes, and the absorbance was measured at 405nm.

Because both luciferase and CAT reporter constructs were used, in some cases, with the same promoter, regions, I performed a comparison of the two different reporter assays (figure 6). The general trends are the same for both reporter constructs. However, the fold induction seen is greater for CAT constructs when comparing the same promoter region in Luciferase.

2.7 Protein -DNA interactions

2.7.1 Preparation of nuclear extracts

A total of 5 plates of 10T1/2 cells (either unstimulated, serum stimulated for 30 minutes, or 3 hours) were scraped in 1mL PBS/0.1% nonidet P-40 (NP40), and transferred to an eppendorf tube. After a brief spin, cells were decanted and rinsed in PBS/0.1% NP40, followed by another quick spin, and aspiration of supernatant. The nuclear pellet was then resuspended in 3 volumes of high salt buffer (25mM HEPES [7.8], 500mM KCl, 0.5mM $MgSO_4$ and 1mM DTT) with protease inhibitors (0.1 mg/mL leupeptin, 0.1mg/mL PMSF, 0.1mg/mL aprotinin) and extracted on ice for 20 minutes. Extracts were then centrifuged at full speed for 2 minutes (4°C), and the supernatant transferred to a new tube. Nuclear extract concentration was determined by Bradford assay using the Biorad assay kit (Bio-Rad Laboratories, Hercules CA) following manufacturers directions.

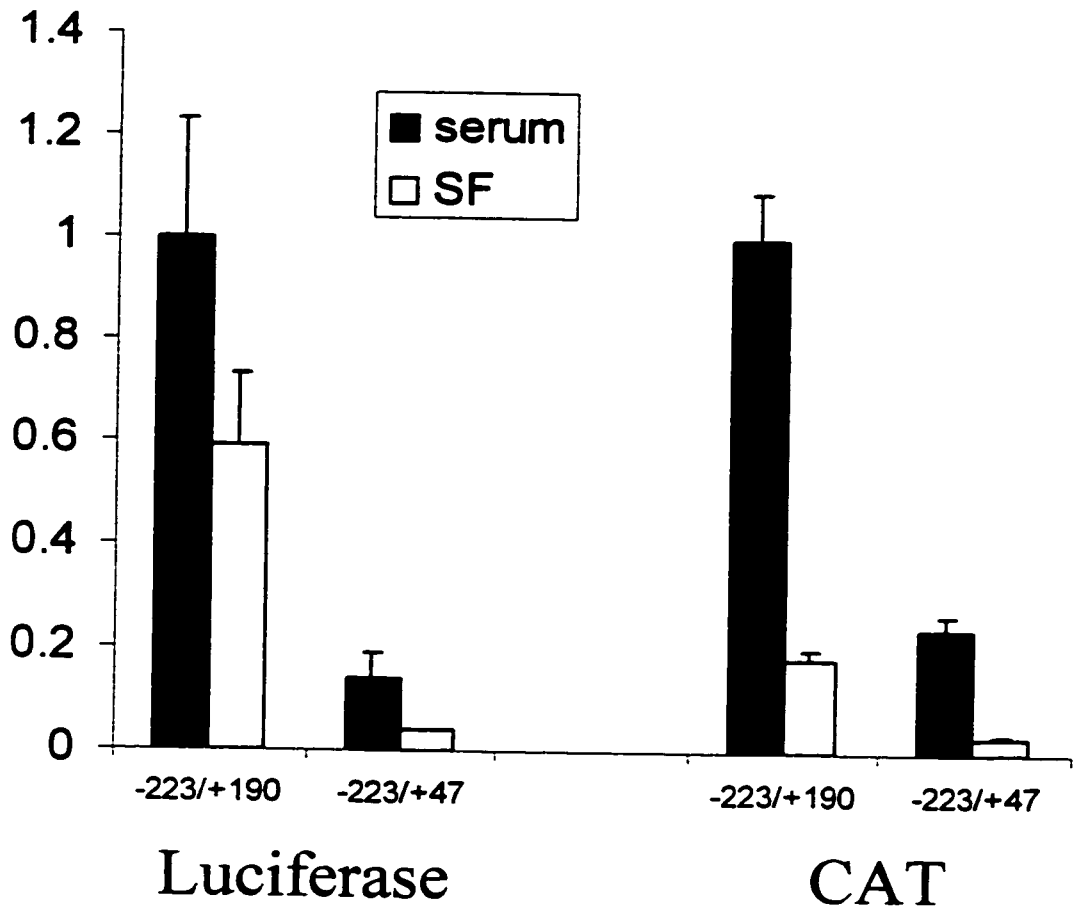


Figure 6. Comparison of luciferase and CAT reporter assays. Parallel transfections were performed using the calcium phosphate precipitation protocol. Cellular lysates were then obtained with the reporter lysis buffer (Promega), and the extracts were subject to Either CAT or luciferase reporter assays. To allow for comparisons, maximal activity was set at 1.

2.7.2 Polynucleotide Kinase End-Labeled Probes

Single-stranded probes were generated by adding 60ng of oligo to a modified PNK buffer (0.5M Tri-HCl [7.6], 0.1 M MgCl2, 10mM DTT, 10mM EDTA, 25% PEG w/v), with 1uL γ-^{32}P-ATP, and 5U T4-PNK. Reactions were incubated at 37°C for 30 minutes, then stopped with 50uL 25mM EDTA. Probes were cleaned by phenol-chloroform extraction, and unincorporated dNTPs removed using G-50 spun-columns. Duplex, fill-in probes were generated by adding 60ng of annealed oligo to 0.5mM dATP, dGTP, dTT, 2uL a-32P-dCTP (10mCi/mL), 1x REACT buffer 2 (Gibco-BRL), 0.5U large fragment of DNA polymerase I. Reactions were incubated on ice for 15 minutes, then terminated by phenol-chloroform extraction. Unincorporated dNTPs were removed by running the reaction through a G-50 column.

2.7.3 Electromobility Shift Assay (EMSA)

40,000 CPM of radiolabelled probe (generated either by end labeling (PNK), or by fill-in (Klenow) as described in section 2.7.2) was incubated with 4ug of 10T1/2 cell nuclear extract in 1x binding buffer (10mM Tris-HCl [7.5], 50mM NaCl, 0.5mM DTT, 5mM MgCl2, and 5% glycerol) in the presence of non-specific competitors (0.5ug Sau3A cut bluescript plasmid and 0.5ug poly (dI-dC)(dI-dC) (Pharmacia)), to a final reaction volume of 20uL. The binding reaction was incubated at 4°C for 30 minutes, then electrophoresed on a 7% polyacrylamide gel (38:2 acrylamide:bisacrylamide) in 1x TBE buffer (90 mM Tris-HCl, 90mM Boric acid, 2mM EDTA) (which had been prerun for at least 30 minutes. The gels were run at 4°C at 230V, then dried onto Whatman paper

using a gel drier (Biorad, model 583 gel dryer) and exposed onto X-ray film (Kodak biomax).

2.7.4 UV-crosslinking

The EMSA reaction was performed as described above (2.6.3), however, after the 30 minute incubation, the binding reaction is UV-crosslinked (1200 μJoulesx100) in a UV-Stratalinker (Stratagene). An SDS-loading dye is then added to the reaction, and the sample is loaded on an 8%SDS-PAGE gel. The gels were then dried, and exposed to X-ray film for autoradiography. Prestained molecular weight markers were used for size comparisons of the complexes.

2.7.5 Southwestern Blotting

Nuclear extracts (30ug) were loaded onto a 10% SDS-PAGE mini-gel (Bio-rad) in an equal volume of loading buffer (5% SDS, 5mM Tris-HCl [6.8], 200mM DTT, 20% glycerol, bromophenol blue), using a lane of pre-stained molecular weight markers as a standard (Gibco-BRL). The samples were then transferred to nitrocellulose (Biorad transblot transfer medium) using the mini-trans-blot electrophoretic transfer cell (Biorad) as per manufacturers directions. The blot was blocked for 1hr at 4°C in BLOTTO (50mM Tris-HCl [7.5], 50mM NaCl, 1mM EDTA, 1mM DTT, 5% skim milk powder) with gentle agitation, followed by soaking overnight at 4°C in binding buffer (25mM NaCl, 10mM Tris-HCl [7.5], 10mM MgCl2, 5mM EDTA, 1mM DTT). The next day, the samples were soaked in binding buffer with single-stranded, end-labeled probe (100,000 CPM/ml) for 6hr to overnight. Following the probing, the blots were rinsed 4

times in binding buffer for 8 minutes at 4°C and air dried. The washed blots were then exposed to biomax X-ray film (Kodak).

2.8 Purification of DNA-binding proteins

2.8.1. Nuclear extract dialysis

The high salt content of the buffer used to harvest nuclear extracts (section 2.6.1) warrants the dialysis of the extract for some applications (such as affinity chromatography). Because our unidentified protein is approximately 54 kDa, a small pore size dialysis membrane (MWCO of 14000) was selected. The tubing was cut to length (about 15 cm) and boiled in 10mM sodium bicarbonate for 5 minutes. The tubing was then washed several times (inside and out) with milli-Q water, then stored at 4°C in 20% ethanol (indefinitely).

For the dialysis of the nuclear extracts, a length of tubing was removed from the storage solution, and rinsed several times with milli-Q water, to remove all traces of ethanol. Then, the tubing was equilibrated in dialysis buffer, which was composed of 50 volumes of EMSA binding buffer (see section 2.6.3) containing protease inhibitors (leupeptin, aprotinin, PMSF, each at 0.05 mg/ml). The tubing was then clamped on one side, and tested for leaks, by filling with dialysis buffer. After draining, the nuclear extract was loaded into the tubing, and it was sealed by clamping on both ends, then placed into the dialysis buffer. The buffer tank was then placed at 4°C with stirring for 4 hours with one change of dialysis buffer during the incubation. The extract was then drawn out of the tubing with a pipette and transferred to a tube. The dialyzed extract was

then tested by EMSA against the pre-dialyzed sample to ensure that the extract did not get degraded by proteases.

2.8.2 Affinity chromatography

The first step in affinity chromatography was an initial purification step for the nuclear extracts to minimize background. I used several different types of matrix for these initial steps. Because I was looking for a single-stranded DNA binding protein, I first used double-stranded DNA cellulose (Sigma), following a batch protocol. First, the DNA-cellulose was equilibrated with EMSA binding buffer (see EMSA, section 2.7.3). Then, 2mg of nuclear extract was added to the matrix, and incubated at 4°C on a spinner. The next day, the mixture was centrifuged, and the resulting 'flow through' collected. The DNA-cellulose was then washed in 1x binding buffer two times (100 μL each), followed by elution in increasing salt concentrations (in binding buffer with increased KCl concentrations).

Another approach was to first use a heparin-agarose column. To a cotton wool plugged 1 ml pipette tip, 90 μL of heparin agarose was added. The heparin agarose was equilibrated with 1.2mL of binding buffer (see EMSA protocol – 2.7.3), then washed again with another 500μL of binding buffer. The sample (dialyzed) was then added to the column, and the flowthrough collected. The matrix was washed twice with 1x binding buffer (100μL each time). Finally, the proteins are eluted off with increasing salt buffers (binding buffer+150mM KCl to binding buffer+2M KCl). The input, flowthrough, and eluate fractions were tested by EMSA for concentration of the unidentified protein.

Affinity chromatography was then performed using either a purified products from above, or with crude, nuclear extract. A biotinylated oligonucleotide was ordered from the U of C DNA synthesis lab (University of Calgary), with the sequence (AGCTTAAATCTTTGGGTTTATCCG AGCTTATCTTTGGGTTTATCCG), which is a doublet of the –115/100 top sequence. A binding reaction was set up with 150 μg of C3H10T1/2 cell nuclear extract, and 15 pmol of biotinylated oligonucleotide in 1x binding buffer (see EMSA, 2.7.3). Poly (dI-dC:dC-dI) was used as a non-specific competitor (100μg), in a total volume of 750 μl. The binding reaction was incubated at 4°C for 2 hours, with occasional mixing. During the binding reaction incubation period, 200 μl of streptavidin agarose was equilibrated in 1xEMSA binding buffer (resuspended in 800μl, then centrifuge the agarose, and repeat 3 times). After the incubation, the streptavidin agarose is centrifuged, and the pellet resuspended in the binding reaction. This was then put on a rotating wheel at 4°C for 30 minutes. The affinity of biotin for streptavidin is so great that the interaction will occur almost immediately. After the 30 minute incubation, the mixture was centrifuged at full speed for 20 seconds, and the supernatant retained as the flowthrough. The streptavidin agarose was resuspended in wash (binding buffer), then the different elution buffers (binding buffer with increasing concentration of KCl) by centrifuging the matrix, retaining the eluate, and resuspending the pellet in the next buffer. The final elution step was to resuspend the streptavidin agarose pellet in 50 μl of 1xSDS-PAGE loading dye (50mM Tris-Cl[6.8], 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol).

2.8.3 Expression Library Screening

A plating culture of E. coli XL1-Blue were prepared by inoculating 50 mL of NZCYM supplemented with 0.2% maltose with a single colony of XL1-Blue, then incubating overnight at 37°C. The next morning, the cells were centrifuged (4000g, 10 minutes, room temperature), and resuspended in 20ml sterile $MgSO_4$ (0.01 M). The plating bacteria were stored at 4°C until needed (up to 1 month).

I used the PCC4 λ-ZAP library (Stratagene), which was diluted down to $3x10^6$ PFU/μl with SM buffer (Sambrook et al., 1989). A total of $3x10^6$ plaques were screened (40 plates with $3x10^4$ PFU/plate). To plate the phage, an aliquot of PCC4 was diluted to $3x10^2$ PFU/μl, then 100μl of diluted PCC4 were incubated with 100μl plating bacteria for 20 minutes at 37°C. After the incubation, 2.5ml of molten top agarose (no more than 65°C in temperature) was added to the infected bacteria, the tube was mixed by inversion and quickly poured overtop of LB-plates. The cultures were then incubated at 37°C until the phage plaques became visible in the bacterial lawn (about 6-8 hours).

While the plates were incubating, nitrocellulose filters are soaked in 10mM IPTG, and then marked for orientation. As soon as the plaques were visible, the filters were placed onto the agar, and incubated for 4 hours. Samples were done in duplicate, so after the 4 hours, new IPTG-soaked filters were overlaid for an additional 4 hours. After being removed from the plates, the filters were treated according to the protocol for southwestern blots. They were immediately placed into BLOTTO (50mM Tris-HCl [7.5], 50mM NaCl, 1mM EDTA, 1mM DTT, 5% skim milk powder) for 1 hour, followed by binding buffer 25mM NaCl, 10mM Tris-HCl [7.5], 10mM MgCl2, 5mM EDTA, 1mM DTT) overnight. The next morning, the buffer was replaced with fresh binding buffer (25

mL per 10 filters) containing $1x10^5$ cpm/mL probe. The filters were incubated for between 4 hours and overnight, then washed 4 times in fresh binding buffer (5 minutes per wash) before being air-dried and exposed to X-ray film.

Chapter 3. Results

3.1 Experimental Strategy

An alteration in TIMP transcription levels has been seen in association with many types of pathologies, such as fibrosis, arthritis, and cancer invasion (section 1.6.2). However, the mechanisms governing TIMP transcription levels are still not understood. I have studied the transcriptional regulatory mechanisms important in controlling murine TIMP expression, and in this thesis, two different murine TIMP genes have been examined: Timp-1, and Timp-4.

There are several different regions of the Timp-1 promoter which play a role in regulating its transcription levels. Accordingly, analysis is most efficiently performed by studying individual units within the promoter. I have been studying two regions of the promoter, the first intron, and a promoter proximal region between –125/-95 which positively regulates basal expression together with the Timp-1 AP1 binding site, which has additional functions besides an interaction with AP1. Two principal methods have been used to analyze these promoter regions. The first is reporter assays of deletion constructs, of which both CAT and luciferase constructs were used. This enables a direct way to study the contribution of a region of a promoter by comparing reporter expression when a region is present to when it is deleted. Similarly, the important regions can be mutated, and the effect can be examined again by reporter expression. I also used a transgenic mouse model to study in vivo expression from a reporter construct. The other technique relied upon is EMSA, which looks at the ability of particular regions to directly interact with nuclear proteins. An important point is that it is necessary to use both types of strategy to analyze promoter elements. EMSA assays are very dependent upon binding conditions, and non-specific interactions, and do not provide information on the

consequence of the interaction (ie, does the interaction result in activation or inhibition). Therefore, any protein-DNA interactions must be deemed functional by demonstrating that the binding site also affects transcription – using reporter assays. These methods, and others, have all been used to demonstrate in several regions of Timp-1 that there are important regulatory regions, as well as interactions between DNA regulatory elements and transcription factors.

I have also performed a preliminary analysis of the regulation of Timp;-4 expression. Because the gene has only recently been identified, there has been no previous work on its transcriptional regulation. First, identification of a putative promoter was performed, using 5'-RACE-PCR. When transcription start points were found, the next step was to locate important transcriptional regulatory elements, using reporter assays of promoter deletions as outlined above.

3.2 Intron-1 of Timp-1 Contains Positive and Negative Regulatory Elements

3.2.1 Deletion Analysis of the Timp-1 Intron 1.

Previous studies from our lab have identified important regulatory regions within the first intron of the Timp-1 gene. This has largely been based on a comparison of the regions, -223/+47 and –223/+190 in CAT reporter constructs. I repeated this deletion experiment (figure 7A), in order to confirm the observation. Two CAT reporter constructs were used, the first ranges from –223/+190, and includes part of the first intron (which extends up to +785). The second construct terminates at the +47 Sau3A restriction site within exon 1. Following reporter analysis, the CAT activity of the –223/+190 construct is almost 3 fold higher than the shorter construct, which confirms the

previously seen result. This was repeated with a different set of reporter constructs. In figure 7B, the same promoter regions were placed in the pGL2-basic luciferase reporter construct (bar 3 and 6 in 7B). The results were of a similar trend – the inclusion of part of the first intron (-223/+190) resulted in a higher CAT activity compared to a –223/+47 construct. The luciferase constructs show a greater fold change than the CAT constructs when comparing the –223/+47, about a seven fold difference for luciferase compared to the three-fold seen in the CAT constructs (compare 7A bars 1 and 2 with 7B bars 3 and 6), which is discussed in detail in section 3.2.2.

On account of the increased activity seen from the +47/+190 region, I made a series of deletions between +47 and +190, driving the luciferase reporter gene (7B, bars 3-6). Following transfection into C3H10T1/2 cells, and reporter analysis, a major regulatory region between +150 and +190 was seen, as there was 2-3 fold decreased reporter activity following its deletion.

Additional regulatory sites are located between +47 and +74, where deletion of the region resulted in a 2-fold loss of luciferase expression. This area is of particular interest because it demarcates the position of the intron-exon boundary. Furthermore, work from our collaborators has shown that this region has positive transcriptional activity within the human TIMP1 gene as well. Further work into this area of the promoter has identified a LBP-1-like site within the first exon which is being pursued by our collaborators (Clark et al., personal communication).

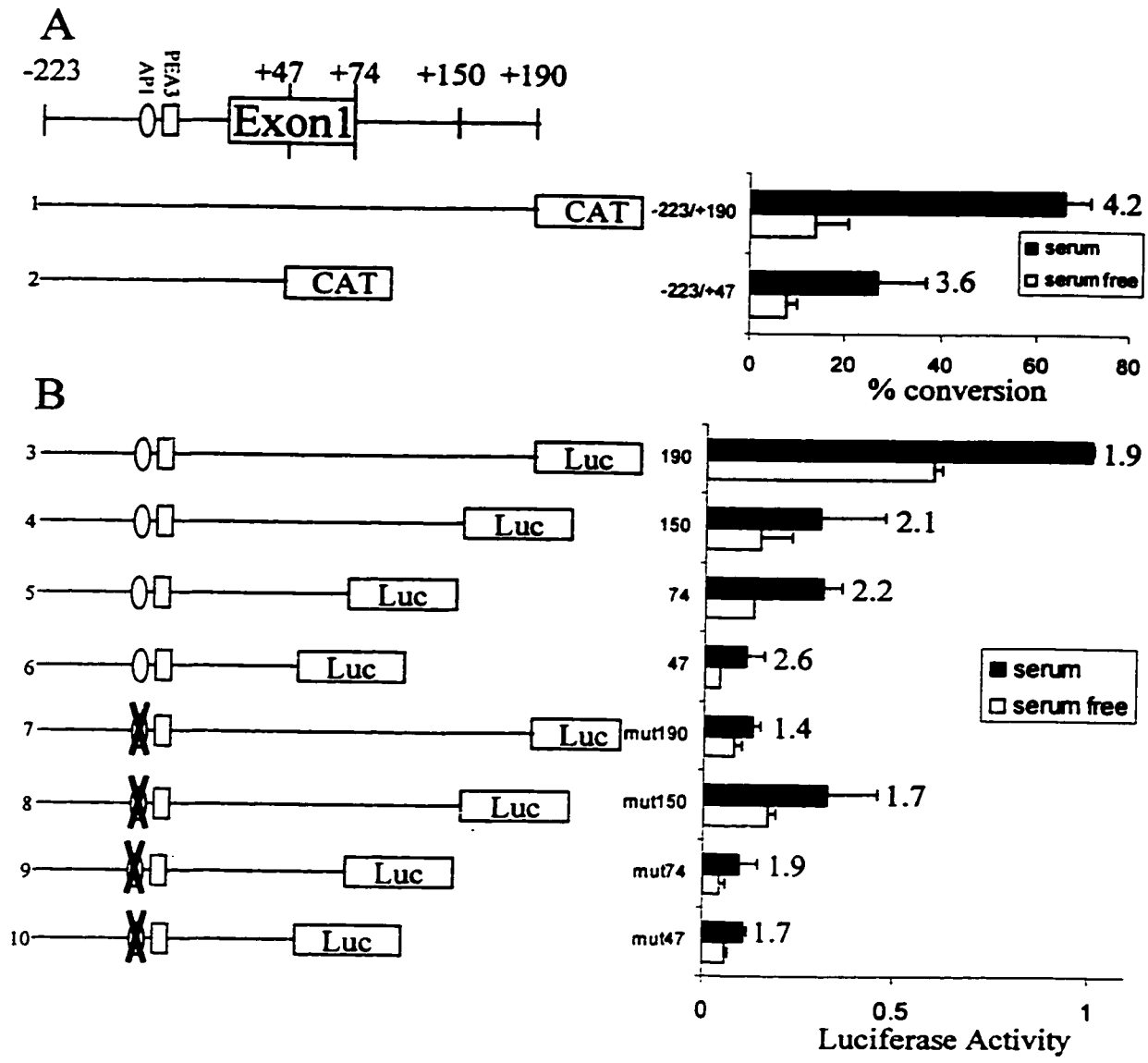


Figure 7. Transcriptional Regulatory Elements Within Timp-1 Intron 1. Transient transfections were performed into C3H10T1/2 cells with the constructs depicted in A and B. A) A comparison between -223/+47CAT and -223/+190CAT showed that including a portion of the first intron increased both basal and serum-induced CAT activity by about 2-fold. To narrow down the cis-acting region, a more defined set of deletions was made (B). The top 4 constructs show positive regulatory regions between +150/+190 and also +47 and +74. The AP-1 binding site was then mutated in the context of the 3'-deletions. The fold induction for each construct is presented numerically beside the serum stimulated bar.

3.2.2 CAT and Luciferase reporter systems

All previous promoter work in our lab has relied upon chloramphenicol acetyl transferase (CAT) constructs to report promoter activity. However, there was recently a switch in reporter systems to the luciferase assay. There were several advantages to using the luciferase reporter system in lieu of CAT assays. The CAT assay is time consuming (two days), uses the hazardous ^{14}C radiolabeled chloramphenicol, as well as several expensive reagants (isotope, and acetyl-coenzyme A). This is in contrast to the luciferase assay system, which is very fast and easy to perform, and does not require any hazardous reagants. However, there were inconsistencies between the CAT and luciferase reporter systems.

In the experiment presented (figure 7), there is an obvious difference between the CAT and luciferase reporter assays. Comparing the fold induction between -223/+190 constructs in pBL-CAT3 versus pGL2-basic, we can see that the fold difference is altered. In CAT assays (figure 7, lanes 1 and 2) there is a 2.6 fold decrease in activity following deletion of the region. However, this difference is much greater, 7.7 fold in luciferase assays (figure 7, lanes 3 and 4). While both assays show a positive contribution from the region +47/+190, the amount of induction will be interpreted differently depending upon which reporter assay is used. All plasmids were sequenced following construction, so the differences are not a consequence of cloning error, or mutation. Instead, I must look to the assay systems themselves to try to rationalize the difference.

Two different reporter vectors were used, pBLCAT3, and pGL2-basic. The difference in expression might be a function of the subcloning into the vector itself, or

possibly structure of the promoter regions within the background of the different vector. Additionally, the expression of the protein product might not accurately reflect the transcription rates due to protein expression, or even at the level of reporter detection. Alternatively, the inconsistencies might be the result of the expression component of the assay system. The long half life of the CAT protein compared to the relatively short half life of luciferase, which has been measured at a few hours at 37°C (Day et al., 1998) might affect the reporter measurements. This will not have any effect if the transcriptional response is unchanging during the 24 hour incubation, however, if there is a change in rate, or a biphasic response, the luciferase assay will measure only the response a few hours prior to measurement, which might be as much as 20 hours after the stimulation. Indeed other growth factors have been shown to have different early and late transcriptional responses. The short half-life of luciferase can therefore enable an analysis of the time dependance of promoter activity following stimulation which would not be possible for CAT (where levels would be constantly increasing). The ideal situation for resolution of these problems would be to study directly the RNA. Thus although direct comparisons between experiments using the CAT and luciferase assay systems may be problematic, results obtained with each reporter are consistent, and meaningful quantification is possible.

3.2.3 Localization of an Intronic AP1 Binding Site

After demonstrating positive regulation within the first intron, I next wanted to characterize the transcriptional response. Previous investigators have suggested that there is a low-affinity AP1 binding site within the first intron, which transactivated by c-

fos and c-jun expression vectors (Logan et al., 1996). While there is a canonical AP1 binding site within the human TIMP1 intron, such a site is not present within the mouse intron.

I tested the presence of an intronic AP1 site using 2 different approaches. In the first case, the promoter proximal AP-1 site was mutated, in the context of the downstream intron 1 deletions in the luciferase reporter constructs (Figure 7B, bars 7-10). This approach has the advantage of neutralizing the promoter proximal AP1 binding site, which will then enable us to see other transcription effects which might otherwise be masked by the strong AP-1 element. Mutating the proximal promoter (-60) AP-1 binding site had the effect of lowering activity of all of the reporter constructs to a low level with the exception of the -223/+150 construct. All constructs have been sequenced to confirm accurate cloning. There are two interpretations to this result. First, the mutation of the AP-1 binding site could be drastically reducing the reporter expression to background levels. The fact that there is no difference between -223/+47 wild-type AP1 compared to mutant AP-1 (figure 7, compare 6 with 10) would support this, because previous studies have demonstrated a clear difference between the expression of these constructs (Edwards et al., 1992). Alternatively, there might be adjacent positive and negative regulatory sites located in the intronic region. Deletion to +150 might allow a positive element located in the +74/+150 region to dominate reporter activity, even when the -60 AP1 site is mutated. There might then be negative regulation between +150/+190 which could negate the positive effect derived from the +74/+150 region. However, this view is inconsistent with the demonstration of what would appear to be a positive element between +150 to +190 when constructs carrying a wild type AP1 site are employed

(figure 7, lanes 3 and 4). Alternatively, it is possible that the linkage of the +150 sequences with the reporter cloning site sequences created an artificial positive element that masks the loss of the AP1 site at -60.

The other approach used to identify a second AP1 binding site was to transfect 10T1/2 cells with CAT reporter constructs corresponding to –223/+47 or –223/+190, again either with a WT or mutant form of the promoter proximal AP-1 site. These four constructs were cotransfected with different combinations of c-fos and c-jun expression vectors (fig. 8).

In constructs -223/+190 and –223/+47 (see 8A and C), cotransfection with c-fos expression vectors alone had no effect on reporter activity. However, cotransfection with c-jun, or both c-jun and c-fos expression vectors resulted in increased reporter activity in the –223/+47 wild-type AP1 construct (figure 8, lane C). This was not seen in the –223/+190 wild type AP1 construct (lane A), but this may be a consequence of depletion of substrate during the CAT assay, which occurs when its conversion exceeds 80%. Alternatively, there might be a problem with the Fos and Jun expression vectors, which would also explain the low levels of induction seen for the -223/+47 reporter construct as well. In the –223/+47 wild-type AP1 constructs, expression of c-Jun almost restored reporter activity of the –223/+47 construct to that of the longer and more active –223/+190. I was disappointed, however, by the absence of any significant increase in reporter activity when the promoter proximal AP1 binding site was mutated (figure 8, lanes B,D). This result was the same for both the –223/+190, and the –223/+47 mutAP1 constructs. The fact that the basal activity level is higher for the –223/+190 (figure 8B) compared to –223/+47 (8D) in the absence of a functional AP-1 site indicated that there

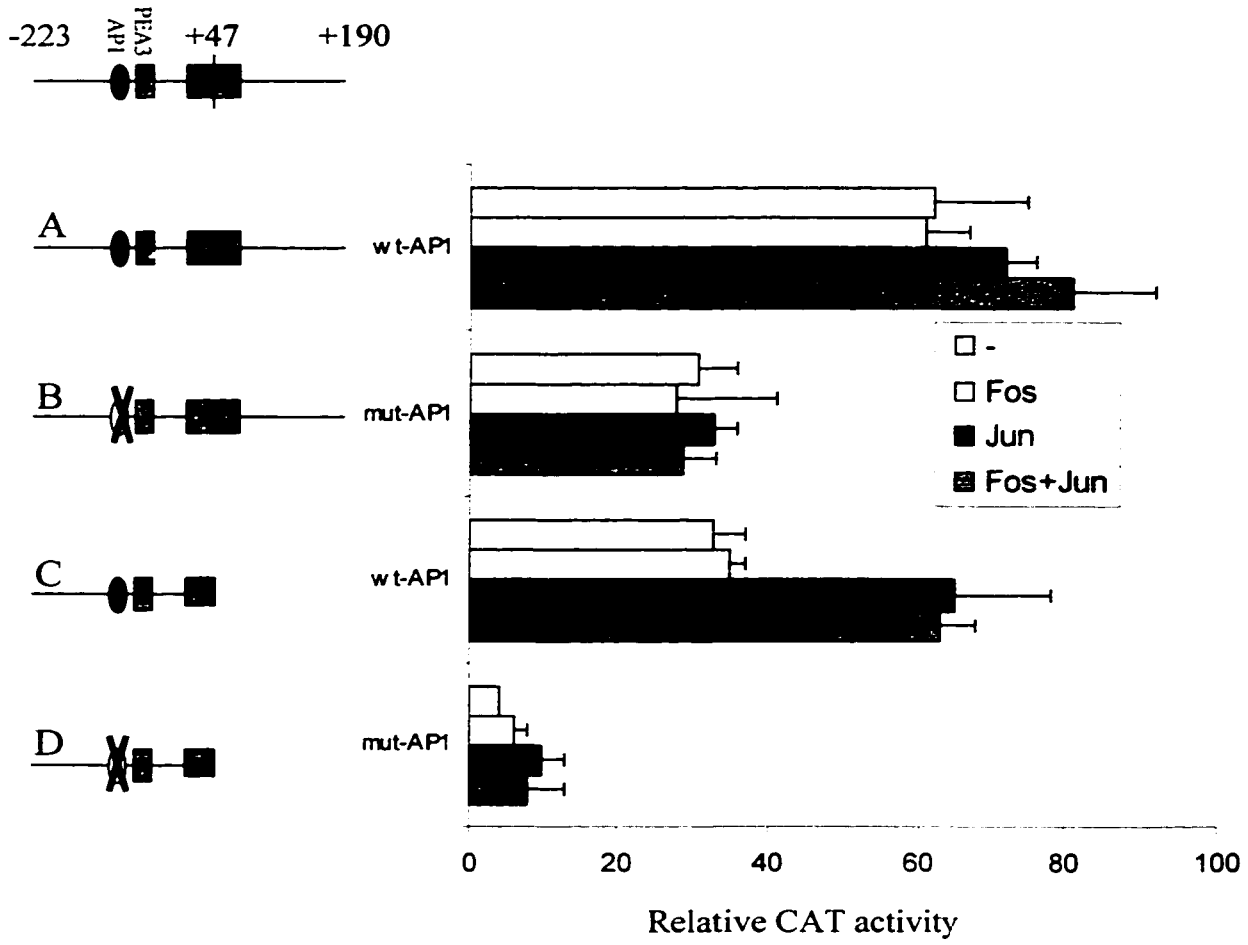


Figure 8. Intron 1 regions of Timp-1 were not responsive to Fos and Jun. Transient transfections were performed into C3H10T1/2 cells with the -223/+190 and -223/+47 constructs in which the AP1 binding site is either wild-type (wt) or mutant (mut). In addition to the reporter constructs, the cells were co-transfected with expression vectors for c-fos or c-jun. Transfected cells were incubated for 24 hours in the absence of serum, then harvested for reporter analysis. The graph shows relative CAT activity.

are positive-acting cis element contained in the +47/+190 region (in agreement with figure 7). though, these elements were not responsive to c-Fos or c-Jun. These results, taken together, confirm that there are positive regulatory elements within the first intron, specifically between +150 and +190 by deletion analysis. However, I was unable to link AP1 to the positive response. Previous studies from Edwards et al (1992) showed that the +47/+190 region of Timp-1, when linked to tkCAT, failed to stimulate activity. This might argue that the positive action of the region may be context or position dependent, or alternatively, might require other factors that associate with the Timp-1 promoter.

My co-transfection study used only c-fos and c-jun expression vectors. Therefore, before an AP-1 response from this region of the promoter is ruled out, it would be interesting and necessary to test expression of the different members of Fos and Jun, and also confirm the expression of the AP1 factors using Western blot.

3.2.4 Developmental Expression of *Timp-1* Using Transgenic Mice

The study of the tissue specific expression of Timp-1 has been examined previously by in situ hybridization (Flenniken and Williams, 1990; Nomura et al., 1989). However, due to limitations in the sensitivity of the techniques, we decided to study the expression of *Timp-1* in transgenic mice. A *Timp-1*-LacZ reporter construct was made using the –2700/+47 fragment of the *Timp-1* promoter. The transgene used for these experiments was constructed previously in our lab (Pamela Leco). The choice of the promoter fragment (-2700/+47) was based upon several criteria. While many studies have demonstrated the importance of the first intron in Timp-1 expression in vitro, at the time of transgene production, it was presumed that the key regulatory regions important

for Timp-1 expression were located upstream from the transcription start sites. Furthermore, reporter analysis demonstrated that *in vitro*, a -2700/+47 CAT construct would recapitulate the expression of endogenous *Timp-1* following a battery of growth factor and cytokine stimulations (Edwards et al., in press). These preliminary experiments, in particular the ability of the -2700/+47 fragment to drive similar reporter expression compared to endogenous Timp-1 following stimulation, suggested that the fragment used contained all the information necessary to drive appropriate Timp-1 expression. After the construction of the transgene, expression of LacZ was confirmed by staining transiently transfected C3H10T1/2 cells with X-gal (Pamela Leco, personal communication).

Following microinjections (U of C transgenic mouse facility) and screening of offspring for the transgene (Leco, P, unpublished data), 3 lines of transgenic mice were generated, and were bred for homozygosity. Of the 3 lines of mice, 2 of the lines express β-gal, designated line 26 and line 32. The analysis of the lines during development is presented by the sagittal sections in figure 9.

The expression of β-gal from both lines of mice was first visible from embryos that were 12.5 dpc in age (figure 9). This corresponds closely with the time at which the endogenous Timp-1 gene can first be detected by in situ hybridization (Flenniken and Williams, 1990; Nomura et al., 1989). The expression levels in the mice changed as the embryos matured. By 14.5 dpc, the LacZ expression from line 32 was both more intense, and localized to a broader range of tissues (figure 9, 14.5 dpc). At the latest stage examined in this thesis, 18.5 dpc, expression from line 32 is similar to that at stage 14.5 dpc, however, LacZ levels in line 26 are very faint, and restricted to only a few areas.

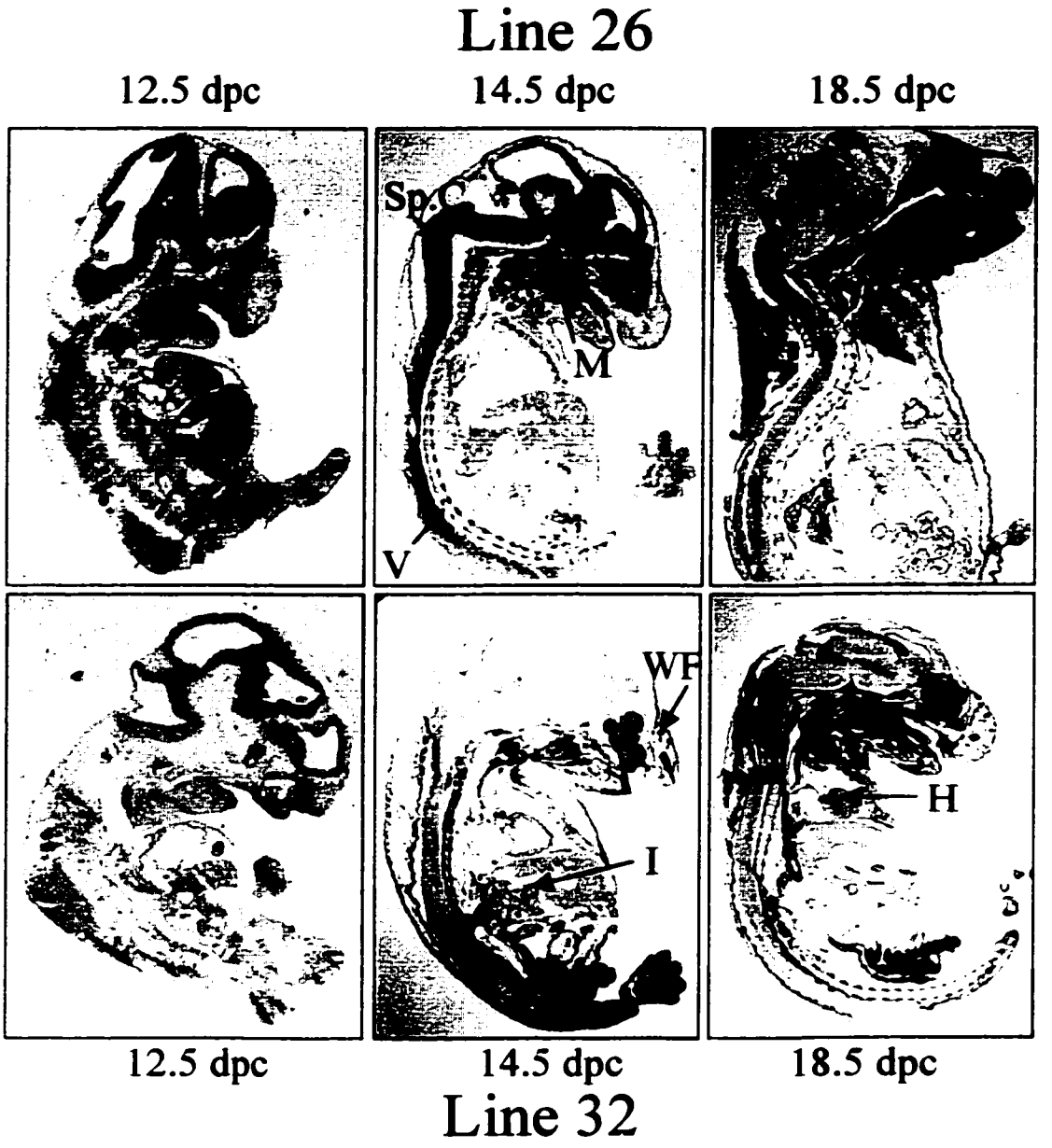


Figure 9. Transgenic mouse analysis of Timp-1 expression. Transgenic mice were produced using a transgene which contains the -2700/+47 region of the Timp-1 promoter controlling the lacZ reporter. Embryos derived from two different lines of homozygous transgenic mice were collected at 12.5, 14.5, and 18.5 days post-coitus (dpc), and snap frozen in OCT embedding media. Cryostat sections were cut at 10um, and stained for LacZ expression. Expression was seen in the Spinal cord (Sp.C.), mandible and tongue (M), whisker follicles (WF), Intestine (I), vertebral column (V), heart (H) and other locations referred to in the text.

This difference in expression could be the result of an increased copy number of the transgene for line 32, or alternatively, could be the result of an insertional effect in either, or both, lines of mice (discussed in more detail in section 4.2). Such an effect would therefore result in transcriptional control from either adjacent transcription factor binding sites, or due to chromatin accessibility of Timp transcription regulatory proteins.

While the age of onset of β-gal expression from the transgene resembles the first appearance of the endogenous gene (12.5.dpc), there are clearly many tissue-specificity differences between both transgenes and the endogenous gene, as well as each other. There are many regions of β-gal expression which are similar in the two lines of mice, and a detailed analysis of specific regions of expression is presented in figure 10. Because LacZ expression was highest at 14.5 dpc for line 26, the majority of comparisons presented (figure 10) used this developmental period. First, there are regions which are thought to be involved in tissue remodelling, and areas where endogenous Timp-1 has been localized by in situ hybridization (Flenniken and Williams, 1990; Nomura et al., 1989), such as within the interdigit space of the developing limb, within the skin, and in areas of the cranium, which display faint but detectable staining in both lines of mice. In addition, there are also regions of reporter activity within both lines of mice where expression has not been detected by other methods (figure 10). While both lines of mice express LacZ in the spinal cord, there is a particularly strong expression from line 26. This occurred despite the fact that overall reporter expression in line 26 was quite low. The mice also express reporter within the developing brain, though at fairly low levels.

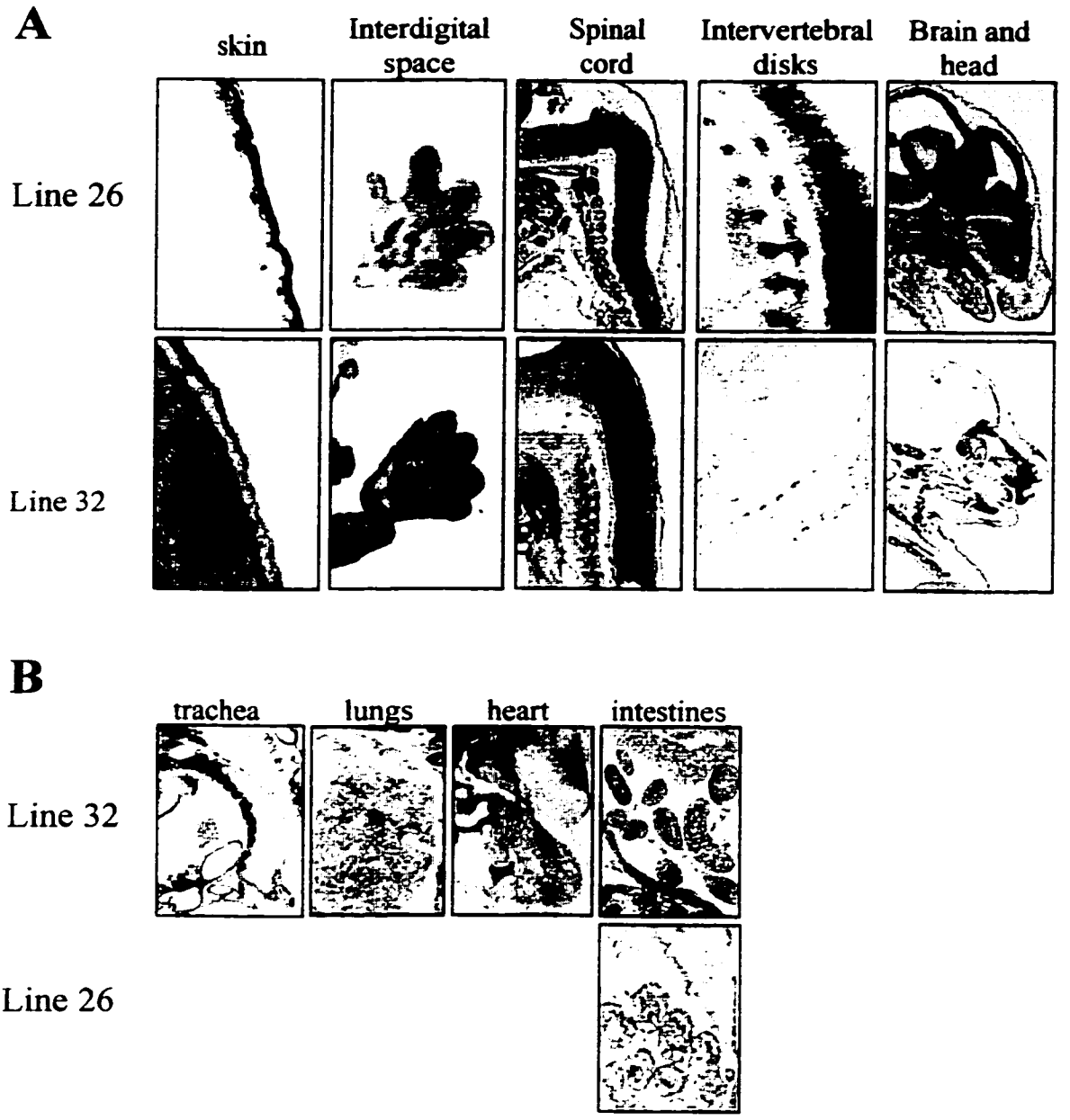


Figure 10. The transgenic mice show abnormal patterns of LacZ expression. Both lines of transgenic mice (described in figure 9) showed staining in atypical locations compared with the endogenous gene, as well as to each other. Depicted are areas of LacZ expression from embryos aged 14.5 dpc. A) In many cases, abnormal lacZ expression was similar between lines 26 and 32. Ssseveral such examples are depicted. B) There are several regions where the two lines of mice had differential expression. These were latgely restricted to expression in line 32 which were not seen in line 26. However, there is differential intestinal expression between the two lines.

These observations demonstrate neural expression from both lines of mice, which is a region that does not express endogenous Timp-1 as detected by in situ hybridization. There is also abnormal transgene expression within the developing vertebral column, which is composed of alternating structures called vertebral bodies and intervertebral discs. While the transgenic mice expressed β-gal in the intervertebral discs (figure 10A), *in situ* hybridization have localized Timp expression to the vertebral bodies (Flenniken and Williams, 1990).

In addition to these areas of overlap between the two lines of transgenic mice, there are also areas where the two mice express β-gal differently (figure 10B). The majority of examples are seen in line 32, which expresses the transgene with higher intensity. In addition to expression within the cartilagenous rings of the trachea, expression of β-gal in line 32 is seen in many internal organs where Timp-1 is not typically expressed during development, such as the heart and intestines. While both lines of mice express LacZ in the intestines, the profile is different between the two lines. Embryos derived from line 32 express the reporter at the periphery of the intestine, while those from line 26 express the reporter within the central region.

There are several possible reasons why there could be differences in expression between the two lines. It could be due to copy number of the transgene, enabling us to visualize regions which typically have a very low output of the gene, or alternatively, it could be due to an insertional effect, which could result in inappropriate expression due to adjacent promoters, or alternatively, due to the dominant effect of chromatin (discussed in more detail in section 4.2). Nonetheless, there are many examples of overlapping expression between the two mice, which suggests that insertional effects

aside, many of the examples of lacZ expression might reflect true Timp-1 promoter driven transgene expression.

An important consideration with our transgene is that by spanning the –2700/+47 region, we did not include the first intron of Timp-1. This alone might explain many of the abnormal expression patterns which I have seen, in light of the intron contributions I have already demonstrated (sections 3.2.1, 3.2.2). However, the requirement of the first intron for tissue specific expression is even more convincing when data from our transgene (-2700/+47LacZ) is compared to that of another group, whose transgene includes the first intron (-1350/+783LacZ)(Flenniken and Williams, 1990). Our mouse shows some correct expression, but many regions of abnormal expression. The intron containing mice, however, express β-gal in regions which have been previously shown to express Timp-1 by *in situ* hybridization with only a few exceptions. This data (discussed in more detail in section 4.2) demonstrate the role of intron 1 in directing correct temporal-spatial expression of Timp-1.

3.3 The Timp-1 Promoter Proximal Region

3.3.1 A Positive Regulatory Region Lies Within the Timp-1 Proximal Promoter Region (-223/-95).

Previous data from the Edwards lab (1992) identified several areas of importance within the promoter proximal region which are required to obtain expression similar to that of the endogenous gene. Aside from the undisputed importance of the AP-1 binding site, there are also several other regions which are necessary for appropriate basal expression, particularly in a region between –223/-95. Earlier CAT reporter studies

showed that the inclusion of this region is required for a greater basal activity compared to a shortened, -95/+47 construct (Edwards et al., 1992). In order to map the positive regulatory elements in the -223/+95 region, I made a series of deletion constructs by PCR amplification and subcloned them into a promoterless luciferase reporter construct (pGL2-basic) (figure 11).

Transient transfections were performed, and reporter analysis revealed the presence of 2 positive regulatory elements (figure 11). The first is located between –125 and –95. Deletion of this 30 base pair region caused a 3-fold reduction in luciferase activity. This three fold change in expression is present in both serum free (basal) and serum stimulated conditions. There are slight changes in the fold induction in the deletions spanning -125 to -195, with the fold induction ranging between 1.7 (-150/+47) and 3.2 (-125/+47). However, these regions were not analyzed further because the overall levels change only slightly, and there are no regions of obvious importance.

There is also a positive regulatory region between –223/-195 (figure 11), though not as dramatic as the region between –125/-95. Unlike the –115/-100 region, this upstream element seems to be involved in a serum stimulatory response, as the fold induction is increased to 3.9 in response to serum (compared to 2.5 fold from the adjacent deletion, -195/+47). The basal levels do not change at all compared to the serum stimulated levels when the –223/-195 region is deleted. This region has not presently been followed up, however, it would be interesting to use a -223/-195 fragment in EMSA analysis to determine whether any nuclear proteins or transcription factors interact with this region of the promoter.

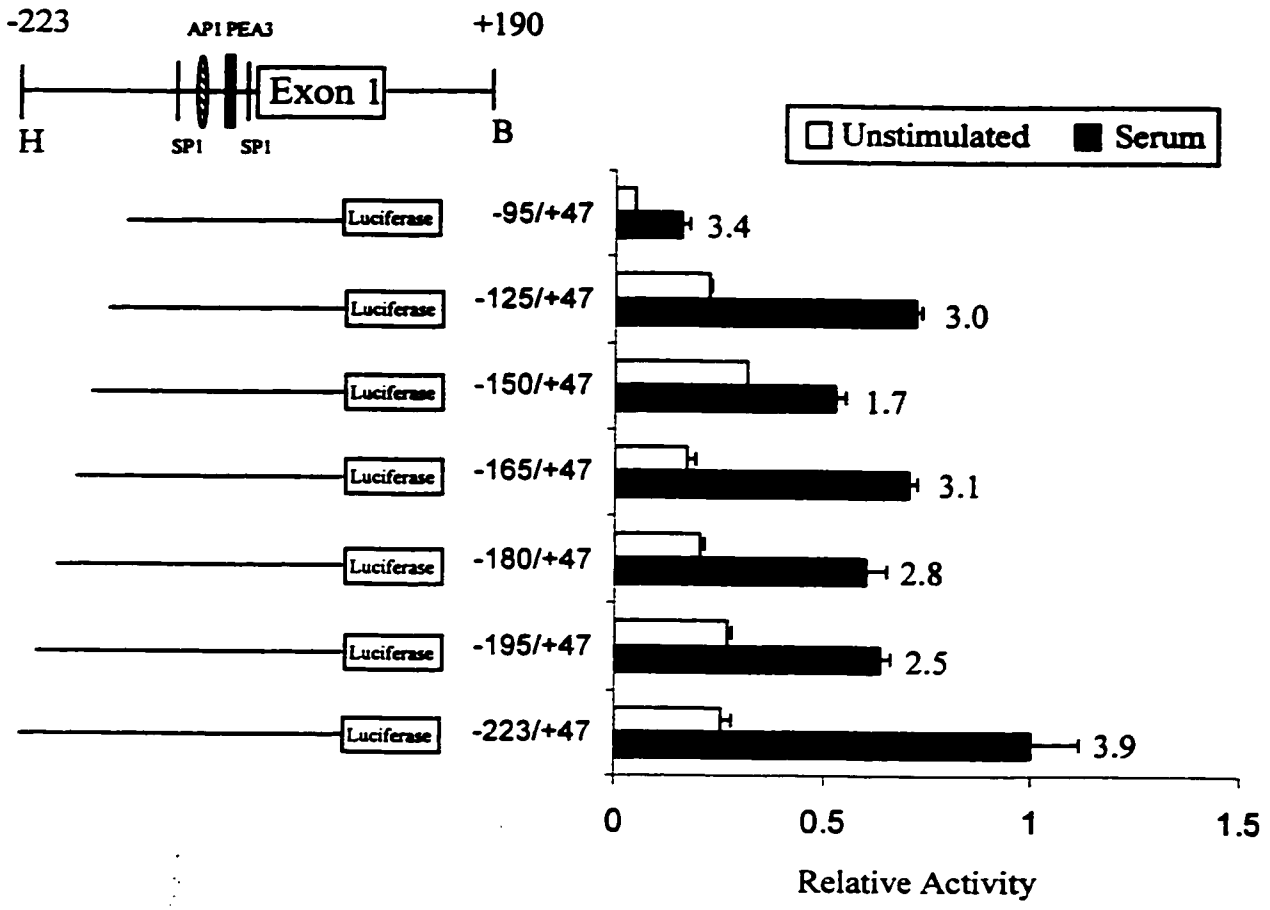


Figure 11. Deletion analysis of the *Timp*-1 promoter region. Deletions spanning -223 and -95 were produced by PCR and the resulting fragments cloned into the pGL2-basic luciferase reporter plasmid (Promega). Following transient transfection into C3H10T1/2 cells the cells were either stimulated with 10% FBS, or left in serum free media (unstimulated). Whole cell extracts were prepared and assayed for luciferase activity. Luciferase activity was standardized by measuring input DNA using the Hirt's assay (described in Methods and Materials). The graph shows relative activity, with the largest value displayed by the -223/+47 construct in serum stimulated cells designated as 1. Fold induction (werum induced compared to unstimulated) is presented numerically beside the serum induced bars.

3.3.2 The –125/-95 Region of the Timp-1 Promoter Interacts With a Single-Strand DNA Binding Protein.

The increase in activity associated with the –125/-95 region of the Timp-1 promoter led to a more detailed analysis of the region. Specifically, I sought to investigate whether the –125/-95 promoter region was able to directly interact with nuclear proteins. A series of oligonucleotides were synthesized (figure 12A) and used for EMSA with nuclear extracts from C3H10T1/2 mouse fibroblastic cells. In order to get the most detailed information from the region, an overlapping series of four probes were used; -125/-95, -123/-110, -115/-100 and –108/-92, which provides a complete coverage of the region. As a control in the experiment, the Timp-1 AP1 binding site (-63/-49) was used, which consistently interacts with AP1 following serum stimulation. A cluster of bands was present in the –125/-95 lanes (figure 12B, lanes 2,7,and 12, labelled 'B'), which were clarified to a single band in the –115/-100 lane (lanes 4, 9 and 14). Shifts were not present in the –123/-110 or –108/-92 lanes. It is difficult to say why there are multiple bands present in the –125/-95 complex, while for the smaller fragment, –115/-100, only a single complex is seen. One possibility is that while the –115/-100 sequence is the minimal requirement for protein-DNA interactions, flanking sequence found in the –125/-95 interact with multiple protein species, or enable a more complicated interaction, resulting in the multiple bands seen in the EMSA. Alternatively, there may be non-specific interactions, which do not occur with the smaller fragments.

In order to test whether the protein factor is induced, a time course was performed by serum stimulating the cells, and collecting nuclear extracts at 0, 30 minutes, or three hours (figure 12B).

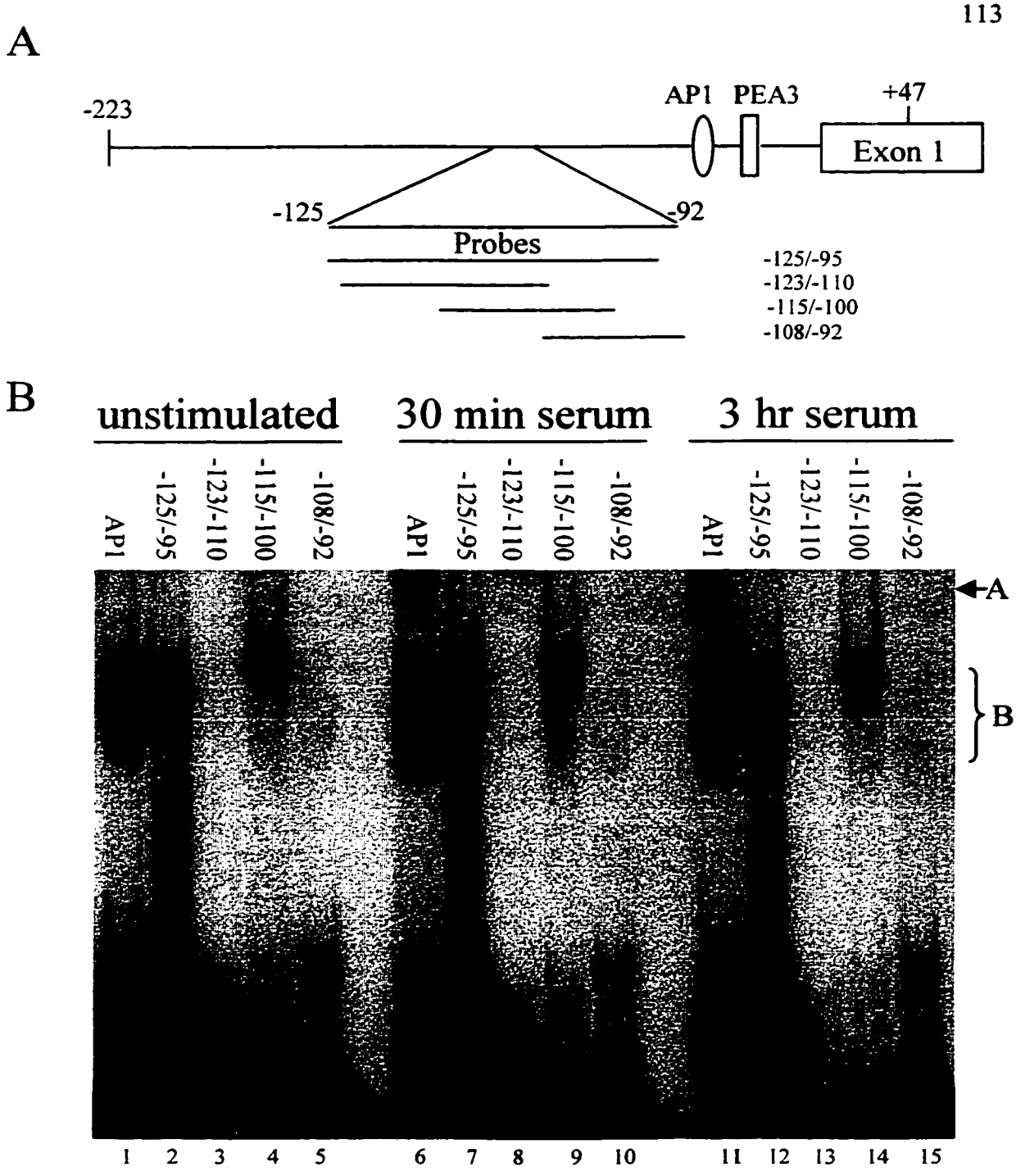


Figure 12. DNA binding factors interact with the -115/-100 region of the *Timp-1* promoter. Annealed probes corresponding to the promoter regions depicted in A were labelled with T4-polynucleotide kinase. The probe was then incubated with C3H10T1/2 cell nuclear extracts which had been stimulated for either 0, 30 minutes, or 3 hours. The EMSA reactions were then separated on a 7% PAGE gel (B). As a control, the Timp-1 AP-1 binding site was used as a probe against the same extract. Two patterns of bands were seen. A band corresponding to AP1 (A) and a cluster of bands, sharpened with the -115/-100 probe, which is an unidentified binding protein (B).

While a protein-DNA interaction was evident at all of the time points for the -125/-95 and -115/-100 construct, the protein species itself was not altered in level following serum stimulation. The apparent levels of the protein in the EMSA was the same in all time points studied, which indicates that the factor was present at constant levels irrespective of induction. Because I have already demonstrated that the interaction at -125/-95 does not alter the overall inducibility of the gene (figure 11), I hypothesize that the complex observed by EMSA is involved in regulating basal expression of the Timp-1 gene. However, other mechanisms of action must not be overlooked, as several transcription factors, though present at constant levels, still affect gene induction through recruitment or activation (NFκB, RAR) (Zandi and Karin, 1999).

While care was taken in preparation of the duplex oligonucleotides to ensure similar molarities of the sense and antisense strands were annealed, I did not gel purify the final labeled product, which gives the likelihood that there will be some single-stranded probe within the probe mix. Similarly, there is a possibility that some of the duplexes will melt, leaving a population of single-stranded probe. A similar phenomenon can be seen in the control, -63/-49 lane used in the experiment. The Timp-1 AP1 binding site (-63/-49) was included because its interaction with nuclear proteins within stimulated 10T1/2 cells has been characterized (Edwards et al., 1992). There are two groupings of bands in the AP1 lanes (figure 12, lanes 1,6,and 11), designated A and B. Previous research from the Edwards lab has focused on the interactions at the Timp-1 AP-1 binding site (Sharma, 1993). In that study, it was demonstrated that the 'A' band is bona fida AP-1 by competition with the collagenase canonical AP-1 binding site (TGAGTCA), as well as by supershifting the 'A' complex with anti-Fos and anti-Jun antibodies. Only

preliminary work on the identification of the protein component of the 'B' complex was performed, and demonstrated that it was not AP-1, but rather a single-stranded DNA-binding protein (Sharma, 1993). Further characterization of the single-stranded interaction at –63/-49 is provided in section 3.3.3. Because of the history of a single-stranded protein/DNA interaction on the Timp-1 promoter (-63/-49), I wanted to characterize whether the interaction at –115/-100 was with single- or duplex DNA.

The EMSAs depicted in figure 12B and 13A used polynucleotide kinase end-labelled probes in the binding reaction. To demonstrate whether the –115/-100 complex was due to single-stranded DNA, I used probes labelled with Klenow polymerase, which will generate only duplex probes. When Klenow labelled probes were used, I saw a great reduction in complex formation for the –125/-95, and a reduction of the –115/-100 band (figure 13, compare lanes 2 with 7, and 4 with 8). However, it is important to note that the cluster of bands (B band, lane 7) did not completely disappear, which might indicate either that there are non-specific interactions, that there is a lower affinity interaction with double-stranded DNA, or that the strands are melting, and interacting with the single-stranded binding factor. Similarly, when using a double stranded probe, the single-stranded complex was no longer detectable, however, a new complex, though faint, is visible (C band, lane 8). This could be due to non-specific protein interactions, which are seen on occasion in EMSA reactions, or there could be an interaction, though weak, with the duplex DNA probe. A similar effect is seen for the –63/-49 Timp-1 AP1 site, in which the AP1 band is unaffected, however, the cluster of faster migrating bands (labelled B) was greatly diminished (lanes 1 and 9). This observation suggests that a

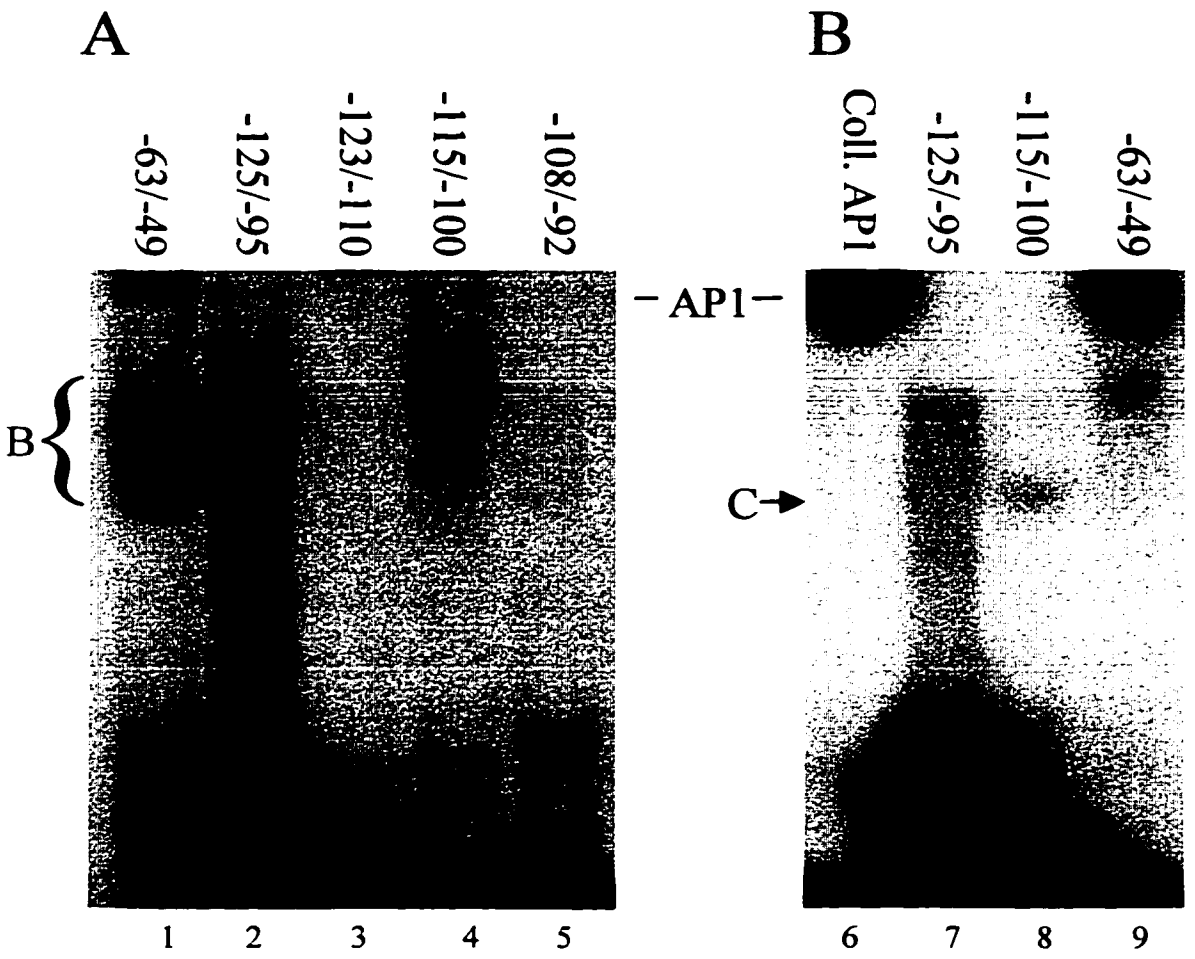


Figure 13. Single-stranded DNA binding factors interact with the *Timp-1* promoter at two different locations. Protein-DNA interactions were assayed by EMSA under two conditions as follows: a polynucleotide kinase end-labeled mixed strand (single- and double-stranded) probe population (A) and a Klenow labeled, double-strand probe (B). Nuclear extracts from C3H10T1/2 cells serum stimulated for 30 minutes were used for the assays with probes corresponding to -63/-49, -123/-110, -115/-100, and -108/-92and the AP1 site, -63/-49 of the *Timp-1* promoter, as well as the consensus, collagenase 1 AP1 site (coll.AP1) as a control. Poly (dI:dC) was used as a non-specific competitor. While the band corresponding to AP-1 was not affected by the method of labeling, only the end-labeled, mix-strand probe was able to effectively form the B complex, indicating its single-strand specificity.

nuclear protein is interacting with *Timp-1* promoter sequences between –115/-100 with single-strand preference.

3.3.3 Both –115/-100 top and –63/-49 bot Bind to a Single-Strand DNA Binding Protein.

It has now been demonstrated that two regions of the Timp-1 promoter interact with single-stranded DNA-binding proteins (-115/-100 and –63/-49), at least one which was functionally important in basal expression (-115/-100). The next step was to characterize the single-stranded interaction. I labelled single-strand probes corresponding to two of the regions of the Timp-1 promoter; the –115/-100 region, and the –63/-49 region (figure 14A). The top (sense) strand of –125/-95 (lane 1), as well as the top strand of –115/-100 (lane 3) interact with a nuclear protein, while neither bottom strand does (lanes 2 and 4). It is important to consider that the -115/-100 bot strand is able to self anneal into a duplex structure, due to a palindromic (..ATCCGGAT..) sequence present in its 5' end. This could interfere with any single-stranded interactions in vitro using the short oligonucleotides. However, since the -125/-95 bot sequence, which does not form the stable duplex structure, is unable to interact with nuclear proteins in EMSA, it is likely that the -115/-100 bot sequence behaves similarly. Therefore, I can assume that the bottom strand of this -125/-95 promoter region does not interact with single-stranded binding proteins. The size of the DNA-protein complexes is variable between the –115/-100 oligonucleotide and the –125/-95 probe. This is not unreasonable if we consider that the –115/-100 fragment provides the minimal elements for interaction with the protein, but such interaction might be stabilized or otherwise differently made in the context of

the larger fragment, which contains flanking sequence. Because there is a low EMSA signal from the bottom strands of both –115/-100 and –125/-95, I concluded that the top strand contributed to the binding activity seen from the mixed strand experiments. For the control, the collagenase consensus AP-1 binding sequence was used, and did not interact with any single-stranded binding factors.

The –63/-49 probes demonstrate a complicated interaction with the nuclear proteins (figure 14, lanes 5 and 6). It has already been shown that double-stranded –63/-49 oligonucleotides interact with AP1 (figure 12, 13)(Edwards et al., 1992). However, a further complexity is revealed when examining the single-strand binding capacity of the promoter region as well. Both strands of –63/-49 interact with nuclear factors, resulting in complexes with differing mobilities. There is a single high mobility band for the top strand (lane 5) compared to multiple complexes which are associated with the bottom strand (lane 6). The difference in appearance of the complexes (compare lanes 5 and 6) in addition to the different sequence between the two strands suggests that each strand binds to different proteins.

To characterize the protein DNA interactions, a series of competitions were performed (figure 14B). For many of the remaining studies on the single-stranded DNA binding protein interactions, the –115/-100 top strand of Timp-1 was used. In the competition experiments, unlabelled strands of –125/-95, -115/-100, -63/-49 and the collagenase AP1 binding sites were used. The top strand of both –125/-95 and –115/-100 effectively competed away the shift, demonstrating specificity (figure 14B, lanes 10 and 12). The bottom strands of both –125/-95 and –115/-100 were also effective competitors of the –115/-100 top shift (lane 11 and 13).

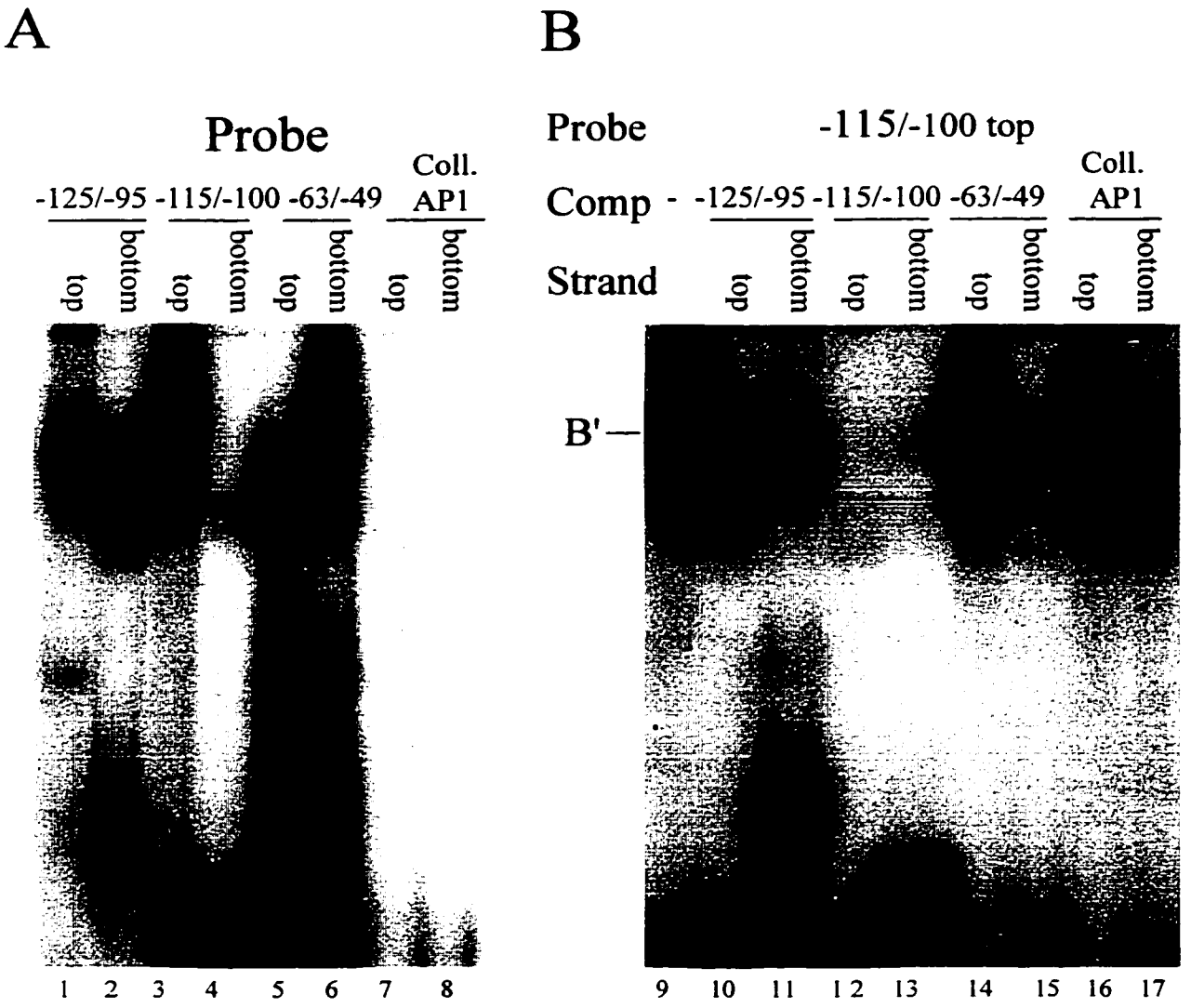


Figure 14. Two regions of the Timp-1 promoter, -115/-100 top and -63/-49 bottom cross compete for the same single-stranded DNA binding protein. Single-stranded probes corresponding to the -125/-95, -115/-100, -63/-49, and collagenase AP1 binding site were used for EMSA analysis with C3H10T1/2 cell nuclear extracts (A). Complexes were formed with the -125/-95, and -115/-100 top and both strands of the -63/-49 regions. B) The -115/-100 top strand was labeled and used for EMSA with the indicated unlabeled competitor oligonucleotides (100x molar excess).

However, this is misleading, since the bottom strand would anneal to the probe to form duplex DNA, which I have already established to have poor binding activity to the single-stranded DNA binding protein (figure 13B). Such an annealing between probe and competitor is seen in the slower migration of the free probe at the bottom of the EMSA gel (figure 14B, lanes 11 and 13).

I was surprised to observe that the bottom strand of –63/-49 also competed away the shift for –115/-100 top. Alternatively, the top strand did not compete, which provides evidence that both strands of the –63/-49 region interact with different single-stranded DNA-binding proteins. These data are important for several reasons. First, it indicates that two regions of the Timp-1 promoter, *in vitro*, are capable of interacting with, and cross competing for the same nuclear factor. Also, it brings more attention to the –63/-49 AP1 binding site. As stated earlier, this site differs from a canonical AP1 binding site by a single base pair change. It appears that this change from the canonical AP1 site enables the region to interact with possibly two single-stranded DNA binding proteins as each strand alone appears to bind to a different nuclear factor.

In order to demonstrate that the –115/-100 top and –63/-49 bot region do in fact, interact with the same binding protein, the reciprocal competition experiment shown in figure 15 was performed. With the –63/-49 bottom strand labelled, the –115/-100 top strand effectively competed the binding factors (lane 5). As expected, neither strand of the –115/-100 region was able to compete with the –63/-49 top strand.

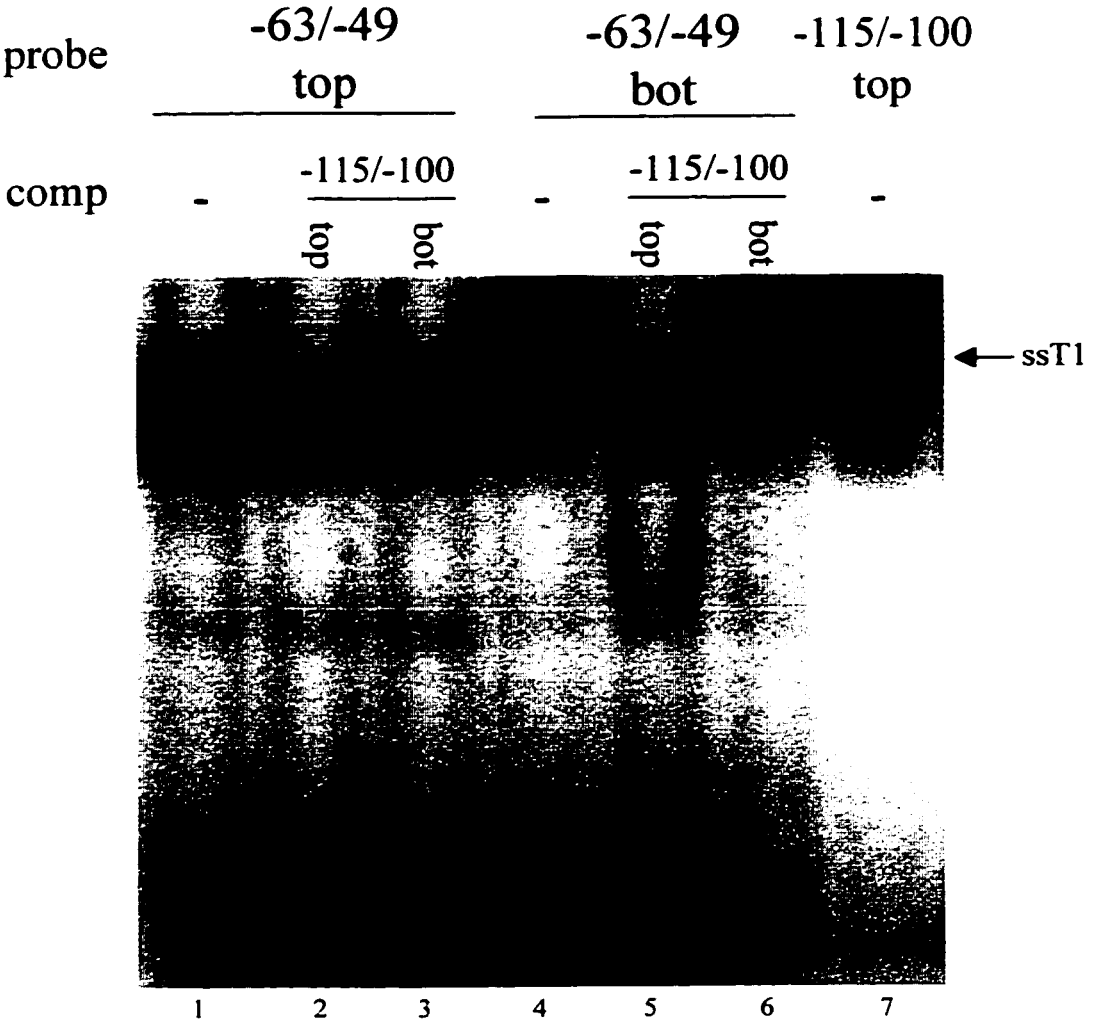


Figure 15. The -63/-49 bottom and -115/-100 top regions of the Timp-1 promoter both interact with ssT1. As an inverse competition experiment (compare with figure 14B), the single-strands of the -63/-49 region were end-labeled, and tested by EMSA with C3H10T1/2 cells. For the competitions, 100 fold molar excess of cold competitor was added to the binding reaction of theEMSA.

3.3.4 The Protein-DNA Interaction is Specific, But Sequence Selective

Both –63/-49 bottom and –115/-100 top compete for the same factor, yet they retain only partial sequence identity. I performed competitions in which a battery of promoter regions and oligonucleotides were used to determine whether the interaction was specific, or if it was a non-specific single-stranded DNA interaction. As seen in figure 16, there is a high degree of specificity in the interaction of the single-stranded DNA binding protein, which will hereafter be referred to as ssT1 (single-stranded Timp-1 promoter binding protein). A mixed probe population (end-labelled annealed duplex probe, resulting in some single-stranded DNA binding, see figure 12) was added to C3H10T1/2 cell nuclear extract in the presence of different mixed-strand competitors (figure 16A and B). As a control, each of –125/-95, -115/-100, and –63/-49 competed for ssT1 binding as seen before (lanes 3-5). Of the other transcription factor binding sites used as competitors, none were as effective as the three control competitors.

Quantification (figure 16 panel B) shows that the CRE-WT sequence resulted in a decreased complex intensity, which I can attribute to a gel artifact (a scratch which reduced the intensity of the complex). There appears also to be competition from the NFκB sequence (lane 13, 3.4 fold reduction in complex intensity), though it is not as effective as the cold Timp-1 AP1 sequence, or the -115/-100 self competition sequence (7.3 and 7.1 fold reduction in complex intensity respectively) (figure 16, lanes 3-5, and 16B). Furthermore, the reciprocal experiment showed that -125/-95 was unable to compete for an NFκB shift (16C, lanes 14-16). These experiments demonstrate that it is not NFκB which is interacting at -115/-100, however it cannot be ruled out that the NFκB sequence does not interact with the ssT1 factor.

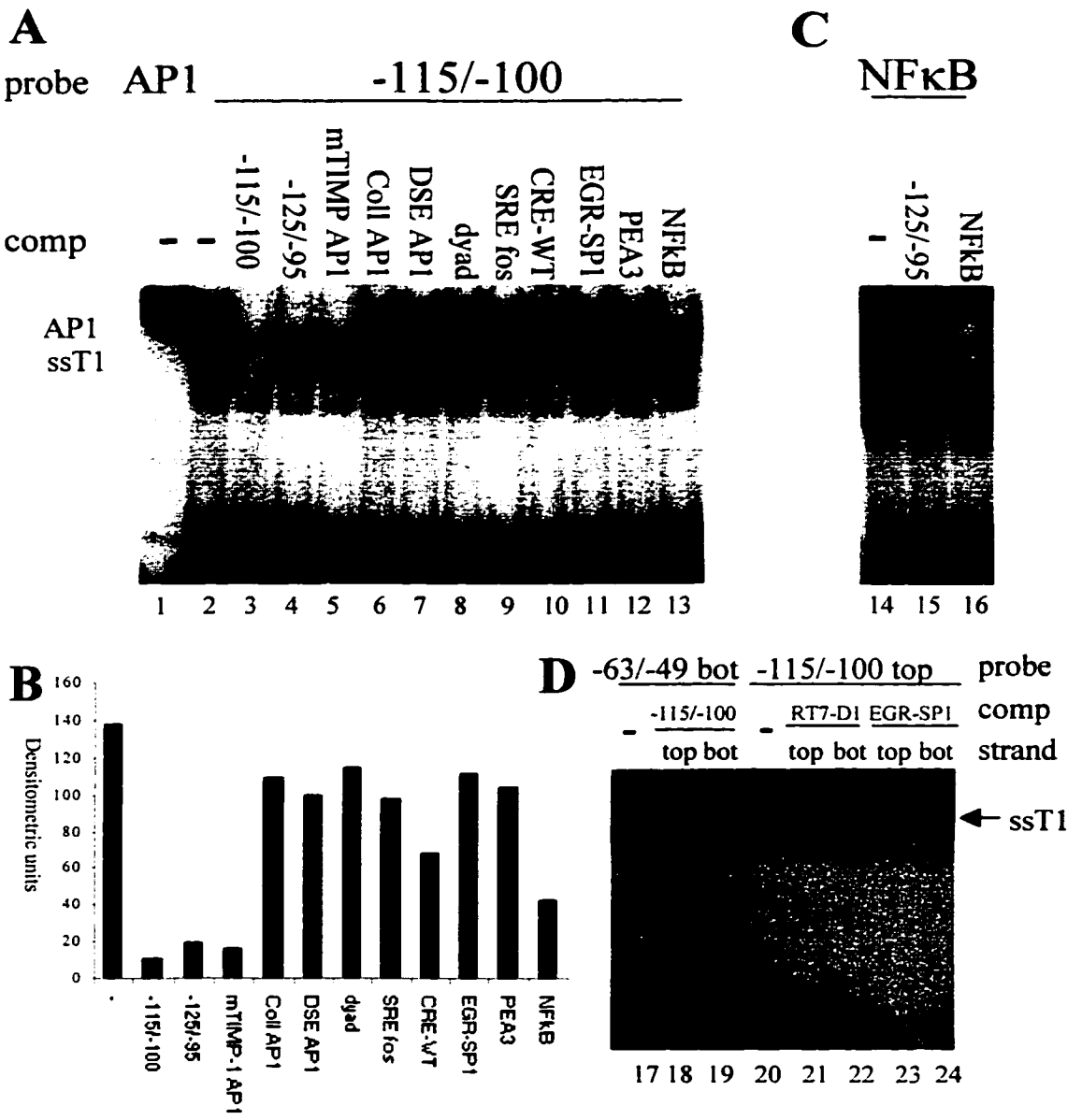


Figure 16. The ssT1 binding factor is specific for regions of the Timp-1 promoter. The -115/-100 promoter region of Timp-1 was used as a probe for EMSA with C32H10T1/2 nuclear extracts stimulated for 3 hours. A) The mixed (single and double strand) probe was end labelled with PNK. As a competitor, 100 fold molar excess of cold mixed strand competitor was added in the binding reaction. The collagenase AP1 binding sequence was used as a positive control (lane 1). B) quantification of the complexes in (A) using the image analysis software, NIH image™. C) NFκB was used as a probe in EMSA using 10T1/2 cell nuclear extracts. Cold competitors were added at 100 fold molar excess. D) Single-stranded competitors were used versus the -115/-100 top sequence at 100 fold molar excess. Competitors used were the RT7-D1 binding site (21&22), and the SP1 site of EGR (23&24). As a control, -115/-100 top and bottom strands were used as competitors against the -63/-49 bottom probe.

This idea is reinforced by the fact that the sequence used for the NFκB EMSA experiments contains a conserved CTTT sequence present in both the -115/-100 and Timp-1 AP-1 sequences (discussed in greater detail in figures 17 and 18).

Additional experiments looked at different single-stranded competitors and their effect on the ssT1 interaction with –115/-100. One competitor was of particular interest, called RT7-D1, because it also had been shown to interact with single-stranded DNA-binding proteins (Oosterhuis and van der Hoorn, 1999). Additionally, it has some sequence similarities with the –115/-100 top sequence, including a 5'-CTT, which proved critical to RT7-D1 interaction with nuclear proteins. However, neither strand of RT7-D1 proved effective at competing for ssT1 against –115/-100 top (figure 16D, lanes 21 and 22). Therefore, it appears unlikely that the RT7-D1 binding factor is the same as the ssT1 factor. Still, because they do share some similarities, including single-strand DNA interactions, and a conserved CTT sequence, the idea that they belong to a class of single-stranded DNA-binding transcription factors cannot be ruled out.

Additional experimentation of the specificity of ssT1 interactions was performed by doing a linker scan, which involves the sequential mutation of a series of bases, along the entire length of the oligonucleotide, in order to localize the important regions for the protein-DNA interactions (see figures 17A and 18A).

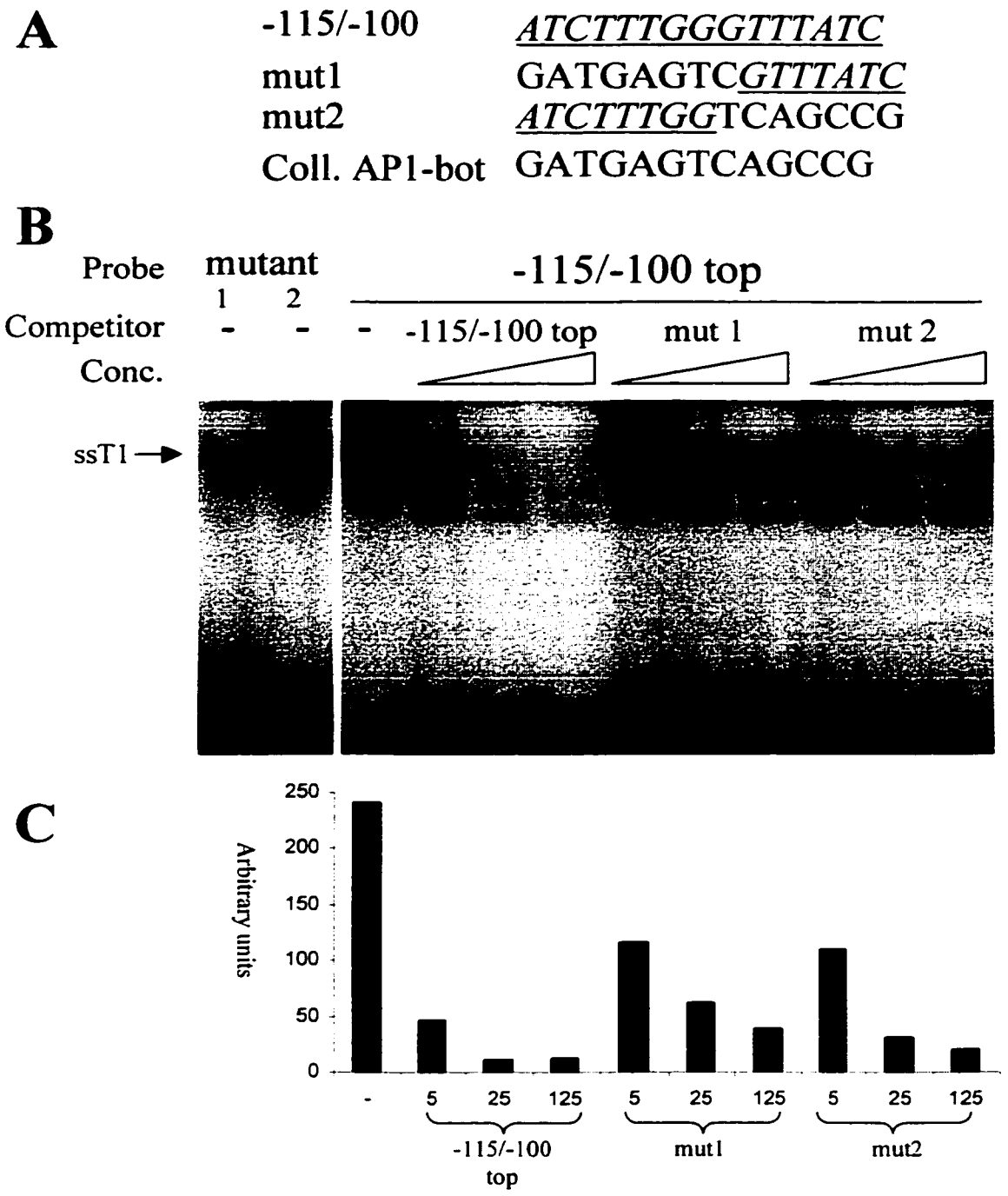


Figure 17. The ssT1 factor displays sequence selectivity. Fusion oligonucleotides were made combining the 5'-half of the collagenase AP1-binding site to the 3'-half of the -115/-100-top binding sequence (mut1), or the 5' half of the -115/-100 top to the 3'-half of the collagenase AP1-binding site (mut2) (A). These mutants were used for EMSA analysis both as probes with C3H10T1/2 cells, and as cold competitors against -115/-100-top probes. Both mut1 and mut2 were less effective at complex formation than the wild type -115/-100 top probe. Cold competitor oligonucleotides were used at 5x, 25x, and 125x molar excesses (B). Both mut1 and mut2 oligonucleotides competed for binding to the ssT1 factor, and the overall competitor effectiveness was WT>mut2>mut1. The complex intensity in the EMSA was quantified (C) using image analysis software (NIH Image ™).

As an initial indication of which region of the –115/-100 oligonucleotide was important, the -115/-100 fragment was broken into 2 halves, where either the upstream or the downstream half was mutated (figure 17A). I used the collagenase-AP1 bottom strand sequence for the mutations because that sequence has been shown to not have any interaction with the ssT1. In this case, mutating the upstream half had the more deleterious effect, though both mutants, mut1 and mut2 were less effective at ssT1 binding compared to the wild-type sequences (lanes 1 and 2, compared with lane 3). These same mutant oligonucleotides were also used as cold competitors against the wild-type -115/-100 top probe, and quantified using NIH Image software ™ (figure 17 B and C). Similar to their effectiveness at interacting with ssT1 in lanes 1 and 2, both mutant 1 and mutant 2 were less effective as competitors compared to the wild type sequence. Quantification showed that at 5x molar excess, there was little difference between the effectiveness of both mut1 or mut2 to compete for ssT1. However, as the concentration increased, mut2 appeared to be a slightly better competitor than mut1, which agrees with the results from lane 1 and 2. However, the difference between the two oligonucleotides was not as great in the cold competition experiments.

The –115/100 region was tested with a more detailed linker scan, in which 3 bases were mutated at a time (figure 18). Quantification of complex intensity when the mutants were used as competitors in EMSA is shown in figure 18B. None of the mutants completely eliminated competition ability, although disruption of the 5'-ATCTT at -115/-114 and -113/-111, as well as the 5'-ATC at –102/-100 had the most deleterious effect on competition ability, and hence on the interaction with ssT1. These data are particularly

A

-115/-100 (WT)	ATCTTTGGGTTTATC
MUT -115/-114	**CG**CTTTGGGTTTATC
MUT -113/-111	AT**TGG**TGGGTTTATC
MUT -110/-108	ATCTT**GAT**GTTTATC
MUT -107/-105	ATCTTTGG**ACC**TATC
MUT -104/-102	ATCTTTGGGTT**CGC**G

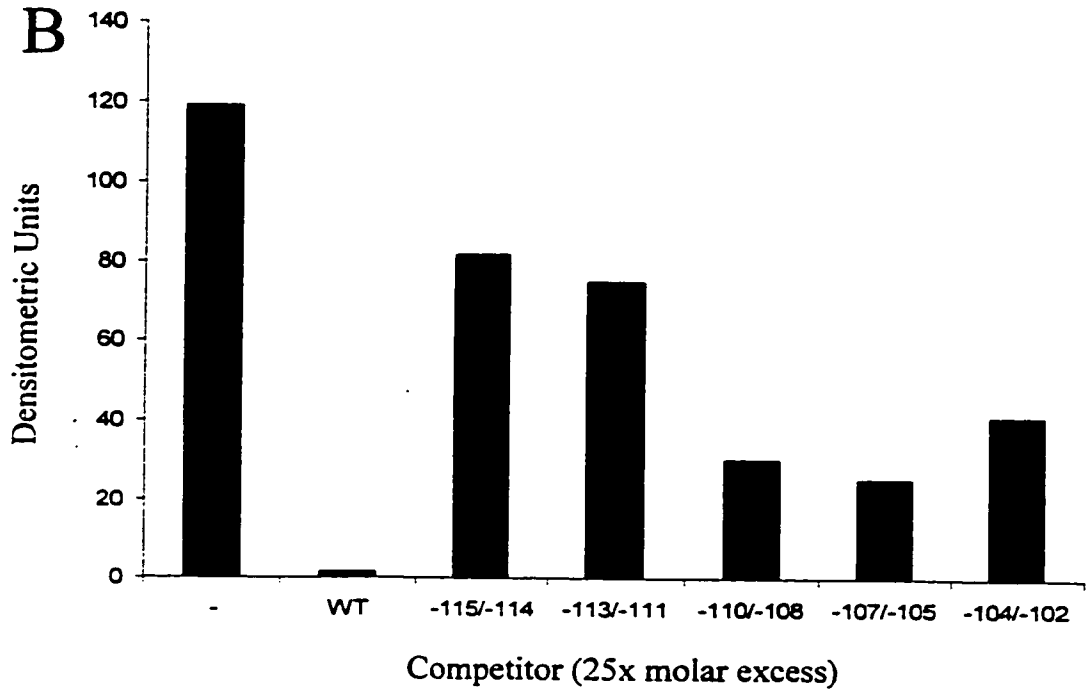


Figure 18. The ssT1 DNA-binding factor displays sequence selectivity. An additional set of mutations (compared to those from figure 16) were designed such that the regions of the mutation were more refined (A). Purine/pyrimidine switches were constructed within the -115/-100-top sequence corresponding to the numerical name of the oligonucleotide (for example, bases -115 and -114 were mutated in the -115/-114 oligonucleotide). This mutant series was used as cold competitors in EMSAs, and the resulting shifted bands were measured densitometrically using NIH Image ™ . The resulting plot shows levels of competition relative to the wild-type oligonucleotide sequence.

important when the sequence of –115/100 top (5'-agcttATCTTTGGGTTTATC) is compared with the –63/-49 bottom sequence (5'-gatccGCATTAACTCATCCA). While initially it appears that there is little similarity between the two sequences, there is a conserved 5'-C(T/A)TT region at the 5'-end of both sequences, and a 5'-ATC at the 3' end. This, in combination with the results of the linker scan, suggest a binding preference for ssT1 as 5'-C(A/T)TT$(N)_{4-6}$ATC-3'. The preference for the C(A/T)TT is particular interesting because the RT7D1 sequence described earlier also has a CTT element which is required for protein interactions (Oosterhuis and van der Hoorn, 1999). However, this identity alone was still not sufficient to allow the RT7D1 oligonucleotide to bind to the ssT1 protein (figure 16B).

I next wanted to test the specificity of ssT1 for artificial components of the probes – namely the restriction tags which were placed on the oligonucleotides for the purpose of cloning. I made a –115/-100 top oligonucleotide which did not have the restriction site, and used it as a competitor in EMSAs in which the previously used, restriction containing construct, was used as a probe (figure 19A). The restriction-less competitor was equal in effectiveness as the restriction containing competitor (compare lanes 2-4 with 5-7). Further support for the notion that ssT1 is interacting with *Timp-1* sequences is seen by end-labelling the restriction-less –115/-100 top sequence (19A, lane 9), which resulted in a shifted complex, although the complex had a slightly higher mobility compared to the restriction-site containing probe. The observed change in mobility is likely a consequence of a shorter oligonucleotide in the complex, but might also reflect an altered configuration of the protein-DNA interaction as well.

In order to test for the cellular localization of the ssT1, extracts prepared from nuclei were compared to whole cell extracts on EMSA using the -115/-100 top sequence as a probe (figure 19C). There is ssT1 complex formation from both types of extracts, however, the concentration in the whole cell extract is lower compared to the nuclear fraction. During the preparation of nuclear extracts, care was taken to avoid contamination of cytoplasmic proteins in the extraction protocol. Microscopic examination of the nuclei (following the initial triton lysis) was performed to ensure lysis was complete, followed by several rinses, prior to the high salt extraction. However, there is the possibility that there is contamination from the cytoplasmic fraction into the nuclear fraction, or visa versa. Following the identification and cloning of ssT1, it would be interesting to perform immunolocalization to more accurately localize the protein. Another possibility might be to probe fixed cells with the -115/-100 top labelled oligonucleotide to localize the protein

As a final test of the specificity of the ssT1 and –115/-100 top interaction, I used single-stranded salmon-sperm DNA as a competitor (figure 19, lane 10). Using a vast excess of the salmon sperm DNA (10ug in the binding reaction) still did not completely abolish the ssT1:-115/-100 top complex. The complex is reduced in intensity compared to uncompeted oligonucleotide (figure 19, compare lanes 8 and 10), which lends support to the hypothesis that the ssT1 is sequence selective, and might therefore be competed by many different sequences. However, the fact that the band remains in the presence of such a vast excess of single-stranded competitor also demonstrates that the reaction is not non-specific.

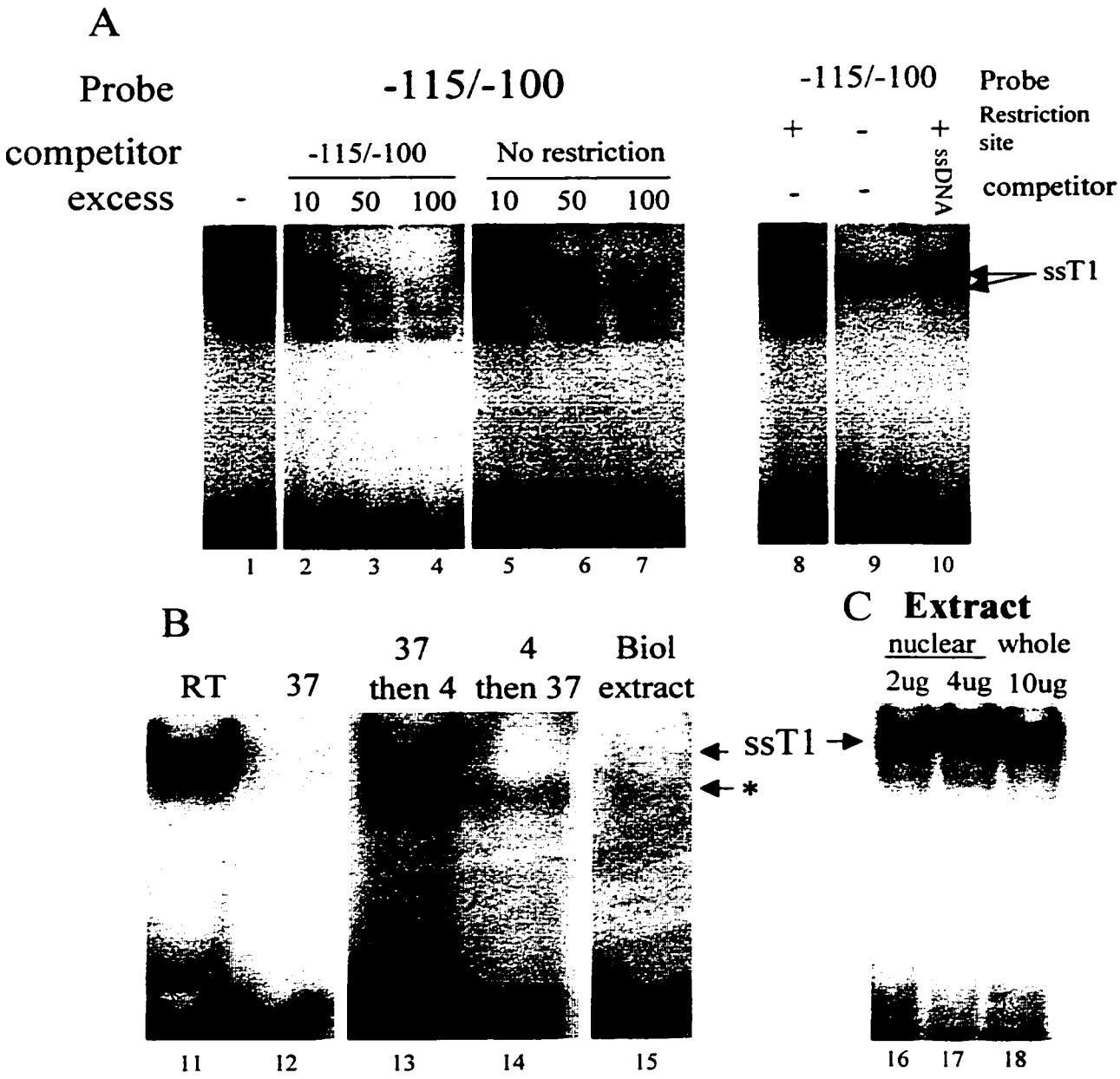


Figure 19. Analysis of the binding conditions of ssT1. Using the -115/-100 top probe for EMSA against C3H10T1/2 nuclear extracts, several conditions were examined. A) The restriction tag on the -115/-100-top oligonucleotide was removed, and the oligonucleotide still effectively worked as a competitor (lanes 5-7) as well as interacting directly with the ssT1 (lane 9). The specificity of interaction is demonstrated by the inability of single-strand salmon sperm DNA (10ug) to completely compete away the interaction (lane 10). B) samples were incubated at room temperature with no change to binding capacity. Incubation for 30 minutes at 37 degrees Celsius eliminated the interaction (lane 12). Incubating at 37°C, then lowering to 4°C or vice versa (lanes 13 and 14) reduced ssT1 interaction. Boiling the nuclear extract prior to the binding reaction resulted in no shift, the consequence of denaturing the ssT1 factor. C) Nuclear and whole cell extracts were used for EMSA with the described protein amounts (2, 4, or 10 ug). The samples were probed with the -115/-100 top oligonucleotide.

3.3.5 Optimal Incubation Temperature of the ssT1:-115/-100 top Interaction

A series of experiments were carried out in order to assess some of the properties of the ssT1-DNA interaction. First, a series of temperature conditions were used during the incubation period to determine the effect on the ssT1-DNA interaction (figure 19). Boiling of the nuclear extract prior to interaction with the DNA destroyed the interaction (lane 15). Additionally, heating the binding reaction to 37°C also impaired the interaction, as did preheating the extract at 37°C (figure 19, lane 12 and 13). While this might indicate that the reaction is temperature sensitive, and works best at room temperature or 4°C (where there was no qualitative difference in the actual EMSA reaction), it might equally show simply that the extract or ssT1 itself is sensitive to proteolysis. Under those circumstances, the ssT1 would be degraded at 37°C, where it is most susceptible to proteolysis, although protease inhibitors were present in the reaction. This might be demonstrated in figure 19 (lane 13), where there is a fast migrating complex (*), which could be either a non-specific interaction, or possibly, a proteolytically cleaved form with altered mobility.

3.3.6 The ssT1 Interacts With Two *Timp-1* Promoter Regions, and has Positive Basal Transcription Effects at Both Sites.

The initial deletion analysis demonstrates a positive basal regulatory effect from the -115/-100 Timp-1 promoter region (figure 11). However, I had not yet shown a similar effect from the ssT1 interaction at the AP1 binding site (-63/-49). This is problematic, because AP1 interaction has such a strong positive regulatory effect within the promoter proximal region. Previous studies attempted to mutate the AP1 binding site

to the canonical collagenase AP1 site (Sharma, 1993), which I have demonstrated to be unable to interact with the ssT1. In those experiments, there was no difference between the *Timp-1* AP1 site or the collagenase AP1 site with respect to reporter activity. However, these mutations were in the context of the -223/+47 promoter regions. Therefore, there will be contributions from the upstream regions, -223/-95, including the -115/-100 ssT1 binding site which might mask any difference between the two AP1 binding sites.

In order to eliminate these confounding variables, I assessed the impact of mutation of the ssT1 site in the context of –115/+47 constructs with either a normal –63/-49 AP1 site, or with the site replaced by a consensus coll.AP1 motif or mutant AP1 (figure 20). Since both the mutant AP1 and the coll.AP1 sequences do not interact with ssT1, this allowed us to focus on the contributions of the –115/-100 and –63/-49 ssT1 sites in basal promoter activity. Incorporation of the mut 1 sequence (used in Figure 17) into the –115/+47 reporter caused a 15-20% reduction in promoter activity (Figure 20, lanes 1 and 2). When the Timp-1 AP-1 binding site was then mutated to the collagenase AP-1 (with the background of a mut 1 ssT1), there was a further decrease of reporter activity. Elimination of AP1 binding ability had a dramatic effect on promoter activity, with the mutant AP1 construct, which eliminates any AP-1 interaction, yielding only 20% of the expression seen with the –115/+47 wild-type TIMP-1 promoter region, which confirms previously seen results. However this low basal level could be further reduced to approximately 10% of that of the wild-type promoter by inclusion of the mut1 sequence at –115/-100.

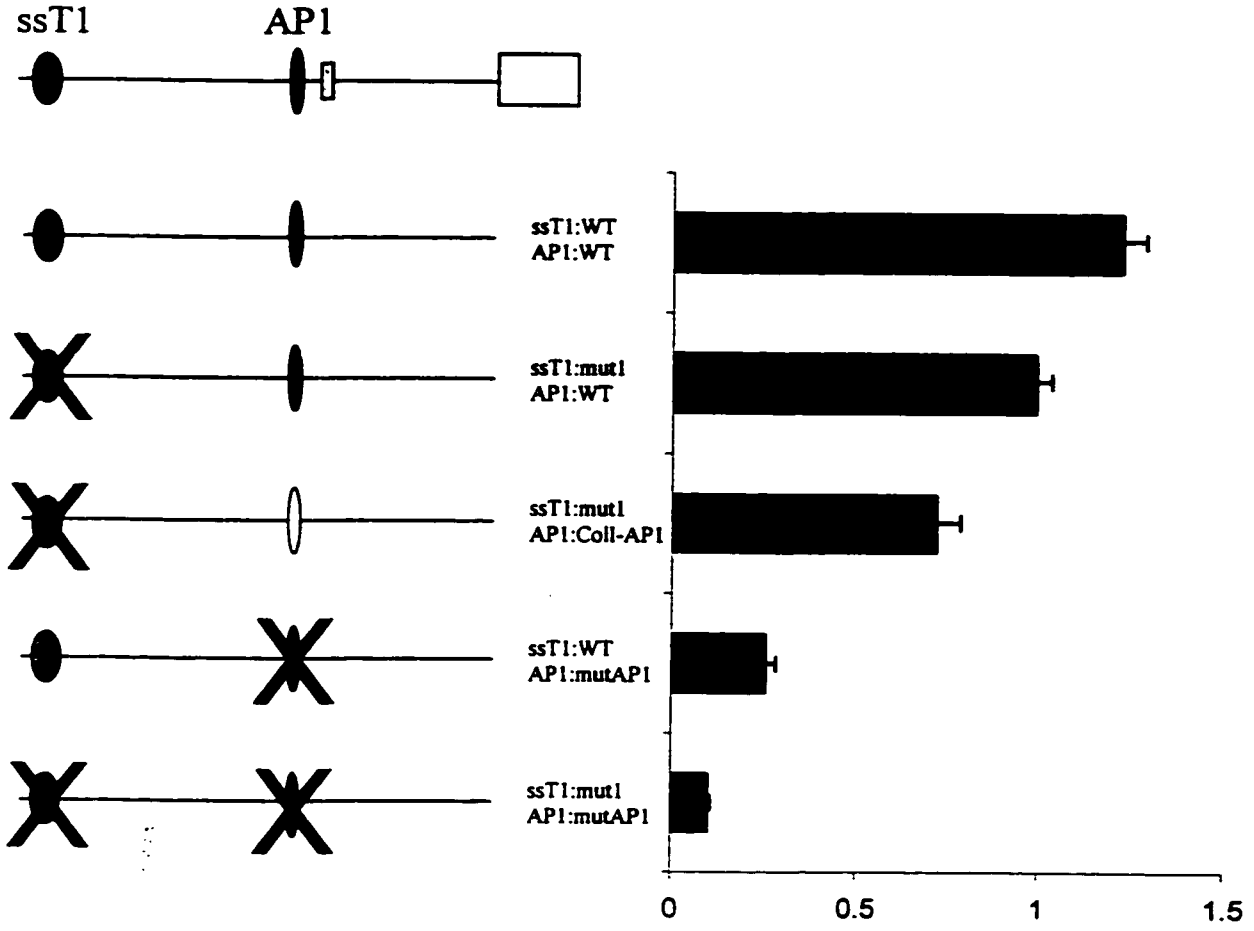


Figure 20. ssT1 interactions at both promoter regions (-115/-100 and -63/-49) positively affect reporter activity. Mutations of the ssT1 site at -115/-100 and the -63/-49 AP1 site were introduced into the -115/+47 *Timp-1* promoter region. The *Timp-1* AP1 motif (WT) was converted to a consensus collagenase or a mutant AP1 site (coll AP1 and mutant AP1 respectively) and placed in the context of wild-type or mutant ssT1 (mut1, figure 16). Relative basal CAT activities following transient transfection of C3H10T1/2 cells are displayed. Mutation of the -115/-100 ssT1 site reduced basal expression relative to the wild-type promoter, and this reduction was exacerbated with either a canonical coll.AP1 site or mutated AP1 at -63/-49.

These data argue that both ssT1 binding sites at –63/-49 and –115/-100 contribute to the basal activity of the TIMP promoter. I suspect that the effect of mutating the ssT1 site was not as severe as deleting it (compare figure 20 with figure 11) because the mut1 mutation still retains some ability to interact with ssT1 (figure 17). It would be informative to use a mutant sequence that lacks any interaction with ssT1 to observe the full effects of the ssT1 at the AP-1 binding site. This experiment provides support that the ssT1 is involved in expression at the -63/-49 promoter region, however, an alternate explanation for the observed reporter expression levels is that the Timp-1 AP-1 binding site (TGAGTAA) binds to a different subset of AP-1 factors compared with the collagenase AP-1 binding site (TGAGTCA), which could then have differential effects on transcription rates. It should be noted that Sharma et al (1993) found no difference between the abilities of various AP1 factors (including c-Fos, c-Jun, JunB, JunD, FosB, and combinations thereof) to bind the -63/-49 AP1 site compared to the collagenase AP1, using reticulolysate-translated proteins.

3.3.7 ssT1 Binding is Conserved in Human TIMP-1.

The AP-1 binding site found in the mouse TIMP-1 is conserved among the TIMP-1 genes identified so far in different animal species. However, there are still differences in the promoter regions of the genes. In order to determine if the human AP1 binding site also interacts with ssT1, oligonucleotides were obtained which contain both the human promoter proximal AP1 binding site (AP1-86) and the intronic AP1 binding site (AP1+144; a gift from Dr. Ian Clark). When used as competitors in EMSA analysis against the mouse –115/-100 top sequence (figure 21), the human sequences were able to

compete in a similar manner as the murine regions. The bottom strand of the promoter proximal human AP1 site competed effectively for ssT1 from the murine probe (figure 21, lane 3). When an oligonucleotide with a mutated AP-1 binding site was used (MAP1-86), it competed for ssT1 as effectively as the wild-type sequence (lane 6), despite no longer containing the C(A/T)TT$(N)_{4-6}$ATC sequence. These data indicate that either flanking sequences are important for ssT1 interaction, or that the sequence specificity is even more selective than we have hypothesized. Nonetheless, the interaction with ssT1 is conserved in the human promoter region. These results indicate a functional significance to the conservation of the unusual AP1 binding site found in most species.

Studies from another lab (David Young, personal communication) have shown that the AP-1 binding site at +144 (within the first intron) of the human TIMP1 gene interacts with a single-stranded DNA-binding protein. I tested the oligonucleotide as a competitor for ssT1, but found that there was no effect on the –115/-100/ssT1 complex (figure 21, lane 7).

A

Timp-1AP1bot	GATCCG**CATT**ACTC**ATC**CA
AP1-86 top	TGGTGGGTGGATGAGTAATGCATCCAGG
AP1-86 bot	CCTGGATG**CATT**ACTC**ATC**CACCCACCA
AP1+144 top	GCAGCCAAGCTGAGTAGACAGGCATCTG
AP1+144 bot	CAGATGCCTGTCTACTCAGCTTGGCTGC
MAP1-86 bot	CCTGGATG**CATC**ACTT**CTC**CACCCACCA
MAP+144 top	GCAGCCAAGCGAAGTGGACAGGCATCTG

B

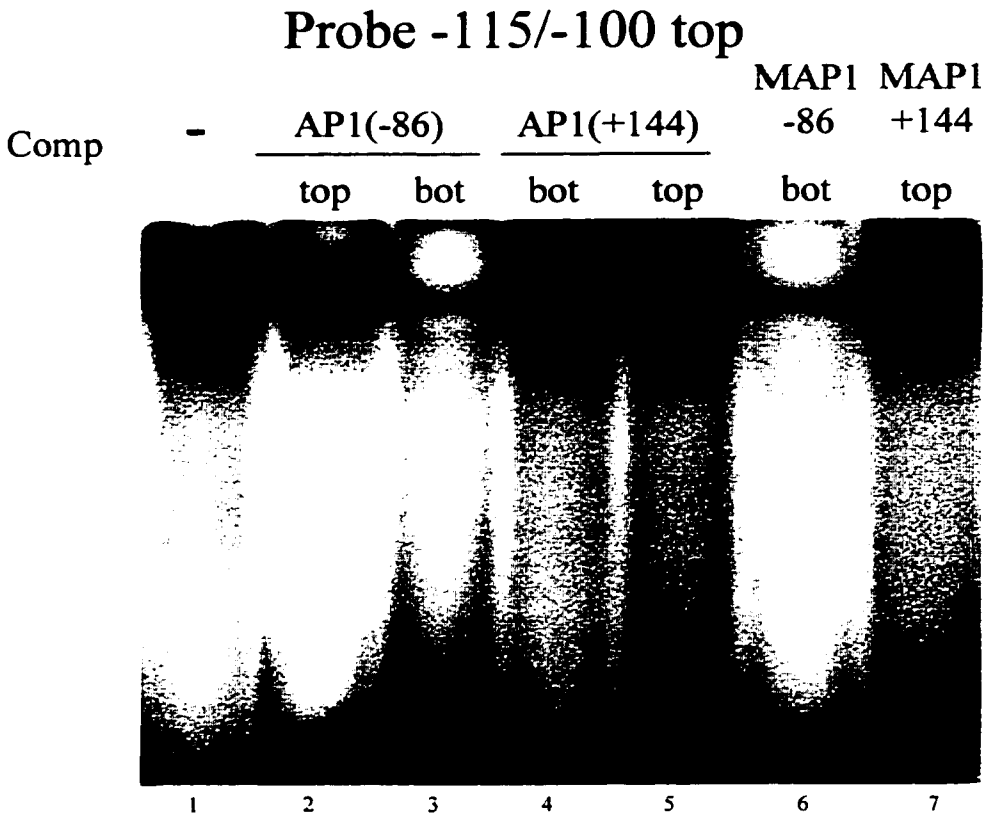


Figure 21. The human AP1 site within the TIMP1 promoter can compete for the murine ssT1 DNA-binding factor. The -115/-100 top probe of the murine Timp-1 was used for bandshifts with C3H10T1/2 cell nuclear extracts. As competitors, human sequences were used from the human promoter proximal AP1 binding site (AP1(-86), as well as an AP1 binding site in the first intron (AP1(+144) (A) The ssT1 consensus sequence, C(A/T)TT$(N)_4$ATC is indicated in bold. Two of the lanes contain mutated AP1 sequences (lanes 6 and 7). The EMSA analysis (B) shows competition (100x excess) for ssT1 from human Timp-1 sequences corresponding to the promoter proximal AP-1 binding site.

3.4 The Properties of the ssT1.

3.4.1 Complex Stability

I was interested in the stability of the ssT1:-115/-100 top complex in vitro. Therefore, I determined the binding efficiency by using cold competitors to measure the rate of complex dissociation. In this case, the EMSA binding reaction was set up for the standard 30 minutes (see methods and materials). Then, cold competitor was added, where different reactions were exposed to the competitor for different periods of time figure 22). The experiment was performed such that the binding reactions were initiated at different (staggered) times, so that all reactions were terminated by loading onto a native gel at the same time. In performing this time course, I enabled the complexes to form, and could then determine the amount of time it takes for the complex to dissociate in the face of excess cold competitor. This demonstrated that there is an approximate half-life for the complex of about 10 minutes, and by 60 minutes the complex was almost completely dissociated.

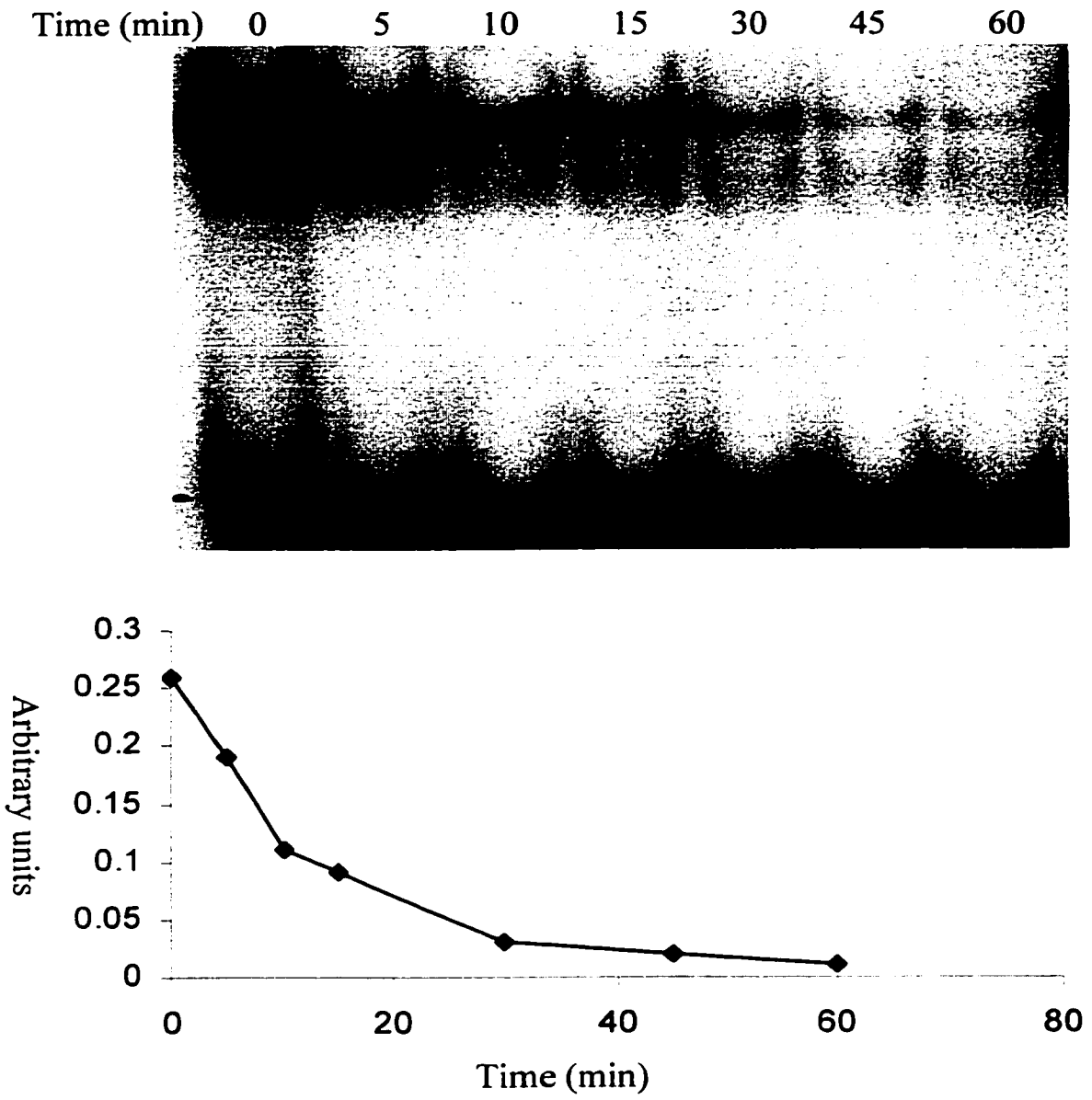


Figure 22. Time course of ssT1 binding efficiency. The -115/-100 top probe was used for EMSA against C3H10T1/2 cell nuclear extracts. The binding reaction was allowed to proceed for the usual 30 minutes (see methods and materials). Then, 100x molar excess of cold competitor was added, at time 0. Competition was allowed to proceed for the following time intervals - 0, 5, 10, 15, 30, 45, and 60 minutes before separating the reaction on an SDS-PAGE gel.

3.4.2 Approximate Concentration of ssT1 in C3H10T1/2 Nuclear Extracts

To determine the approximate molar concentration of the ssT1 in 10T1/2 nuclear extracts, I did a titration with increasing concentrations of probe. I started with 0.002 pmol up to a maximum amount of 0.09pmol (figure 23). Using the image analysis program NIH image™, I then determined the net intensities of the EMSA bands that appeared. Two things were being examined. First, the point at which the ssT1 complex did not increase in intensity despite increasing amounts of probe. This means that the majority of ssT1 was already complexed, and occurred at approximately 0.0175 pmol of probe. The second criterion was based on an examination of the free probe. When ssT1 concentrations are greater than probe concentrations, all of the probe will be complexed, and no free probe will be visible. As the concentrations become more equal, there will be increased amount of free probe visible. When 0.004 pmol of probe was added to 2 μL of nuclear extract, all of the probe was complexed and there was no free probe at the bottom of the polyacrylamide gel (figure 23, lane 2). However, a slight band of free probe is visible when 0.01 pmol of probe is used, and the free probe then increased dramatically with each subsequent increase in probe added to the binding reaction. Using this analysis, I estimated the concentration of ssT1 in C3H10T1/2 cell nuclear extracts to be between 0.01 pmol/μL and 0.0175 pmol/μL.

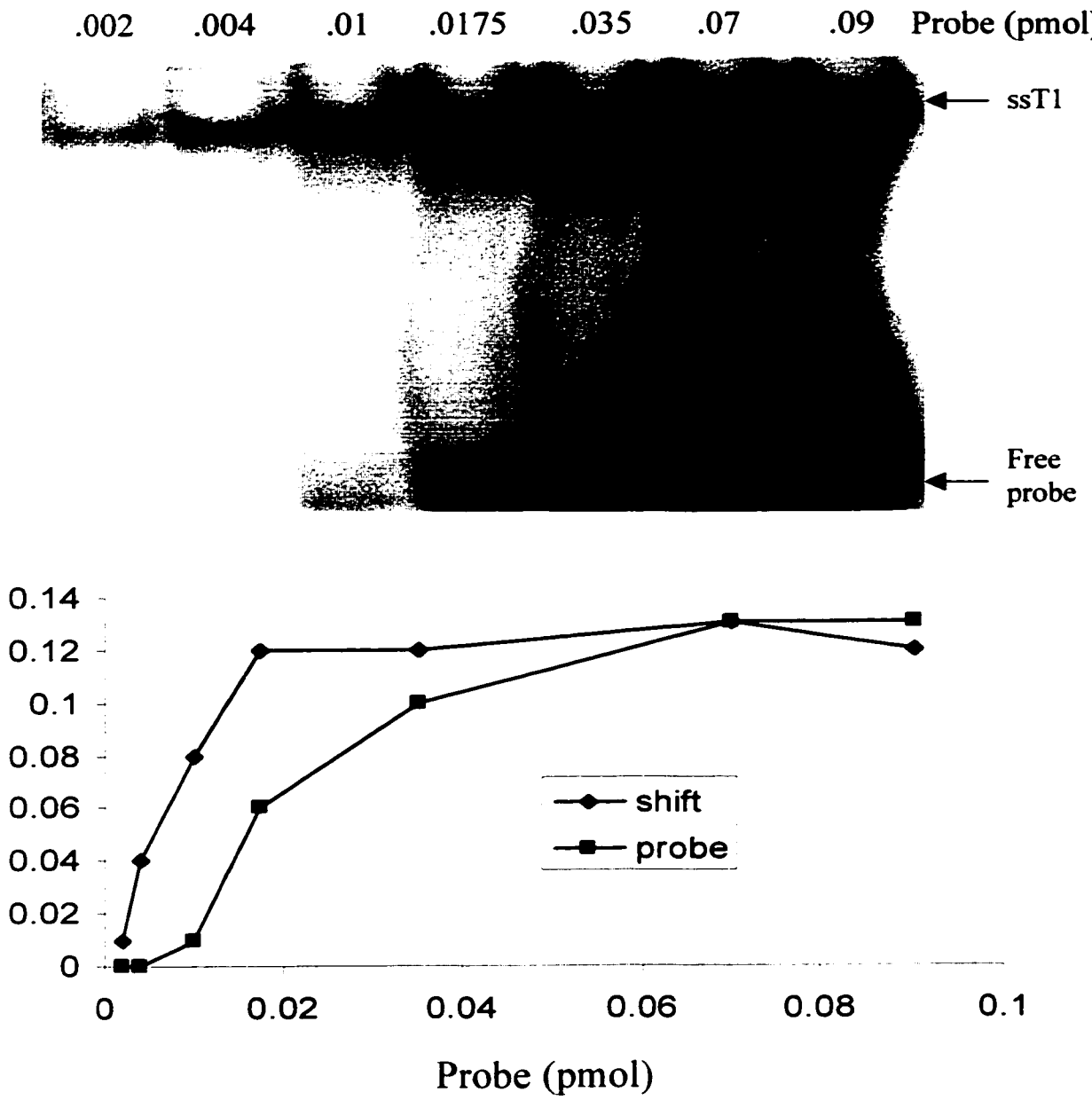


Figure 23. Estimate of ssT1 molarity within the 10T1/2 cell nuclear extract. Increasing amounts of probe were used in EMSA binding reactions to determine the approximate levels where ssT1 is titrated out of the C3H10T1/2 cell nuclear extract. Two measures can be examined, the appearance of free probe at the bottom of the gel, and the point at which the ssT1 band no longer increases in intensity.

3.4.3 Optimal Ionic Concentration for ssT1:DNA Interaction

I examined the effect of salt concentration on the interaction between –115/-100 *Timp-1* top and ssT1 (figure 24). A range of KCl was included in the EMSA binding reaction, from a low of 32.5mM (the amount brought in from the high salt nuclear extraction protocol) to a high of 2M. The ssT1 was able to interact with the –115/-100 top over the entire range of KCl concentrations. There seemed to be slightly less-optimal conditions for interaction between 150mM and 300mM of KCl, however the complex still formed under these conditions. While a complex formed even at the highest range of ionic concentration, it was difficult to tell if there was a qualitative difference compared to the lower salt concentrations because the mobility in polyacrylamide gels was disrupted by the high salt levels. The stability of the complex in such high ionic concentration was surprising, especially in light of the fact that the protein is competed off the DNA with a half life of around 10 minutes (figure 22). There is also a possibility that the high ionic concentration is being diluted in the gel following loading, which would then allow the complex to form. While the altered mobility of the free probe does indicate a change in conditions, it is difficult to maintain a 2M KCl concentration through the entire course of the experiment.

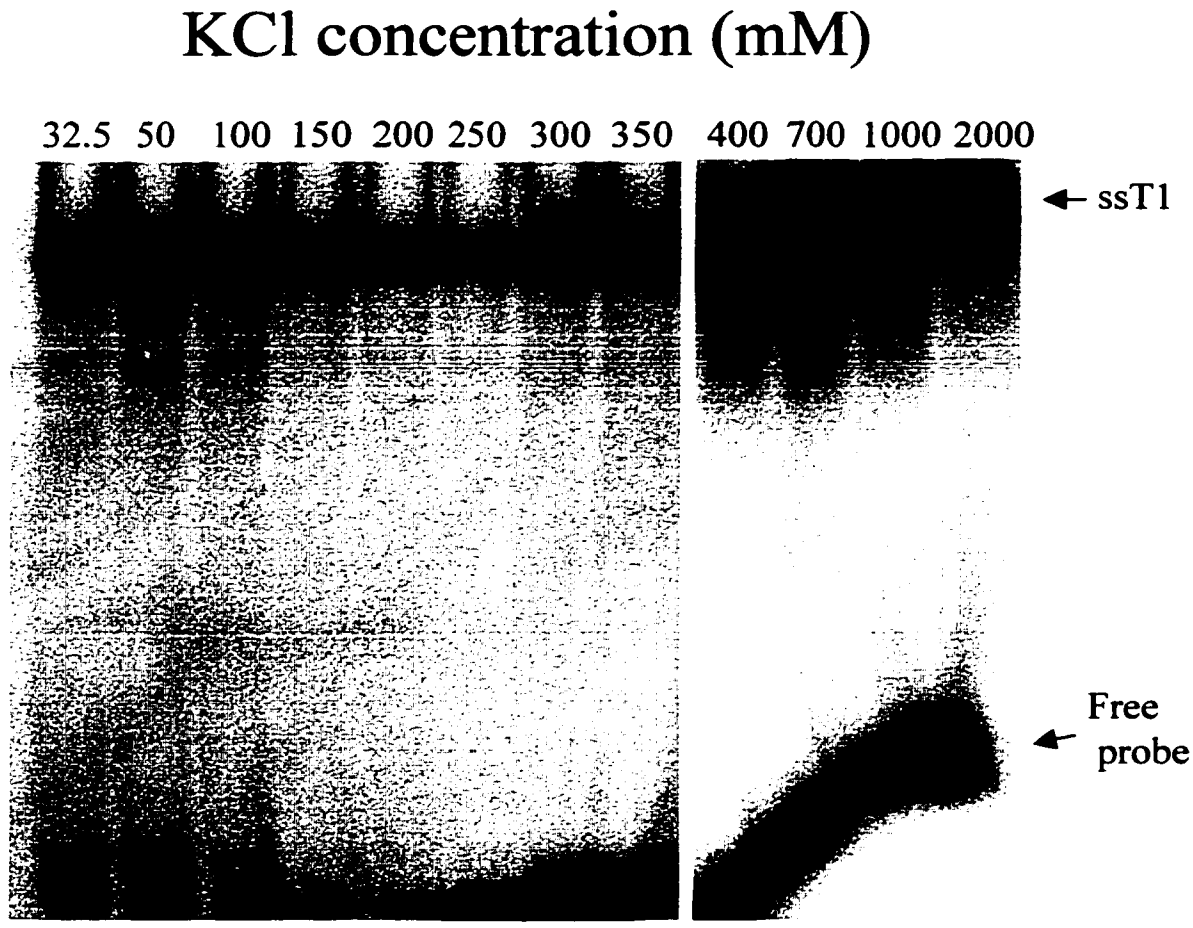


Figure 24. The ssT1 binding factor interacts with the -115/-100 top *Timp-1* sequence in a wide range of salt concentrations. The concentration of KCl was altered over a range of 32.5mM to 2M in the typical EMSA binding reaction. The change in KCl concentration had little effect on the ability of the ssT1 to interact with the DNA. However, the high salt did effect the overall migration of the free probe, and the ssT1 complex in the PAGE gels.

The stability of the complex at such high salt concentration also questions the ability of the factor to be extracted from nuclei using a high salt extraction protocol. This stability in high salt could be a consequence of the artificial in vitro assay system (EMSA buffer is not the same as the in vivo cell microenvironment). Alternatively, there is no evidence that the ssT1 factor is in contact with the DNA in general situations. It has been identified in cytoplasmic extracts, though in lower concentration compared to nuclear extracts (figure 19C). Furthermore, the ssT1 requires single-stranded DNA, and is unable to interact with duplex DNA, which means that it might require other proteins to open the DNA. This dependence upon other proteins might limit its ability to interact with the DNA under many circumstances, which would result in a largely soluble protein. Finally, the complex might not be stable at such high salt concentrations, and the result might be the result of complex formation following a dilution of KCl in the electrophoresis tank prior to loading.

3.4.4 ssT1 is a Protein With an Approximate Molecular Weight of 54 KDa.

To determine the approximate molecular weight of the proteins which interact with the –115/-100 and –63/-49 regions of the Timp-1 promoter, the EMSA binding reaction was performed and the complexes bound irreversibly by UV crosslinking. Each complex could then be separated by electrophoresis in an SDS-PAGE gel without the risk of dissociation (figure 25). Similar to what was seen by EMSA analysis, the top strands of both –125/-95, and –115/-100 interact with nuclear proteins (lanes 4 and 7). The range in size for the multiple bands is between 30 and 65 kDa, as compared to standard

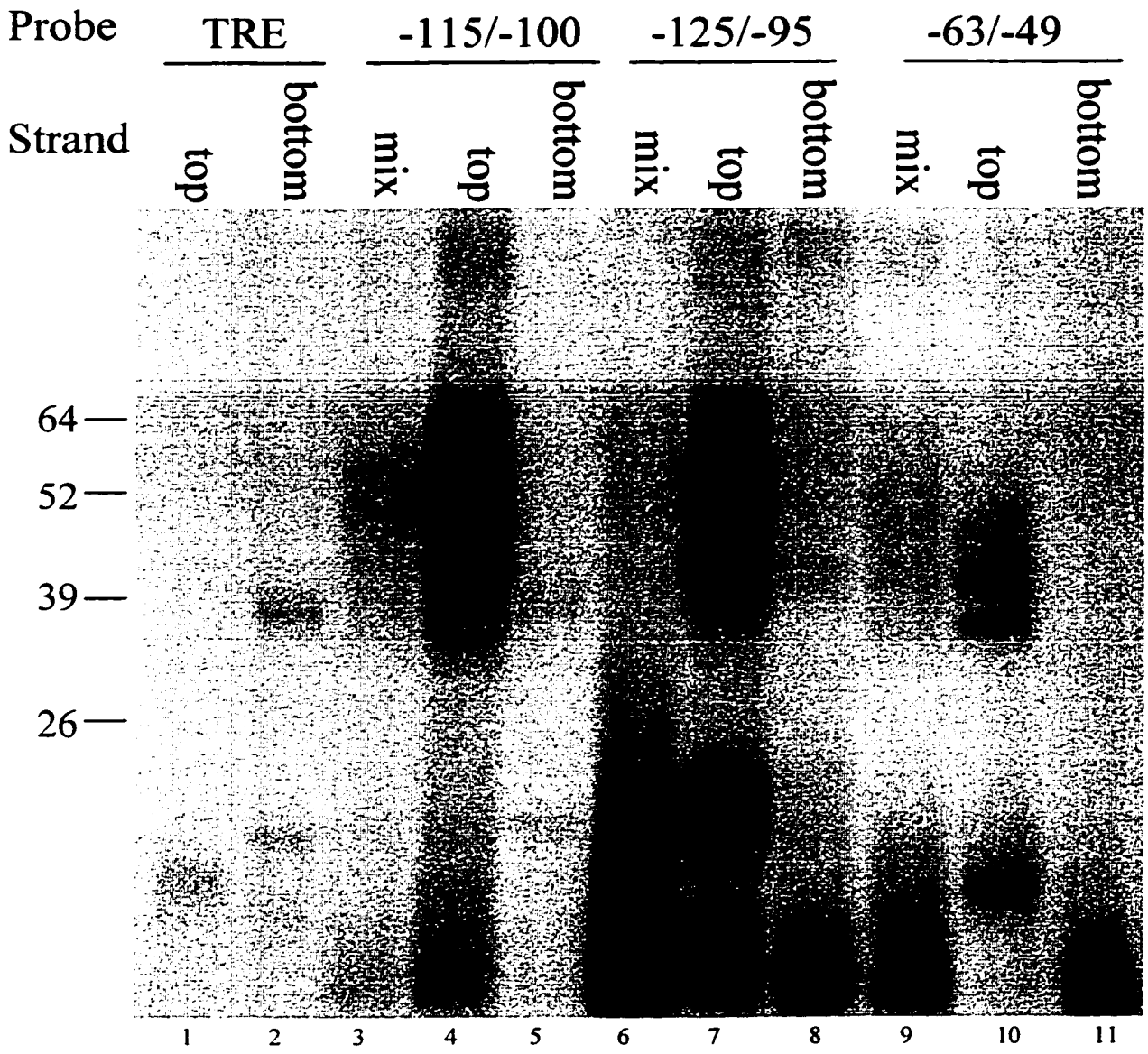


Figure 25. SDS-PAGE analysis of protein-DNA interactions within regions of the *Timp-1* promoter. A series of probes within the Timp-1 promoter (-115/-100, -125/-95, -63/-49, and the consensus collagenase AP1 site (TRE)) were used in either mixed, top or bottom strand form for EMSA analysis. The protein-DNA complexes were then UV crosslinked, and separated on an SDS-PAGE gel. By comparing to protein size markers, an approximate molecular weight for the ssT1 factor can be obtained.

molecular weight markers. There were no complexes seen for the bottom strand of either –125/-95 nor –115/-100 (lanes 5 and 8).

Neither strand of the control collagenase AP1 sequence (TRE) interacted with any nuclear proteins (figure 25, lanes 1 and 2), which is consistent with previous experiments. The *Timp-1* –63/-49 region, however, gave mixed results. The top strand, as seen previously in UV-crosslinking studies, gives a triplet of bands which are between 35 kDa and 45 kDa in molecular weight. However, the bottom strand does not show any complex under these conditions. I have already demonstrated that the region does interact with ssT1 by EMSA and competition experiments, but unfortunately, size determination is not possible in this manner, because a UV-crosslinked complex did not form. UV-crosslinking may depend on close proximity of critical bases in DNA and side-chains in the protein. Therefore it is not unreasonable that all complexes are not able to crosslink.

Competitions were then performed in UV-crosslinking experiments using the –115/-100 top as a probe (figure 26). The competitions were virtually the same as was seen in the EMSA experiments. –125/-95 top and –115/-100 top were all able to compete with the probe (lanes 3 and 5), as would be expected. The bottom strands of each of the aforementioned probes were also able to compete, again likely because of duplex formation (lanes 4 and 6), or possibly because of self-annealing secondary structure. In this experiment, the –63/-49 top strand as well as the bottom strand were able to compete.

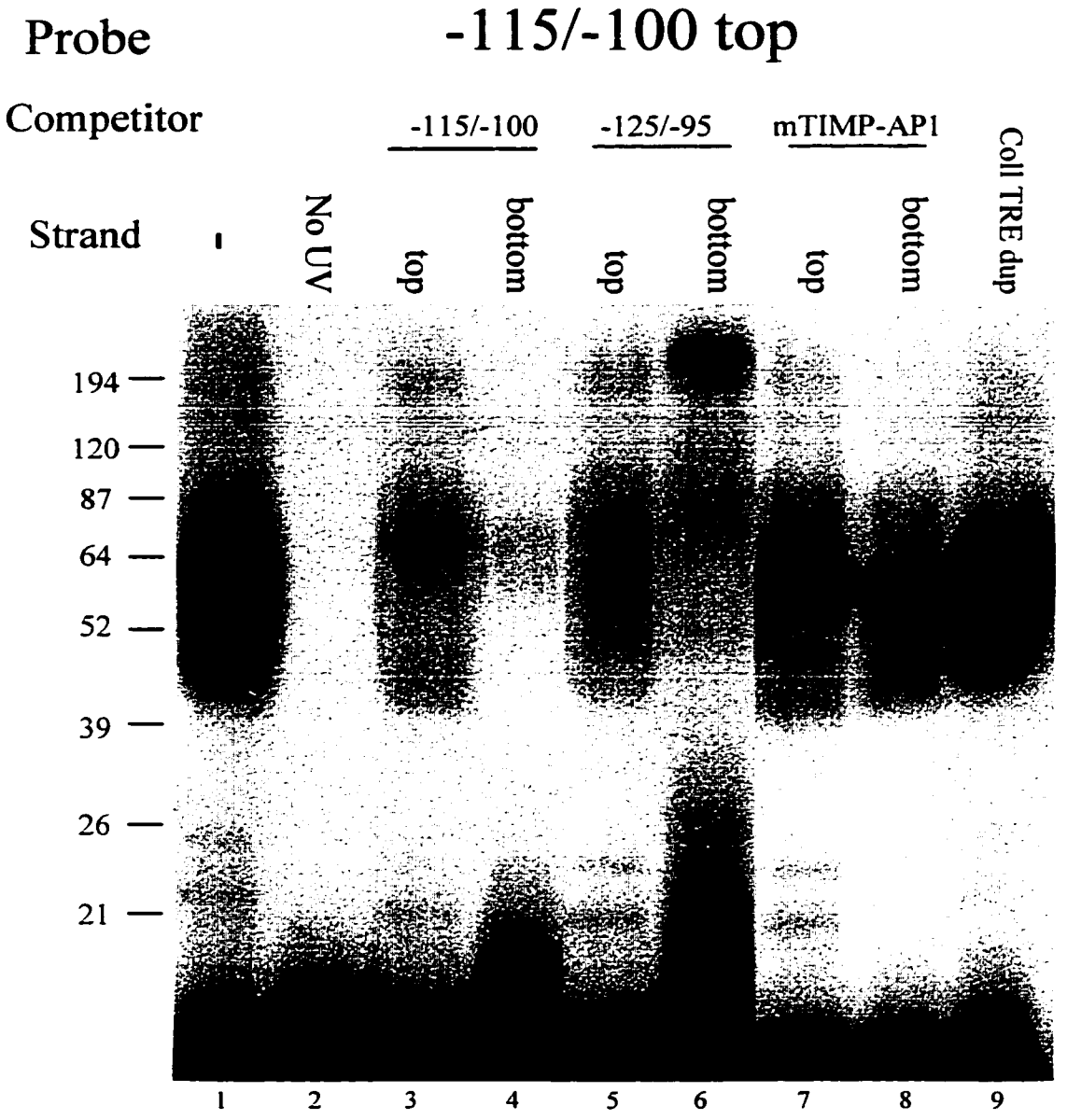


Figure 26. Specificity of the ssT1 interaction with UV-crosslinking, SDS-PAGE. The -115/-100 top oligonucleotide was used as a probe against C3H10T1/2 cell nuclear extracts. The interactions were made irreversible using UV-crosslinking and separated on a 10% SDS-PAGE gel. Competitors were added at 100x molar excess in the binding reaction. Protein standards were run as size markers to determine the approximate molecular weight of the complexes.

The top strand of –63/-49 is not effective at competing against –115/-100 top in EMSAs (figure 14B), therefore this observation must therefore be a function of the UV-crosslinking. As a control, the collagenase AP1-binding site was unable to compete against –115/-100 top.

As further characterization of the ssT1 factor, Southwestern blots were performed (Figure 27). The -115/-100 top and -63/-49 bottom oligonucleotides both interact with proteins from nuclear extracts of 10T1/2 cells and rat liver, and different binding activities were revealed. The -115/-100 top strand interacted strongly with a protein of approximately 54 kDa in nuclei from mouse 10T1/2 fibroblasts, identified as band 2 (Figure 27A). This band is specific because it is competed away by 100x molar excess cold competitor (-115/-100 top, self) in competition experiments (Figure 27B). Furthermore, similar to what was seen in EMSA assays, the protein species identified as band 2 did not change in quantity following serum stimulation (Figure 27B) of the 10T1/2 cells. There are also weaker interactions with the -115/-100 top probe described as band 1, and 3 at 90 kDa and 32 kDa molecular weight respectively. When using the –63/-49-bottom probe with 10T1/2 nuclear extracts, I observed a similar banding pattern, however, there is an approximately equal distribution of signal between bands 1-3, with an additional band 4 at 22 kDa being detected. Band 2 co-migrated for both -63/-49 and -115/-100 probes, which likely accounts for the cross-competition I have observed. However, for the -63/-49 probe, the 54 kDa band 2 appears to be a doublet, which might be the consequence of glycosylation or some other post-translational modification of the protein.

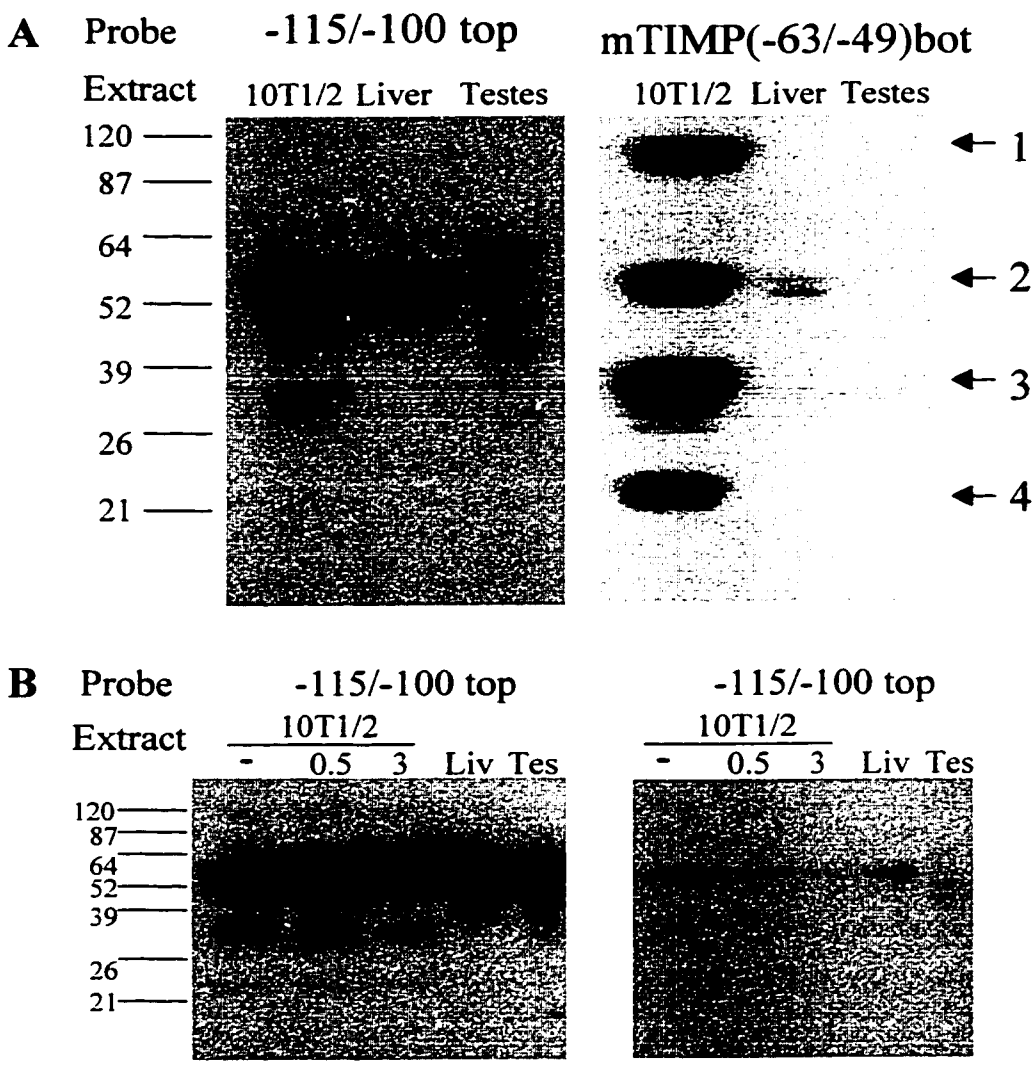


Figure 27. The ssT1 factor is approximately 54 kDa, and shows differential tissue expression. A more accurate size determination of ssT1 was performed with SW-blotting. C3H10T1/2, liver, and testes nuclear extracts were separated on a 10% SDS-PAGE gel, then transferred to a nylon membrane, and treated for SW-blotting. A) Blots were probed with single-stranded, end-labeled oligonucleotides corresponding to the -115/-100 top and -63/-49 regions of the *Timp-1* promoter. Four primary bands were seen, labelled 1 through 4, which were seen in the 10T1/2 extracts probed with either oligonucleotide. Additional bands were seen in the liver, and testes extracts. B) SW-blots were performed upon 10T1/2 cell nuclear extracts which were serum stimulated at 0.5 and 3 hours, with a unstimulated control with the -115/-100 top probe (left panel). To test for specificity, 100x molar excess cold oligonucleotide was included in the SW-blot probing reaction (right panel).

Based on the results from the Southwestern blot, in comparison to the EMSA results and cross competitions, I can deduce that the 54 kDa protein (band 2) is the ssT1 factor. The identity of the other bands seen on SW blot in the 10T1/2 cell extracts, are uncertain. Both -115/-100 top and -63/-49 bot interact with the same proteins, however, they differ in specificity for them. The -115/-100 top probe has a distinct preference for the 54 kDa protein, while -63/-49 bot interacts with all four proteins with approximate equal specificity. This could mean that there are non-specific single-strand interactions, involving factors that might interact with either single-strand probe. Alternatively, as the sequences between the two probes differ significantly, it is likely that these differences may allow preferential binding of related, non-identical factors.

In addition to the interaction with proteins in C3H10T1/2 cell nuclear extracts, there is also interesting results from other nuclear extracts. There is only one main liver nuclear protein which interacts with the both probes in SW-blot (figure 27A), which is slightly smaller than the 10T1/2 interacting protein (53 kDa). The size difference might be a consequence of post-translational modification, or alternatively, might signify a related, but different protein present in the liver. The other extract studied was derived from mouse testes. Unlike the other extracts studied, the -63/-49 probe did not interact with anything in the testes extract. However, the -115/-100 top probe reacted with several proteins between -40 and -60 kDa. These could represent cleavage products, or post-translational modifications of a single protein, or multiple, but related proteins.

3.5 Efforts at the Purification of ssT1

3.5.1 Cold Competitions With Known Single-Strand Binding Proteins

As a first approach to identify the ssT1 protein, I searched the literature to see if there were similar single-stranded DNA-binding proteins found. This strategy was problematic because of the broad sequence binding specificity of the ssT1 factor. However, knowing the approximate size of ssT1 (54 kDa), several candidate genes for single-stranded DNA-binding proteins were identified. Accordingly, oligonucleotides were synthesized for the binding sites of the two factors, called YB-1 (MacDonald et al., 1995; Mertens et al., 1997), and CEprMss (Coding Element-Purine-rich Motif, single-stranded)(Kelm Jr. et al., 1997). Both strands of the YB-1 binding site are able to interact with single-stranded binding proteins, therefore, both were used as a competitor during EMSA analysis (Mertens et al., 1997)(figure 28A, lanes 3 and 4). The results show that there is no detectable competition from either strand of the YB-1 binding site. The inverse experiment was also performed, in which the YB-1 bottom strand was labeled, and the –115/-100 top sequence was used as a competitor (there was no shifted bands when YB-1 top was used as a probe, data not shown). In this case, -115/-100 top appears to have a slight competitive effect, lowering the YB-1 band to about half (figure 28, lane 6). However, because of the inability of YB-1 to compete away the ssT1, and only a modest competition of –115/-100 top against YB-1, I ruled out the ssT1 factor as YB-1. Another factor that was tested was the CEprMss factor, which showed a complete

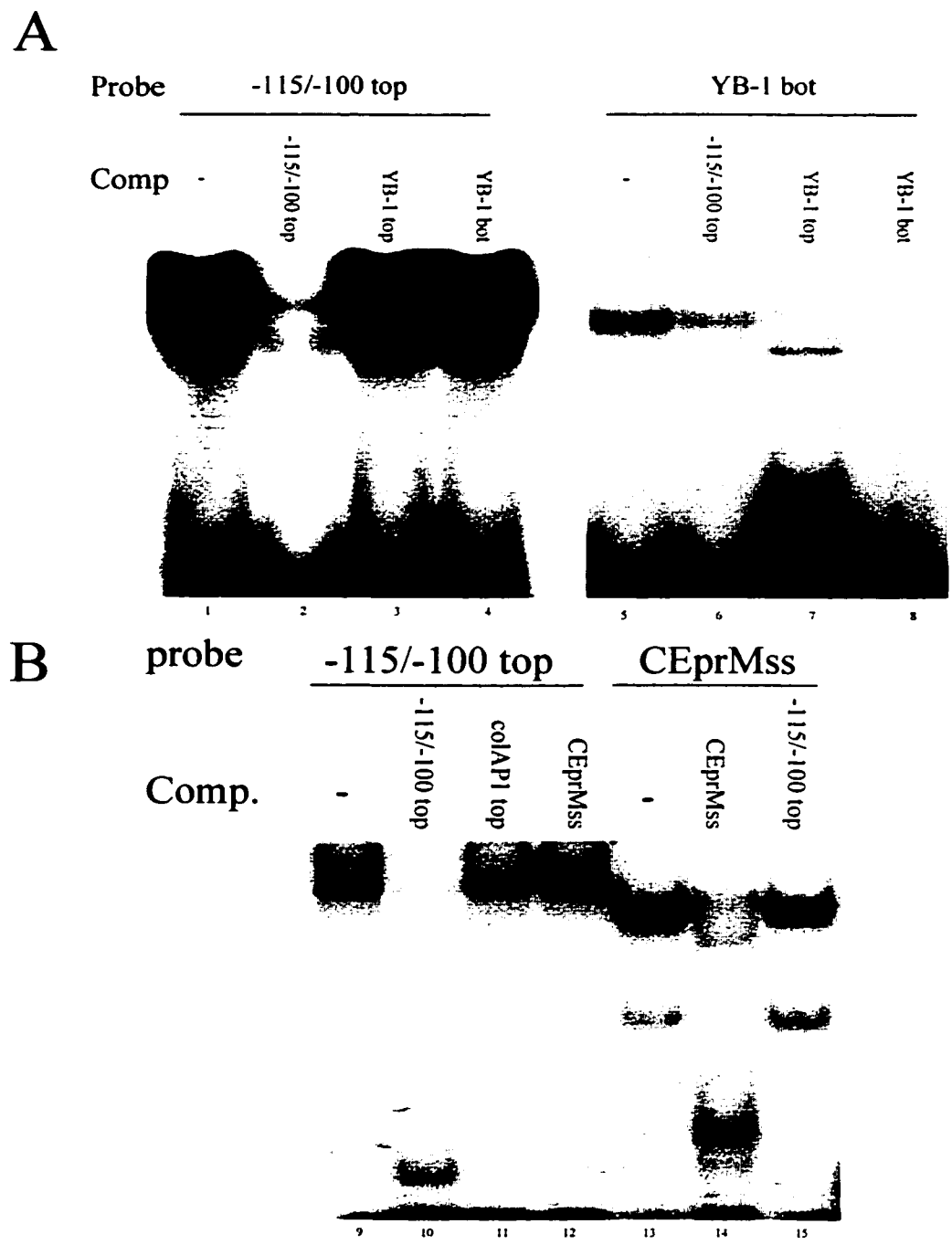


Figure 28. Comparison of ssT1 with known single-stranded binding factors. Cold competitor oligonucleotides for known single-stranded binding proteins were used as competitors against the ssT1 probes in EMSA analysis. In C3H10T1/2 cells, -115/-100 *Timp-1* sequence was used as a probe. 100x molar excess competition of the YB1 (lanes 2-4) and CEprMss (lanes 11,12) were used as competitors. Alternatively, YB1 or CEprMss were used as a probe, while -115/-100 top *Timp-1* as a competitor (lanes 5-8;13-15 respectively).

inability to compete against the –115/-100 top sequence for ssT1 binding (figure 28B). Also, the –115/-100 oligonucleotide did not compete for binding of CEprMss to its target.

3.5.2 Affinity Chromatography

A chromatographic approach to the purification of ssT1 was undertaken for several reasons. The first is that by bandshift analysis, there is a relative abundance of ssT1 in the nuclear extracts of C3H10T1/2 cells. Additionally, liver tissue also expresses ssT1, or a closely related protein, and nuclear extracts from mouse liver should have enough ssT1 protein for effective purification. It was hoped that enough ssT1 could be obtained in a pure enough form for N-terminal microsequencing.

The initial step in affinity purification strategies was a crude purification of the nuclear extracts. I used a double-stranded DNA-cellulose column, which I hoped would remove any double-stranded DNA-binding proteins, and provide a reasonable degree of purification. All reporter and EMSA experiments had been performed using C3H10T1/2 cells and extracts, and therefore, 10T1/2 cell nuclear extracts were used for chromatography efforts. While an extract from liver tissue would provide a greater quantity compared to one from 10T1/2 cells, I was concerned that the liver might express a different, though related protein from the ssT1. This was based upon the liver protein having a slightly different mobility on SW-blot (figure 27A), as well as by EMSA (figure 30B). The input and flowthrough from the DNA-cellulose column were both assayed by EMSA (data not shown), and unfortunately showed that a large portion of the ssT1 was depleted in the DNA cellulose. Also, it was not released during salt elutions in sufficient quantities to proceed with the affinity purification. I abandoned using DNA cellulose and

opted instead to use the ion exchange matrix heparin agarose. Unfortunately, I had the same problem as with the DNA cellulose. Too much of the ssT1 was lost in the initial purification step to attempt affinity chromatography. This loss of material may be due to rapid degradation when the ssT1 is removed from stabilizing factors in whole nuclear extracts despite the presence of protease inhibitors in the buffers used for purification. Alternatively, the proteins could be binding irreversibly to the chromatography matrix.

Many protocols for affinity chromatography suggest that in some cases, an initial purification step is not necessary. I therefore attempted to use the affinity chromatography with the crude nuclear extracts. C3H10T1/2 cell nuclear extracts were used in the protocol outlined in figure 29A. The procedure involved setting up a large scale binding reaction with conditions the same as in the EMSA reactions, using a biotinylated oligonucleotide corresponding to the –115/-100 sequence. I had synthesized a 5'-biotinylated oligonucleotide which was composed of 2x-115/-100 sequences in tandem. This probe was tested in EMSA and interacted with ssT1 as effectively as the –115/100 probe previously in use (data not shown). After the binding reaction, the complex was removed from the solution by streptavidin agarose, either in a test tube (batch) or by running the binding mixture through a streptavidin-agarose column as depicted in figure 29A. Increasing salt concentrations were then used to elute fractions. The fractions and input were tested for the presence of ssT1 using EMSA. As seen in figure 29, using both column and batch, the ssT1 was depleted in the flowthrough (see the FT lane in panels B and C). This was an encouraging result, because it meant that the ssT1 was therefore kept within the column substrate. However, the salt elutions were unable to remove the ssT1 from the column (29B and C).

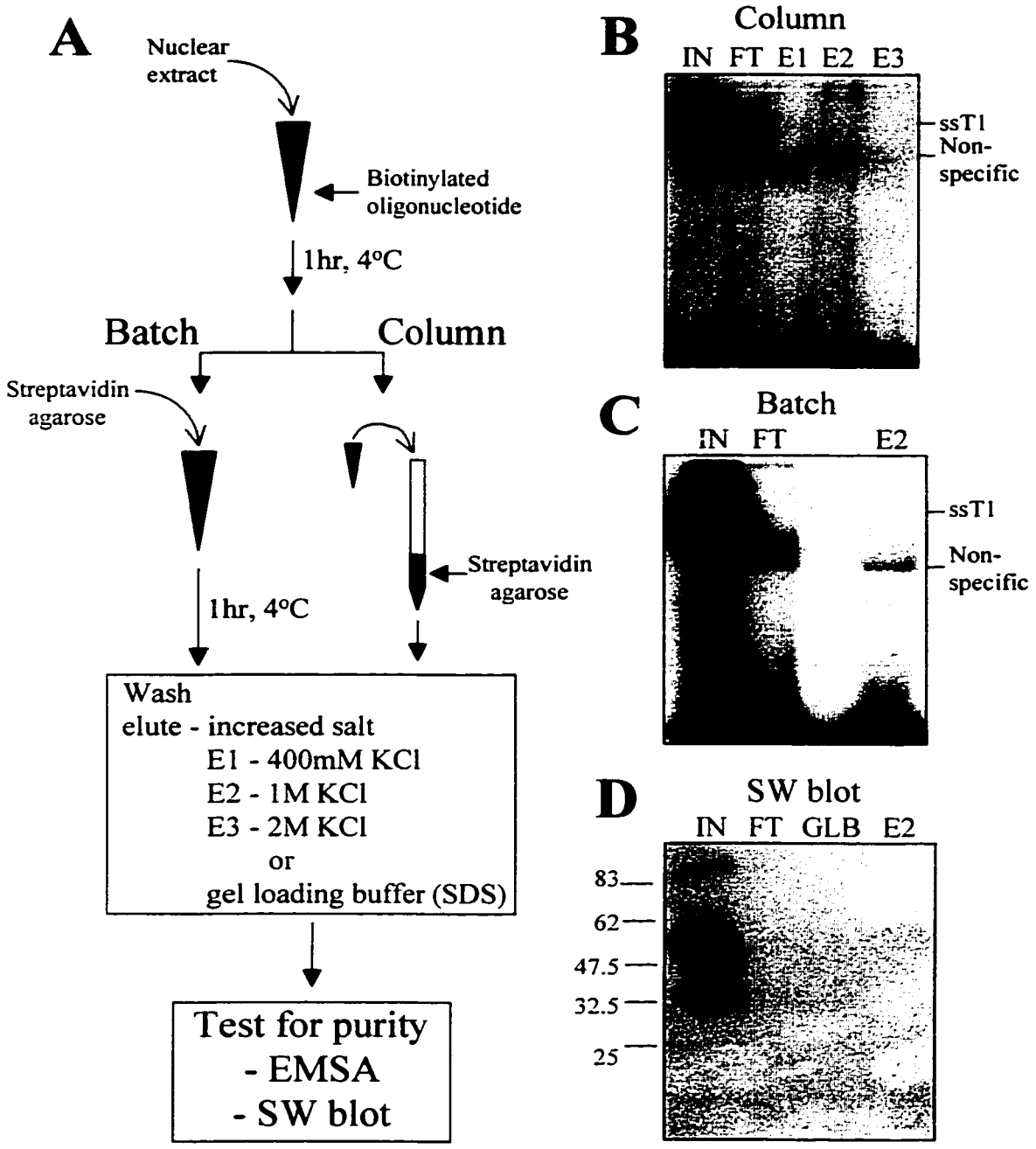


Figure 29. Attempt at ssT1 purification using affinity chromatography. A) Schematic representation of batch, and column methods of affinity chromatography to purify the ssT1. Both methods utilized a 2x-115/-100 multimer with 5' biotinylation. B) EMSA analysis of input (IN), flowthrough (FT) and 3 salt elutions (E1 - E3). Similar testing was done for the batch chromatography (C). D) Southwestern blot testing of batch chromatography. Because the ssT1 complex is stable on a range of salt concentrations, the complex was dissociated with 4xgel loading buffer, and SW-blot was used to identify the product. Unfortunately, all affinity approaches for purifying the ssT1 were unsuccessful.

I have already demonstrated that in vitro, the ssT1-DNA complex is resistant to salt concentrations which are used for affinity chromatography elutions (figure 24). Therefore, the inability to elute the protein with these salt concentrations was not wholly unexpected.

In order to avoid the problem with salt stability of the protein-DNA interaction, I used gel loading buffer containing SDS to try to elute the factor from the column. It was demonstrated in the UV-crosslinking and SDS-PAGE experiments (figures 25 and 26) that the –115/-100:ssT1 complex is sensitive to SDS. If the EMSA binding reaction is not UV-crosslinked, no shifted band is present if the reaction is separated on SDS-PAGE. Therefore, a batch affinity chromatography strategy was implemented (figure 29A), but after the washes, the complexes were eluted by adding 4xgel loading buffer (containing SDS) to the pelleted matrix. The gel loading buffer eluate, along with the input, flowthrough, and a subsequent salt elution were separated on SDS-PAGE, and transferred to nitrocellulose before being treated for SW-blot analysis. Similar to what was seen before, the ssT1 factor was depleted in the flowthrough, but again, there was no recovery in any of the elution fractions, even in the gel loading buffer fraction (figure 29D).

As a final attempt at affinity purification, mouse liver nuclear extracts were used. Because a higher volume (and concentration) of nuclear extract can be obtained from tissue compared with cells, it was hoped that this approach could more easily tolerate the loss of material incurred during chromatographic procedures. However, as has been seen previously with the C3H10-T1/2 cell extracts, there was no recovery of ssT1 from the affinity column (figure 30).

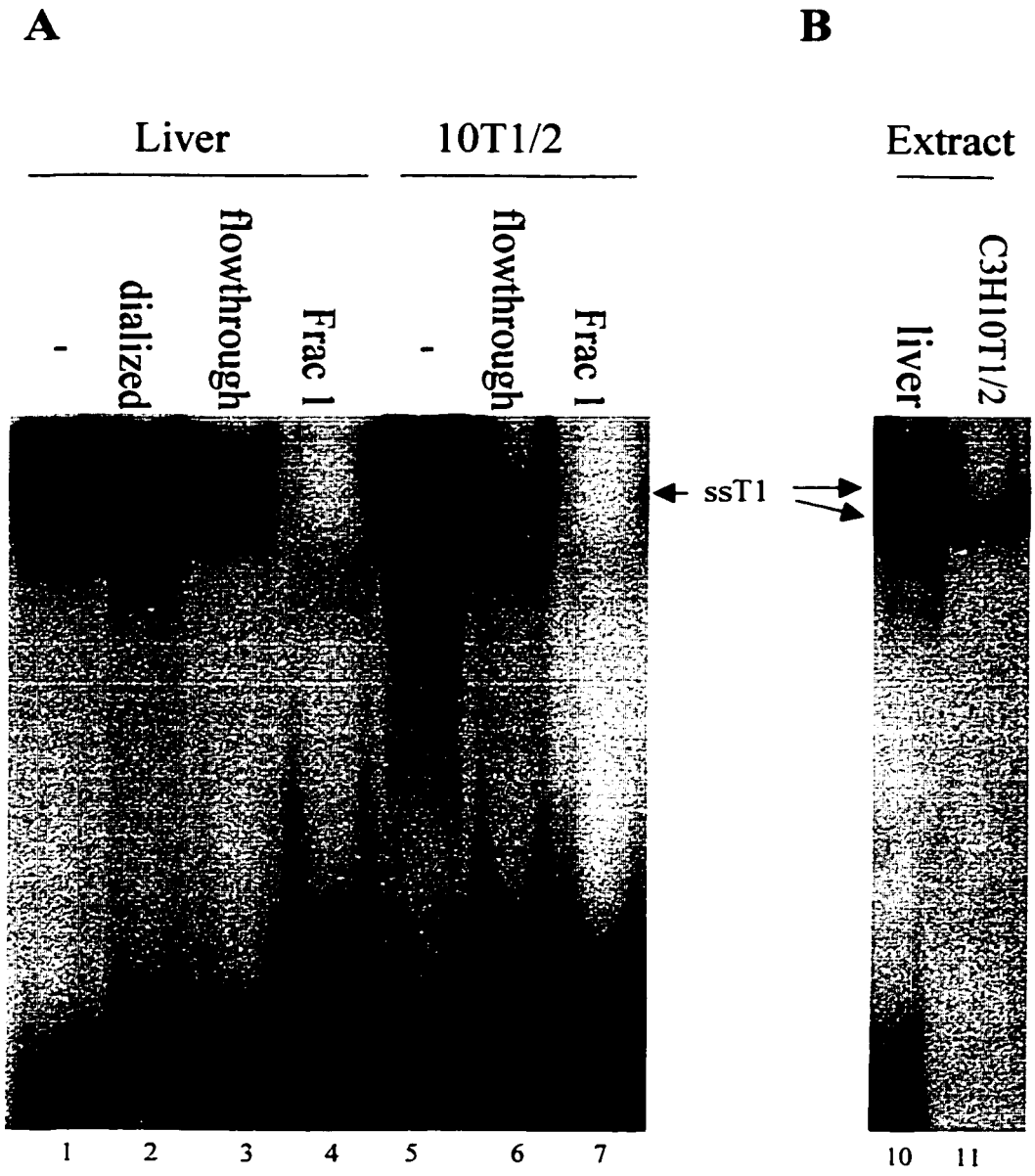


Figure 30. Purification of ssT1 in mouse liver, and C3H10T1/2 cell nuclear extracts. A) Due to an increased abundance of nuclear extract in tissue compared to cultured cells, an attempt to purify ssT1 from rat liver tissue nuclear extracts was made. First, the crude nuclear extract was dialyzed, then added to the biotinylated -115/-100 oligonucleotide. Samples of flowthrough, and elution fractions are presented. B) liver and C3H10T1/2 cell nuclear extracts were both tested in EMSA as probed with -115/-100 top probe to determine the similarity in protein species at the level of protein-DNA complex.

No further attempts were made to isolate ssT1 using affinity chromatography. The initial purification strategies were not able to generate any enrichment of the ssT1. Furthermore, while affinity chromatography was able to deplete the ssT1, I was never able to get any recovery. It is possible that the ssT1 was interacting non-reversibly to the streptavidin agarose, or alternatively, it could be degraded or denatured from the conditions of the treatment. Future efforts might be aimed at examining different types of matrix for chromatography, such as cellulose instead of agarose. Also, it might be possible to pre-block the streptavidin agarose (or cellulose) with extract prior to the experimental purification.

3.4.3 Expression Library Screening

Cloning and isolation of the ssT1 by expression library screening seemed promising because several criteria were met. First, with the successful SW-blotting of ssT1 (figure 27), I knew that I was dealing with a single protein species, rather than a complex. This is important because a multiprotein complex would not be generated in a cDNA library-based expression system. Additionally, the ssT1 is an abundant protein, as determined by EMSA.

We were unable to obtain an expression library for C3H10T1/2 fibroblasts. However, we had a mouse λ-ZAP PCC4 tetracarcinoma library which had been previously been used for the cloning of full-length TIMP-3 (Leco et al., 1994). A total of $1x10^6$ PFU were screened with the –115/-100 top probe, but unfortunately, positive colonies which were not background were not obtained from the library.

While this approach still holds some promise, I suspect that the problem lies in the choice of library. There has been no indication that tetracarcinoma cells express functional ssT1. Other problems could be that the bacteria used to propagate the library are unable to process the recombinant ssT1 properly, and the protein may be unable to recognize its recognition sequence due to an absence of required post-translational modifications. Future attempts at the cloning of ssT1 using expression libraries should utilize a library derived from C3H10T1/2 cells. Presently, such a library is not commercially available, and must be constructed. In addition, more experiments are required to determine if bacterially expressed ssT1 is able to interact with the *Timp-1* –115/-100 promoter region.

3.6 Preliminary Mapping of a putative Timp-4 Promoter

3.6.1 Timp-4 Expression in Cultured Cells

We already know from preliminary data that Timp-4 is expressed in several types of tissues (breast, heart, brain, smooth muscle cells), yet still is not expressed in a widespread manner (Dollery et al., 1999; Fata et al., 1999; Leco et al., 1997). However, a comprehensive screening of cultured cells has not been performed. These data were of particular interest to me because I required a model system in which to study Timp-4 expression. A panel of cultured cells (figure 31A) was screened by RT-PCR for TIMP-4 expression (representative PCR shown in 31B).

A

Cell Line	Species	Cell Type	TIMP-4
32d	mouse	lymphoma	-
ECR3T3	mouse	fibroblasts	-
3T6	mouse	fibroblasts	-
C3H10T1/2	mouse	fibroblasts	
3d heart	mouse	tissue extract	+++
C6	rat	glioma	-
ARBEC	rat	rat endothelial	-
A10	rat	smooth muscle cells	-
PC12	rat	adrenal gland	-
PC3	human	prostate	+
DV1	human	prostate	-
U251N	human	glioma	-
MRC5	human	lung fibroblast	+++
HCEC	human	endothelial	-
HUVEC	human	umbilical vein endothelial	-
MDCK	dog	kidney fibroblast	-
BHK	hamster	kidney fibroblast	-

B

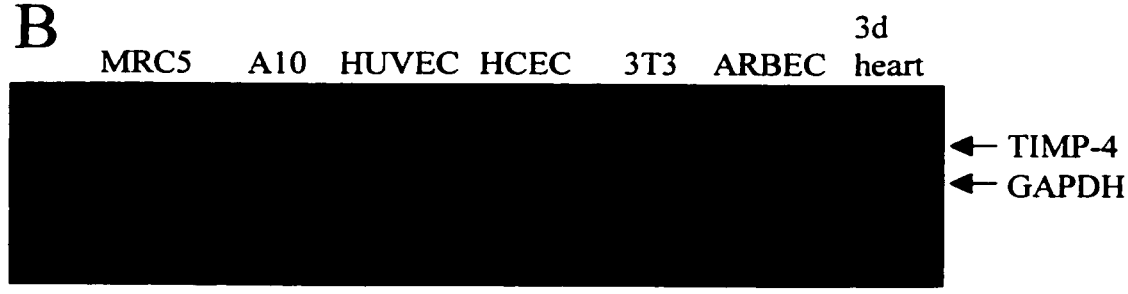


Figure 31. Expression of TIMP-4 in cultured cells. Many types of cells from different species were screened by RT-PCR for TIMP-4 expression. Both mouse and primers were used in each cell lines, and the results tabulated in panel A. Representative RT-PCR is shown in B.

Different types of cells from human, mouse, dog and hamster were tested, though only a few showed TIMP-4 expression. Expression of TIMP-4 by this screen was restricted to PC3 (human prostate), and MRC5 (human lung fibroblastic) cells. This result is in agreement with the expression data from adult mouse tissue (Leco et al., 1997), which suggests that Timp-4 has a restricted tissue distribution. Both murine and human PCR primer sets were used for the experiments (which might explain the absence of TIMP-4 detection from the dog and hamster cells). The expression in MRC5 fibroblastic cells was unexpected because all other fibroblastic cells screened were negative for TIMP-4. This result indicated that there are cell type differences in the expression of TIMP-4.

3.6.2 Localization of a Putative Timp-4 Promoter

A Timp-4 genomic clone was obtained by screening mouse 129 ES cell genomic DNA libraries (Hogan, A., and Leco, K., personal communication). However, at this point, there was no indication of where the promoter elements were located although the translation start site has been identified. By examining the gene structures of the other Timp family members, it was possible to estimate the location of a putative promoter. Should Timp-4 resemble Timp-1, there would be an untranslated exon which would be located upstream from the translation start site, where in the case of Timp-1, the untranslated exon is almost 800 bases upstream (see figure 4). Alternatively, if the Timp-4 gene resembles Timp-2 or Timp-3, then the promoter would be located fairly close to the translation start. In either scenario, the Timp-4 promoter would be located within an EcoR1/HindIII fragment (E/H) of the genomic Timp-4 clone, which spans about 4 kb

upstream from the translation start site. The only possibility not accounted for is that there might be an untranslated exon located farther upstream than the EcoR1 site.

Because of the likelihood that the Timp-4 promoter lies within the E/H restriction fragment, sequencing of this large region was performed. The translation start site was the only confirmed landmark at the time of the sequencing, therefore, all numbering was made relative to that, which was designated as +1. There are several interesting features within the sequence of the suspected Timp-4 promoter (figure 32). There are two TAAA sequences, which are possible TATA-box elements. However, there are few sequences which resemble known transcription factor binding motifs within the region of the promoter near the putative transcription start points (identification is described in detail later), as determined by the search of a transcription factor data base. A possible c-ets binding site was found at -540 and potential Sp-1 binding sites at -120 and –670. There are several areas which are peculiar in the setting of a promoter region. A $(CA)_n$ repeat is located at –3000, which is a characteristic reminiscent of the MMP-9 promoter, and might possibly play a role in gene expression. Although in the case of MMP-9, the $(CA)_n$ repeat is split, with part located quite close to the transcription start points, and the others located quite far upstream. Additionally, there is a long stretch of $(T)_{23}$ at –325, and $(C)_{21}$ at –755 (figure 32). The function of these regions at this time is unknown.

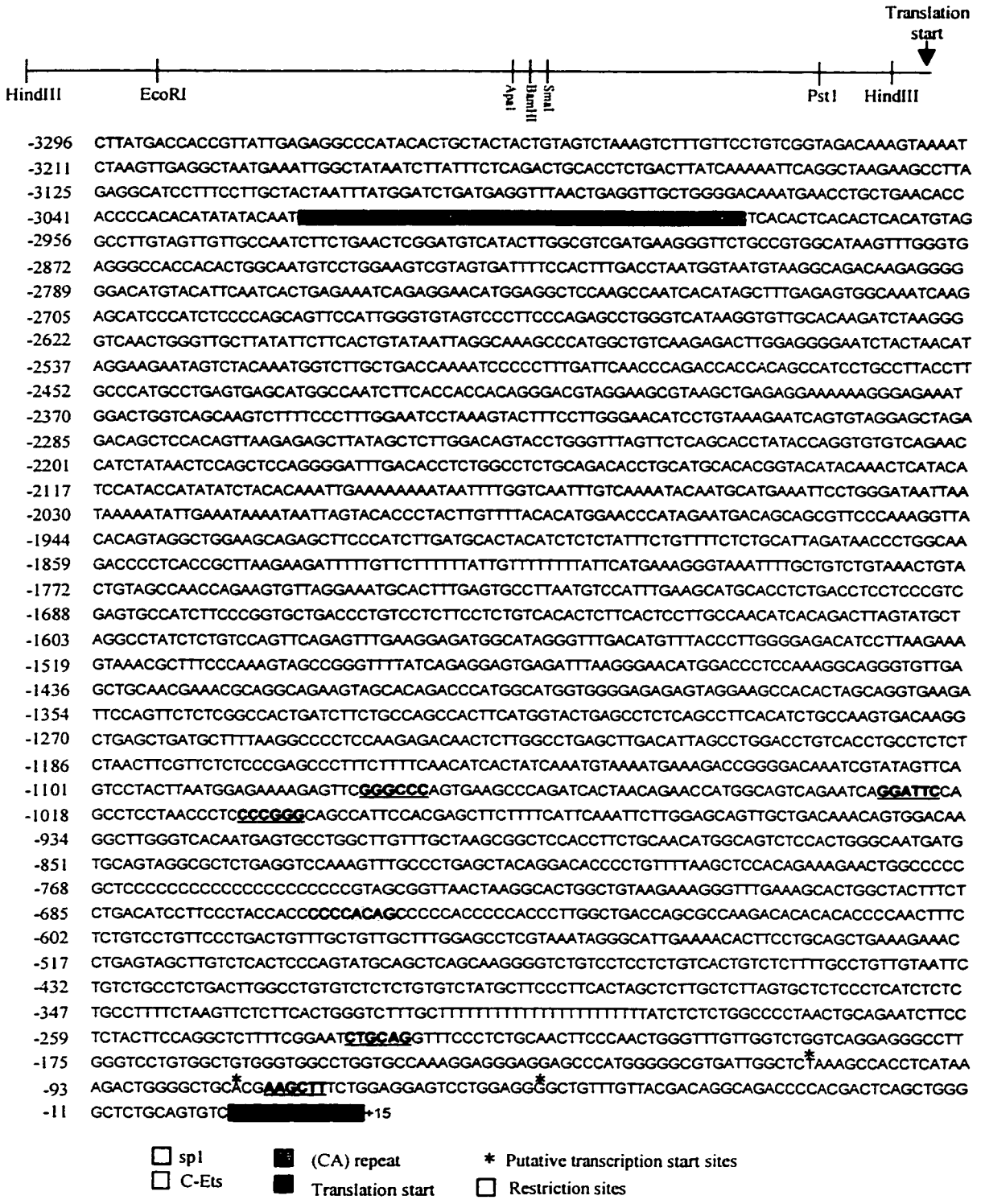


Figure 32. The sequence of the murine *Timp-4* promoter region. The region from -3296 to the translation start site are shown. Important features such as restriction sites, potential transcription factor binding sites or regulatory elements, and transcription start sites are highlighted as defined in the legend

In order to identify putative transcription start points initially, 5`-RACE-PCR was performed on the Timp-4 gene, using a series of primers in close proximity to the translation start point. This was undertaken because preliminary reporter constructs of large fragments of the Timp-4 upstream region were inactive (data not shown), and we feared that the promoter was located further upstream.

The RACE protocol utilized three different primers (see methods and materials, and figure 33) which promotes specificity of the product. After the initial PCR reaction, there was not a clear band, but rather, a broad smear which extended from about 100bp to 600 bp (figure 33). Using a second round of PCR with the SP3 primer, however, then resulted in a sharper band at around 300bp in size. The band was gel purified, eluted, and then subcloned into an AT-PCR cloning vector (Promega). Five individual clones were sequenced, resulting in three possible start points. The start points occur between 52 and 108 bases upstream of the translation start point (figure 33B, and 32). This result confirms that the transcription start point is upstream of, but close to the translation start site, an observation that has recently been confirmed by primer extension (Ian Clark, personal communication). Note that the use of RACE-PCR is prone to problems as the reverse transcriptase can terminate prematurely in situations dependent upon sequence and specificity. This problem was minimized by using the AMV reverse transcriptase enzyme (which is active at a slightly higher temperature, 42°C).

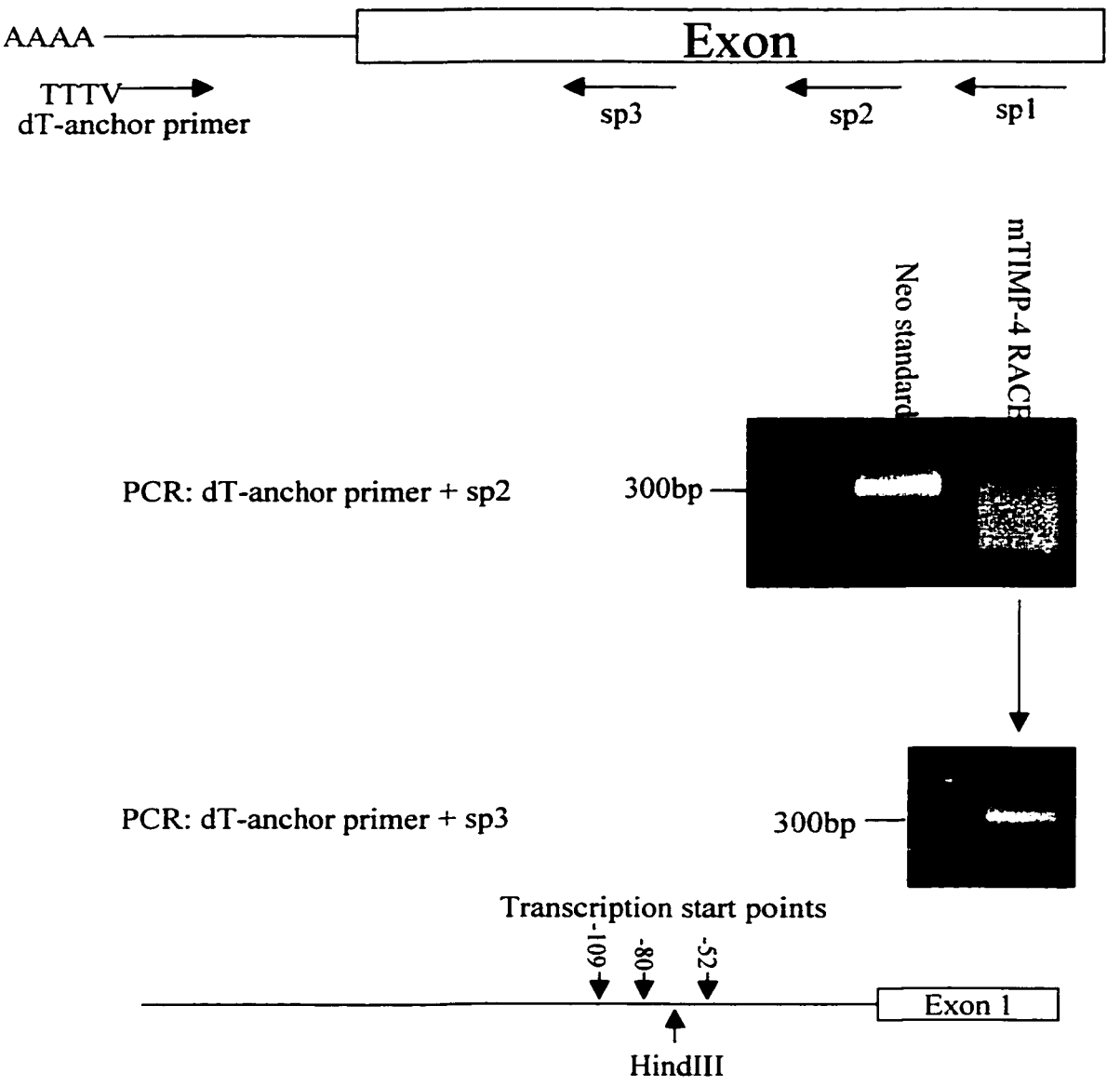


Figure 33. Localization of the Timp-4 transcription start sites using RACE-PCR. Three antisense primers were synthesized based on the exon sequence, called sp1, sp2, and sp3. Sp1 was used to reverse transcribe 3 day mouse brain RNA. Then, after synthesis of a poly dA tail using terminal transferase, 2 rounds of PCR were performed. First, the sp2 primer was used with the dT-anchor primer, which primes from the polyadenylated region. Then, the product is re-amplified using sp3 with the dT-anchor primer. The resulting fragment was subcloned into an AT-PCR cloning vector and several clones were sequenced, thereby giving 3 transcription start sites.

Another approach to confirm transcription start points is to use RNase protection. This was not used as an initial step because it is first necessary to know where the start points are in order to test by RNase protection. RACE-PCR has identified candidate tsp sites which may now be subject to confirmation in the near future.

3.6.3 Transcriptional Regulatory Elements Within the Timp-4 Promoter

Following the localization of the promoter region, we next sought to determine if there were any positive acting transcriptional regulatory regions. Because the transcription factor database search was not fruitful, we proceeded to make a series of deletion constructs. Originally attempting to clone a range from short regions (-87/-28) to long regions (-3000/-28), I found that there was difficulty in generating longer PCR fragments. The PCR reaction used to generate restriction-tagged fragments, was incapable of amplifying fragments which were upstream of the $(C)_{21}$ region at –755. Several attempts were made, including with different PCR-kits that were supposed to be effective at high CG content sequences (Roche high fidelity PCR kit), however, no attempts yielded any products for subcloning. Accordingly, the longer constructs are presently being generated using restriction fragments, which is difficult due to the unfortunate location of restriction sites within the promoter region therefore the cloning procedure needing several blunt-ending steps. Despite these cloning difficulties, several informative constructs were still generated, as depicted in figure 34A.

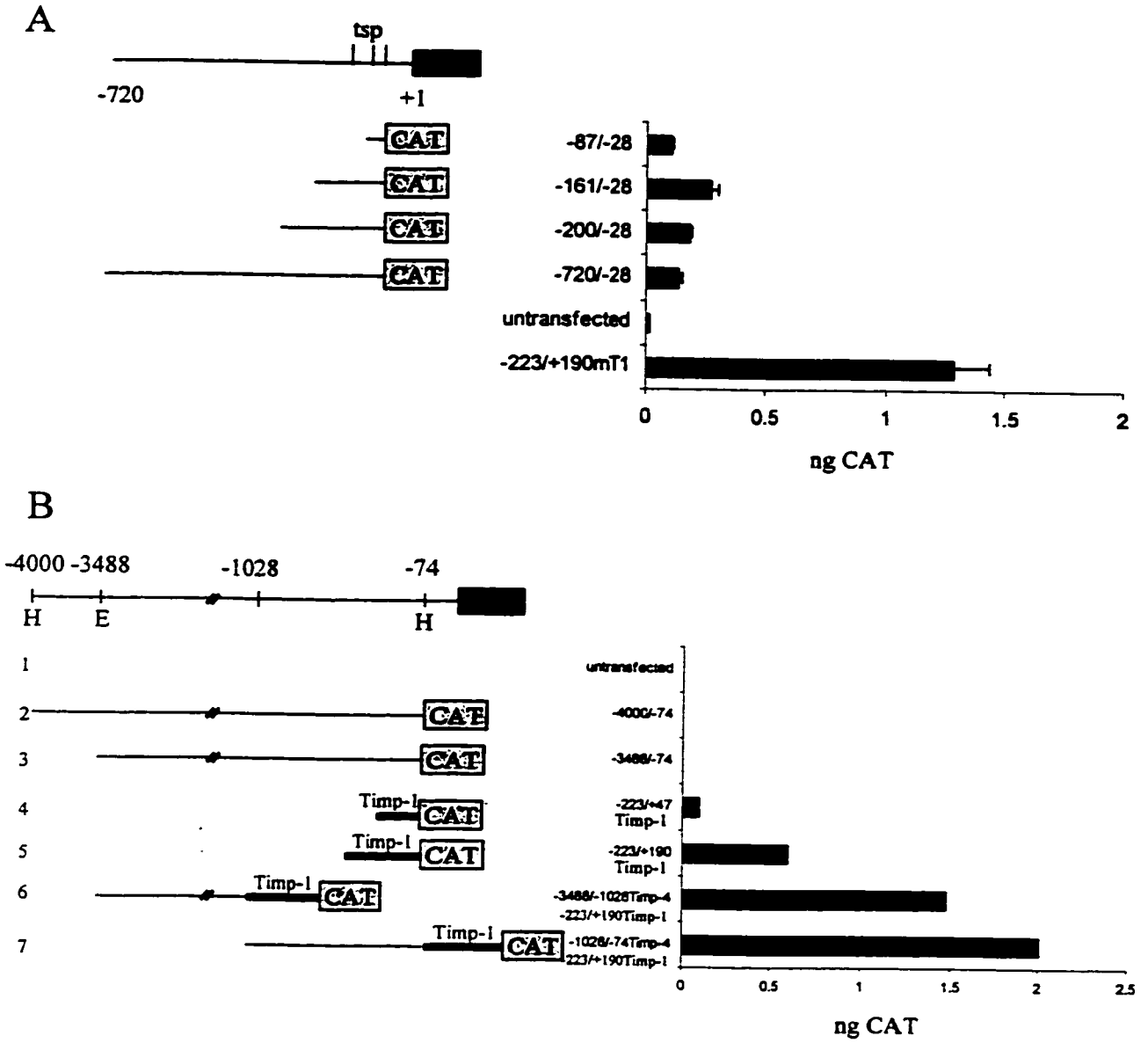


Figure 34. Positive regulatory elements are located within the Timp-4 promoter region. Transient transfections were performed using deletion constructs of the promoter fragment depicted in A. C3H10T1/2 cells were transfected, serum stimulated, and then the protein extract collected and assayed for CAT activity. B) upstream regions of the Timp-4 promoter were analyzed for reporter expression. Bars 2 and 3)-4000/-74 and -3488/-74 CAT Timp-4 reporter constructs. Bars 4 and 5) -223/+47 and -223/+190 Timp-1 reporter constructs. Bar 6) fusion construct: -3488/-1028Timp-4+-223/+190 Timp-1. Bar 7) fusion construct:-1028/-74 Timp-4+-223/+190 Timp-1. All graphs depict ng of CAT per uL of whole cell protein extract.

A region proximal to the transcription start points of Timp-4 was studied by deletion analysis (Fig 34A). Four constructs, spanning the promoter region between –87 and -720, were subcloned into the pBL-CAT3 reporter vector, and used for transient transfections. Three different cell lines were utilized for transfections. Based upon the results from my cultured cell screen, I used the TIMP-4 expressing cells, PC3 and MRC5 for transient transfections of my constructs. Unfortunately, reporter activity was barely detectable (data not shown), indicating either that the promoter regions are inactive, or the cell lines are ineffective at interacting with the constructs. I believe that the problem might be the usage of murine promoter fragments within human cell lines. Accordingly, I used murine C3H10T1/2 cells for all future transfections.

Unlike the transfections into the human cell lines, there was informative reporter activity from transfections into C3H10T1/2 cell extracts. The experiment, which measured CAT activity from cells which were serum stimulated, demonstrate two regulatory regions within this area of the promoter (figure 34A). Deleting the region between –720 and –161 results in an increased reporter activity, which indicates negative transcriptional regulation from this area. A further deletion up to –87 causes a 3-fold reduction in activity (compare 34A, bars 1 and 2). As a control for the transfection, I used a Timp-1 –223/+47CAT construct, which has a fairly strong promoter. The Timp-4 constructs show an increased expression compared to the background (fig 34A, bar 5), however, the most active Timp-4 promoter region, -161/-28, is still 4-fold lower than the –223/+47. While the expression of Timp-4 is still strong evidence of enhancer elements, the levels might be artificially low in this model because C3H10T1/2 cells are unable to

express endogenous Timp-4. It will be useful to transfect these constructs into Timp-4 expressing cells to confirm these cis-acting elements identified within C3H10T1/2 cells.

I have also studied a large upstream region for promoter activity, spanning from approximately 4 kb upstream from the translation start site (figure 34B). In the first instance, the large fragments were subcloned into pBL-CAT3, and tested by transfection into C3H10T1/2 cells. However, the results (34C, bars 1 and 2) show that there is only background reporter activity. This indicates that either there is no activity from this promoter region, or that there are suppressor elements. To test for the possibility of inhibitory elements in these fragments, they were subcloned in front of the –223/+190 Timp-1 CAT constructs, which have already been demonstrated to have high activity (figure 34B, bar 6). The result showed that the large promoter elements actually enhanced activity of the –223/+47 Timp-1 promoter region (34B, compare bars 6 and 7 with 5). There are several possible reasons why the upstream Timp-4 promoter regions are inactive at driving CAT alone (fig 34C, bars 1 and 2), yet enhance activity from the Timp-1 promoter region. The first is that the fragments terminate at 3'-28, which includes only two of the three identified transcription start points. Perhaps there is a requirement for the other tsp regions to optimize gene expression. Alternatively, there could be effects from DNA looping, or protein-protein interactions which are enabled by the inclusion of the Timp-1 promoter region.

One problem with the Timp-4 expression data presented here is that I have identified only a putative promoter. RACE-PCR, as described earlier, is prone to errors, and there is still the possibility that I am analyzing expression from an intron. If this

were the case, my data would identify intronic enhancer elements which would still be important in regulating Timp-4 levels.

Chapter 4. Discussion and Future Considerations

4.1 Summary

The different pathological conditions which are accompanied by an alteration in the expression levels of the TIMPs are testimony to the complexity as well as the necessity of ECM regulatory mechanisms. I have shown here that, in the case of *Timp-1*, there are a multitude of factors which affect the expression of this gene. Although it could be argued that the AP-1 binding site within the promoter proximal region is the most critical transcription regulatory element, there are clearly other regions which are important. This is reinforced by the observation that the promoter proximal region (-223/+47), which includes the AP1 binding site, requires additional regulatory regions in order to display the full gamut of responses of which the *Timp-1* gene is capable (Edwards et al., 1992). I have demonstrated two regions of particular importance within the *Timp-1* promoter. The first intron of the *Timp-1* promoter appears to contain elements which contribute to both the expression levels, and site-specific localization of the *Timp-1* gene. Furthermore, I have identified a novel binding protein, ssT1, which interacts with the *Timp-1* AP-1 binding site. The ssT1 also interacts with another region of the promoter upstream from the AP-1 site. In both cases, ssT1 interaction results in an increased basal activity of reporter genes. Finally, I have performed the initial identification and characterization of the *Timp-4* promoter. These results significantly expand our knowledge of the regulatory mechanisms controlling this important gene family.

4.2 Tissue distribution of *Timp-1*: the Role in the First Intron

Expression of the *Timp-1* gene is specific to a number of tissues, and to sites within the body which are undergoing tissue remodelling, such as in developing bone and cranial structures (Flenniken and Williams, 1990; Nomura et al., 1989). Two lines of transgenic mice, both derived from the -2700/+47 *Timp-1* promoter driving LacZ reporter expression, confirmed this pattern, showing expression in cranial structures, as well as in both the mandible and maxilla. The development of bone in the cranium is highly dependent upon ECM remodelling and matrix deposition, and the expression of Timp-1 would reflect a contribution to the regulation of this process (Nomura et al., 1989, Flenniken and Williams, 1990). While these expression patterns are highly indicative of Timp-1 expression, there were also regions of inappropriate transgene expression. We saw strong reporter expression within the spinal cord and developing hindbrain, which are locations where *Timp-1* expression has never been documented by in situ hybridization. An example of other atypical regions which showed transgene expression were the heart, and intestine.

The inappropriate expression of β-gal controlled by the –2700/+47 *Timp-1* promoter is particularly interesting when compared to a Timp-1-LacZ transgenic mouse line derived from a different lab. Flenniken and Williams (1990) used a mouse in which the LacZ reporter was controlled by the -1350/+783 region of the Timp-1 gene. Aside from very few exceptions, expression from their transgenic mouse mirrored endogenous *Timp-1* gene expression during development (Flenniken and Williams, 1990; Nomura et al., 1989). They saw expression in developing long bones, and crania, however, there was abnormal expression in whisker follicles (Figure35). This led to several possible

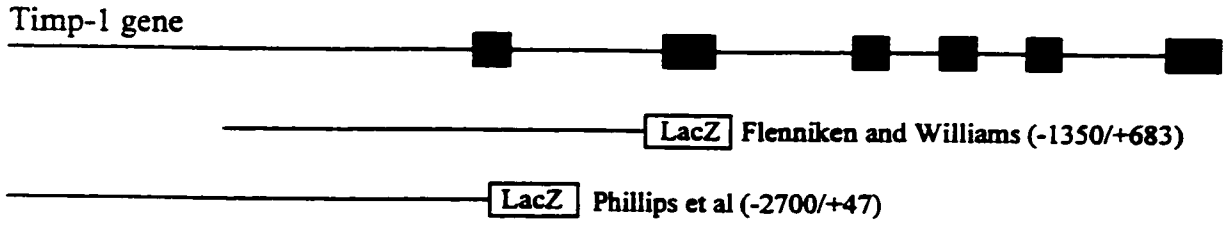
explanations for the difference in expression seen between our group, and Williams group. First, the transgenic analysis from Flenniken and Williams was based on a single line of mice, and their results should be confirmed in other lines derived from the same transgene to rule out insertional effects. Another reason for the different expression is that both groups utilized different promoter fragments in construction of the transgene. In both cases, the *Timp-1* minimal promoter was present, however, our transgene extended further upstream than the Flenniken and Williams, and their transgene included the first intron, only a few bases short of the beginning of the second exon. This 3'-downstream point might actually be problematic, because it does not include the splice site at the intron 1/exon 2 boundary.

An inherent danger in production of transgenic mice is the possibility of insertional effects, which can result in abnormal gene expression resulting from the site of transgene integration. It has been suggested that transgene expression can be determined in large part by the heterologous chromatin environment at the site of integration (Stamatoyannopoulos et al., 1997). Several studies have demonstrated that the use of additional regulatory information included with the promoter and reporter can reduce or alleviate positional effects. The locus control region of β-globin has been used in previous transgenic mice constructs, and been shown to direct expression in a position dependant manner (Garrick et al., 1996; Grosveld et al., 1987). While the mechanism of action is presently unknown, it is thought that the LCR is able to establish an open chromatin configuration (Festenstein et al., 1996). Because neither our lines of mice, nor those of Flenniken and Williams (Flenniken and Williams, 1990) used any means of controlling position effects, it is possible that all of the lines display inappropriate

expression due to such an effect. However, we studied 2 lines of mice, and both lines show abnormal regions of expression. Furthermore, in several areas, the abnormal expression is represented in both lines. Based on this, I can hypothesize that some of the areas of atypical expression might be caused by specific regulatory elements located within the Timp-1 promoter fragment used for synthesis of the transgenic mice.

While there is overlap within the transgenes used by myself and Flenniken and Williams (figure 35), there are also two regions with different regulatory information. The construct used to produce our mice extends from -2700 bases upstream of the tsp, while that of Flenniken and Williams begins at -1350. Our transgene does not include the first intron, which is present in the other groups transgene. With this reasoning, regulatory elements could be located within the first intron, or in regions far upstream of the transcription start sites. However, there are several observations which make the first intron to be the most likely candidate for regulating the *Timp-1* tissue specific expression. The evidence linking the +47/+190 region of the *Timp-1* promoter to positive transcriptional regulation of the gene implicated this region in regulating tissue-specific expression. The details of this promoter region are discussed in detail in section 5.2.

A



B

	Flenniken and Williams	This Thesis	Endogenous Timp-1
long bones	++	++ (data not shown)	++
heart	-	+	-
brain		+	
12.5 dpc embryos	+	+	+
Vertebral column	Vertebral bodies	intervertebral discs	Vertebral bodies
Spinal cord	-	+++	-
Whisker follicles	++	++	-
mandible	+	+	

Figure 35. Summary of *Timp-1* transgene expression during development. A) The *Timp-1* gene is presented along with the promoter and gene fragments used for contruction of transgenic mice. The Flenniken and Williams mice were made from a -1350/+683 LacZ transgene, and the Phillips et al mice were made from a -2700/+47. B) A summary of similarities and differences between both sets of transgenic mice and the endogenous *Timp-1* gene expression during development.

Despite that the transgenic mice derived by Flenniken and Williams (1989) showed reporter expression which resembled the endogenous gene, the existence of other important regulatory regions is quite likely, such as enhancers or repressors far upstream, or 3' of the Timp-1 gene. These issues can not be easily resolved with this sort of transgenic analysis, as it is impossible to include all regulatory regions in the construct, especially if there are unknown regions. However, the use of gene targeting to knock-in a reporter into exon 1 of a gene can serve to both knock-out the gene, and provide a reporter for gene regulation. Such an approach demonstrate Timp-1 expression in the context of the entire promoter and regulatory regions, as well as to enable the study of the contribution of specific regions following mutagenesis and deletion. Another concern with this analysis is that the protein turnover for β-gal and endogenous TIMP-1 will differ, which makes a direct comparison between the two proteins difficult.

There are also many regions within the intron which are able to affect transcription, as demonstrated by reporter-based deletion assays. As I have previously described, there are a series of transcription factor binding sites, including several CCAAT boxes (Ponton et al., 1992), which affect the stimulation of a reporter gene. While our transgene did not contain these elements, they were present within the Flenniken and Williams mice (Flenniken and Williams, 1990). Additional data implicating the importance of the intronal region was generated by Susan Logan (in preparation). In her study, it was shown that the inclusion of the first Timp-1 intron (+190/+780) drastically reduced reporter expression in several different cell lines compared to a construct with a 3'-endpoint of +190, suggesting the presence of silencing elements between +190 and +780. This would agree with our transgenic mouse data, as

the absence of the putative silencer region in our transgene would likely result in expression within regions where *Timp-1* is usually not expressed.

A functional role for introns in both reporter activity and tissue specific expression has been demonstrated in many different genes. *In vitro* assays have shown the importance of intron regions within the GATA-3 gene (Gregoire and Romeo, 1999), the EGF-receptor gene (EGFR) (Gebhardt et al., 1999), the manganese superoxide dismutase (Mn-SOD) (Maehara et al., 1999), and collagen Type1 (I) (Bornstein, 1996). In all of these genes, either deletion of the intron region, or fusion of the intron region itself to a reporter construct, demonstrated an effect on transcription, be it as an enhancer or repressor. These in vitro studies show directly that certain regions of the intron are able to regulate transcription, however, the in vivo relevance of the studies must be considered.

In addition to the *in vitro* experiments, the large number of transgenic mice that have been produced in the last decade have also given insight into the roles that introns play in regulating gene expression. Just as we have demonstrated by comparing our intron-less transgenic mice to the Flenniken and Williams mice, other studies have shown expression differences between transgenes which differ only by the presence or absence of the first intron.

The role of the first intron in collagen 1(I) gene regulation is controversial (Bornstein, 1996), with some groups showing a positive contribution, while others not being able to reproduce the effect. Furthermore, there are cell-specific responses to the first intron. An *in vivo* analysis of the intron regions contribution to collagen 1 (I) expression has been performed (Hormuzdi et al., 1998). Using gene targeting, an intron-

1 knockout mouse was produced by deleting a 1.3 kb region of the collagen 1 (I) gene. While the mice displayed no apparent abnormalities, RT-PCR analysis of the transgene expression showed that in many tissues, but in the lung in particular, the expression was greatly reduced compared to wild-type collagen 1 (I) (Hormuzdi et al., 1998). This effect has been demonstrated more dramatically in several experiments which have shown that LacZ reporter transgenes containing the first intron of the genes in question have different expressions throughout development compared with either the endogenous genes, or with intron-containing transgenes. For the VEGF-receptor-2 (Flk-1), it was shown that the endothelium-specific expression is regulated by elements within the first intron (Kappel et al., 1999). Mice with a transgene containing specific elements located at the 3'-end of the first intron show proper vascular expression, which is lacking in mice where this region is absent. Another example of intron regulation of transgene expression was seen for the promoter of α-actin (Kawada et al., 1999). Similar to the Flk-1 gene, it was shown that a large fragment of the α-actin gene, -891/+3828 driving β-gal, showed LacZ expression identical to the endogenous gene. However, deleting the intron-1 region from the transgene abolished the tissue specific expression (Kawada et al., 1999). Gene enhancers located within an intron is not confined to mammals, as similar results have been seen in transgenic zebrafish as well. Enhancers have been localized to the first and second intron which are required for the tissue specific expression of the gene sonic hedgehog (SHH) (Kawada et al., 1999). The authors also demonstrated that similar intronic regions were required for expression within transgenic mice.

Collectively, it appears that cis-acting elements within the first intron are not unique to the *Timp-1* gene. While in most cases the intron elements are not the critical regulatory sites within the promoter, they are still necessary for the appropriate expression patterns and tissue-specific localization of the gene. It has been the recent use of transgenic mice which has demonstrated the complexity of gene regulation, and shown how regulatory sites outside of the minimal promoters are required for the minute details of gene expression.

4.3 Transcriptional regulatory elements within the first intron

Several groups have demonstrated transcriptional regulatory elements within the first intron, most of which are located in close proximity to the second exon. Preliminary data from our lab has indicated that there are also cis-acting elements within the first intron between +47 and +190, because deletion of this region slightly reduces basal activity, and impaired the serum induction response of the gene (Edwards et al., 1992). Accordingly, when a deletion-reporter analysis of the gene was performed, two regions of positive activity were shown, the first being located at the junction of the first exon, and the other between +150/+190.

Clark et al., (1997) have demonstrated cis-acting, positive regulation from within exon 1 and the first intron of the human TIMP1 gene and are investigating a leader-binding protein (LBP-1) site in exon 1 which appears to regulate the positive response. I have analyzed the sequence between +150 and +190. Based on work in the group of Zena Werb (Logan et al., 1996), it was thought that a second AP1 binding site was present within the first intron between +47 and +190. However, my attempts at

identifying the site, using both deletion reporter analysis and coexpression of c-Fos and c-Jun failed to demonstrate any AP1 response within the region. Although the Werb group have demonstrated an AP1 response, the absence of an canonical AP1 binding site, in addition to my negative data argues against a direct response. However, a more detailed analysis of expression of different members of the AP1 transcription factor is warranted before any direct AP1 interaction is ruled out.

4.4 Transcriptional regulation within the promoter proximal region

Several groups have now demonstrated that the AP1 binding site in the promoter of mammalian TIMP-1 genes is of critical importance in serum-inducible transcription in fibroblastic cells (Campbell et al., 1991; Edwards et al., 1992; Logan et al., 1996). I have shown that this site, which differs from a consensus AP1 binding motif by a single base (5'-TGAGTAA-3'), confers additional protein binding properties on single-stranded versions of the sequence covering -63/-49 of *Timp-1*. My data indicate that the top- and bottom-strands of the sequence bind distinct nuclear single-stranded DNA binding factors. Double-stranded -63/-49 AP1 probes either do not bind these factors, or bind them very inefficiently. Likewise single- or double-strand versions of a consensus collagenase AP1 site with core motif 5'-TGAGTCA-3' do not bind either factor. The bottom strand of the sequence binds a 54kDa protein that has been termed ssT1. The ssT1 factor binds to a second site in the *Timp-1* promoter between -115/-100, deletion of which results in a 3-fold decrease of both basal and serum-stimulated transcription from the promoter. These data argue that ssT1 may be involved in maintaining efficient transcription of *Timp-1* in unstimulated basal conditions in mouse fibroblasts, which in

turn may affect the overall level of gene activity that can be attained following stimulation.

The binding of ssT1 to ssDNA shows clear sequence preferences. Although the -63/-49 AP1-bottom strand and the -115/-100-top strand were able to cross compete effectively for binding of the ssT1 factor, and they both detected a 54kDa protein by south-western blot analysis, the -63/-49 sequence bound strongly to other proteins at 90, 32 and 22 kDa that were only weakly bound (if at all) by the further upstream site. Thus ssT1 may be one of many different proteins, each of which may prefer particular sequence motifs.

Comparison of the -63/49 AP1-bottom and the -115/-100 top sequences suggests a possible consensus motif for ssT1 binding as 5-CA/TTTn$_{4-6}$ATC-3'. Within this motif the 5'-sequences may be the most critical for several reasons. First, the underlined T residue indicates the distinguishing difference between the unusual TIMP-1 AP1 site and the consensus collagenase AP1. Second, mutational analysis involving fusing either half of the -115/-100 sequence to the inactive collagenase AP1 site indicated the loss of the first half containing the 5'-CTTT was more deleterious, and this was supported by additional mutations involving triplet replacements through the sequence. Third, this sequence is most conserved between mouse and rat (5'-CTTTGGGTTTATC-3' versus 5'-CTTTGGGCTCAGC, respectively; (Bugno et al., 1995)). Finally, the RT7D1 sequence, which has been shown to interact with a single-stranded binding protein (Oosterhuis and van der Hoorn, 1999) also has a CTT sequence, which is required for the protein-DNA interaction to occur. This might indicate a family of transcription factors with a common DNA-binding motif. However, my mutational studies also show that multiple sequences

participate since disruption of the 5'-CTTT still allowed some binding. Furthermore, either half of the -115/-100-top sequence alone was insufficient to confer ssT1 binding, as shown by the failure of the -123/-110-top and the -108/-92 top sequences to bind. As well, when I used human sequences for competition, the mutant AP-1 binding site was able to compete, even though it lacks a C(T/A)TT sequence. Thus sequences around the motif may also contribute to binding preferences, and as a consequence we prefer to term the binding of ssT1 'sequence-selective' rather than sequence-specific.

The DNA-ssT1 interaction data from EMSA studies complement the functional analysis of promoter activity from transient transfection studies of the various deletions of the *Timp-1* promoter. Loss of the -125/-95 region lowered basal activity from the promoter without a profound effect on the fold-induction following serum stimulation. Mutation of the ssT1 site located at either -115/-100, or -63/-49 are both associated with a decrease in promoter activity under basal conditions. Likewise, ssT1 was present at equal levels in nuclei isolated from unstimulated and serum-stimulated mouse fibroblasts. This supports the idea that ssT1 may function in a cell specific fashion to maintain the basal level of TIMP-1 expression required for ECM homeostasis.

The involvement of single-strand sequence-selective DNA binding proteins in transcriptional regulation has been documented for other genes. The muscle factor 3 (MF3) single-stranded binding activity (Santoro et al., 1991) binds to three individual sequences which show few significant regions of identity: the cARG motif of MRE ($CC(A/T)_6GG$), the E-box of creatine kinase (TCAGGCAGCAGGTGTTGGGGG), and MCAT (CATTCCT), which is found in many muscle gene promoters. However, the relevance of these interactions remains unknown.

At present, we can only speculate about the function of the ssT1-DNA interaction. A number of genes are regulated in part by interactions with single-stranded DNA-binding activity through a number of different mechanisms. Control of the adipsin gene bears similarity to what we have found for *Timp-1*, as it is regulated by two factors each specific for single-stranded DNA, with little dsDNA binding activity (Wilkison et al., 1990). One of the two single-stranded DNA-binding proteins is expressed in a differentiation-dependent fashion, and is thought to play a role in establishment or maintenance of the differentiated state. A 40bp regulatory region upstream of the gelatinase-A (MMP-2) promoter is involved in high level expression of the gene in glomerular mesangial cells (Mertens et al., 1997). It has recently been shown that this site binds transcription factors AP2 and YB-1 (Mertens et al., 1998), with YB-1 showing preferential binding to the isolated single strands of the response element (Mertens et al., 1997).

Other identified single-stranded DNA-binding proteins provide additional possible modes of action. The ssDNA-BP, DBSF interacts with purified estrogen receptor, enabling it to bind to its response element (ERE) (Mukherjee and Chambon, 1990), which implicates transcription factor recruitment as a mechanism of transcriptional activation. Such recruitment is also seen for the Aα core protein, which is involved in regulation of the Aα fibrinogen gene. The Aα core protein, which is related to the mitochondrial ssDNA-BP, P16 (Liu et al., 1997), has been shown in overexpression studies to be involved in IL-6 induced transcription, possibly through recruitment of STAT signaling molecules (Liu and Fuller, 1995). Work on the rat TIMP-1 promoter identified the sequences between the AP1 and PEA3/Ets sites (which are

precisely conserved in the mouse promoter, corresponding to –53/-45) as an oncostatin-M/IL-6-responsive element that binds STAT3 (Bugno et al., 1995). It will be interesting to determine if ssT1 is in any way involved in STAT3 recruitment.

Another mechanism of activity for ssDNA-BP activity is through a direct recruitment of RNA polymerase (RNAP), as shown by the coliphage protein N_4SSB, which activates σ70, and does not bind to dsDNA (Miller et al., 1997). It has been demonstrated that the protein interacts directly with the RNAP B' subunit, which has relevance to eukaryotic transcription because the region of interaction is conserved in the largest subunit of the eukaryotic RNAP II. There are several possible functions of interaction with RNAP subunits. The protein could act as a tether to link the RNAP to a promoter (Barberis et al., 1995; Farrell et al., 1996). Alternatively, subsequent steps of RNAP function could be targeted (Miller et al., 1997). Another mechanism of action, as demonstrated by the EcoSSB single-stranded binding protein is the enhancement of transcription by establishing adequate DNA secondary structure (Glucksmann-Kuis et al., 1996). Finally, a single-stranded DNA-BP might be involved in establishment of single-stranded regions at sites of transcription. Such activity might serve to enable open DNA complex formation during initiation by the RNAP, or alternatively, to maintain an open state in order to relieve torsional stresses during the act of transcription itself (Duncan et al., 1994; Wittig et al., 1992). It is possible that ssT1 induces some conformational change in DNA once bound, since this may explain why the EMSA complex of ssT1 with the –125/-95-top probe migrates faster than the corresponding complex with the –115/-100 oligonucleotide, despite the larger size of the former.

The inability of ssT1 to bind to dsDNA appears to rule out a function in denaturation of the promoter region. However, the possibility remains that the proteins may stabilize a single-stranded conformation. Other possibilities exist for preinitiation complex recruitment and binding being aided by the ssDNA-BP. Such a function is seen for PC4, which is a positive transcription cofactor (Ge and Roeder, 1994; Ge et al., 1994; Wu and Chiang, 1998). PC4 interacts with both free and bound VP16 activation domains, and also with TFIIA. However, there is no interaction with TBP. Similar to ssT1, PC4 interacts with single-stranded DNA (Werten et al., 1998).

Conversion of the unusual TIMP-1 AP1 motif to a consensus collagenase AP1 site did not affect basal or serum-inducible expression from the promoter in transient transfection assays with a reporter driven by the –223/+47 promoter. This may be due to compensation via the presence of the –115/-100 ssT1 binding site. However, it is clear that the –223/+47 *Timp-1* region does not recapitulate the full range of expression of the endogenous gene; for instance, expression from these constructs is unresponsive to phorbol ester stimulation. Thus the –63/-49 AP-1-bottom ssT1 binding may be more important in the context of other regulatory elements upstream or downstream from the promoter. It is also possible that the full effects of ssT1 will be evident only with the chromatin template, and it would thus be interesting to study the effects of the promoter deletions, and mutant ssT1 fragments in both integrated, stable cell lines, or alternatively, in transgenic mice. Another potential area of involvement of ssT1-like factors is tissue-specific expression. Only one cell line (fibroblastic) and two tissue types (liver and testes) were analyzed by southwestern blot, yet significant differences in binding activities were seen. The 54kDa ssT1 that is recognized by both the –115/-100-top and

the –63/-49 AP1-bottom probes is present in fibroblasts and liver, but the testis pattern was more complex. It will be important to determine the relationships between these various binding activities.

In conclusion, I have identified a 54 kDa nuclear single-stranded binding activity which is involved in establishing basal expression of *Timp-1* through interaction with at least two regions of the promoter. The ssT1 factor and related molecules may be involved in the regulation of a number of genes.

I have used several different approaches to purifying or identifying the ssT1 factor. The first method involved using affinity chromatography to attempt to purify ssT1 from a large quantity of 10T1/2 or mouse liver nuclear extracts. I was able to deplete ssT1 from the nuclear extracts using my biotinylated –115/-100 top oligonucleotides, however, I was unable to get any recovery of the ssT1 from the column. My data has shown that the ssT1-DNA complex is salt stable, so there was no reason why increasing the KCl concentration would elute the protein. The complex is definitely SDS-sensitive, however, yet no ssT1 was eluted by SDS-PAGE loading dye. There were several different types of protease inhibitors present at each step of the chromatography protocol, but I cannot rule out that the ssT1 is being proteolytically degraded. Alternatively, it appears that the ssT1 is binding irreversibly to some component of the column, either the oligonucleotide itself, or the agarose (from the streptavidin agarose). Future efforts might utilize other types of column matrix, such as streptavidin cellulose.

Expression cloning strategies were also used to isolate the ssT1. However, positive clones were never obtained from a mouse tetratocarcinoma library. I believe that the best chance of success would be to use an expression library derived from

C3H10T1/2 cells. This is the cell type in which all experiments have been performed, and definitely express ssT1. Southwestern blotting gave a strong ssT1 signal in liver tissue, however, closer examination (data not shown) suggests that the protein detected in liver tissue is slightly smaller in size compared to that detected in the C3H10T1/2 cells.

4.5 Transcriptional Regulation of *Timp-4*

4.5.1 Identification of a putative *Timp-4* Promoter

Both the murine and human TIMP-4 genes were recently cloned (Greene et al., 1996; Leco et al., 1997), however, there is still very little published on their expression patterns and regulation. Using RACE-PCR, I was able to map 3 potential transcription start points (tsp) of *Timp-4* to -52, -80, and -109 bases upstream of the translation start point. The presence of multiple tsp sites bears similarity to *Timp-1*, however, *Timp-4* clearly does not have a 5'-untranslated exon. An analysis of the sequence upstream of the tsp sites reveals an absence of any region resembling a TATA-box and there are relatively few known transcription factor binding sites, except for c-Ets, and Sp-1 sites at –540 and –560 respectively (see figure 32). It remains to be seen whether these sites are functional, and if they actually bind to these transcription factors. Furthermore, a confirmation of the multiple transcription start sites must be performed by an independent technique, such as RNAse protection assay.

Aside from the lack of any obvious regulatory elements within the promoter regions of Timp-4, there are some interesting features. There are two mononucleotide repeats in close proximity to the tsp sites. The closest is a poly T tract $(T)_{23}$, followed by a poly C region $(C)_{21}$, located at approximately -315 and -755 respectively. Furthermore,

there is a dinucleotide repeat $(CA)_{21}$ present far upstream at -3010. The significance of these sites is not certain at this point. However, at least for the (CA) repeat, there is precedence in the literature for transcriptional regulation by such a region. The murine MMP-9 gene contains two stretches of (CA) repeats, the first 130 bases upstream of the tsp, and another far upstream at -1700 (Manaut et al., 1999). While no direct demonstration of transcriptional regulation was shown for the region, reporter assays with the proximal (CA) repeats deleted showed a loss of activity (Mohan et al., 1998; Shimajiri et al., 1999). Additional evidence of transcriptional regulation by a (CA) repeat is seen in the Epidermal Growth Factor Receptor gene (EGFR), which has a (CA) repeat within its first intron (Gebhardt et al., 1999). It was shown that in the case of the EGFR gene, the size of the dinucleotide repeat is inversely correlated with reporter activity, suggesting a gene silencer effect from the (CA) repeat. While the mechanism in which such a region could regulate transcription is uncertain, it is possible that it has to do with DNA bending. Lengthy (CA) repeat will be a highly bendable region (Gebhardt et al., 1999) or alternatively, has the propensity to form Z-structure DNA (Shimajiri et al., 1999), either of which might then affect transcription factor interactions.

4.5.2 Cis-acting transcriptional regulatory region of the Timp-4 promoter

A tissue expression screen by Northern blot has shown a very restricted pattern of expression of Timp-4, to heart and neural tissue (Leco et al., 1997). Expression has also been seen to be associated with invasive glioma tissue (Laurie Groft, personal communication). However, for the purposes of an in vitro model, I needed to find cultured cell lines which express Timp-4.

A screen of 15 cell lines by RT-PCR showed that very few cell lines express the TIMP-4 gene. Three neuronal cell lines (U251N, PC12 and C6, see figure 30) did not express any Timp-4, and we did not have access to cardiac muscle cells. A prostate cancer cell line (PC3) and MRC5 fibroblasts expressed detectable levels of Timp-4, but there was no consistency in the expression with respect to the tissue origin of the cell lines; for example, all fibroblasts did not express Timp-4. This might indicate that the expression has to do with the transformed status of the lines, or in some cases, the lines were derived from tumours, and might reflect genetic changes during tumour formation. Another problem was in the primer specificity. We were using human and mouse TIMP-4 primers to screen tissue from rat, dog, and hamster, although we are not certain that the primers will be able to anneal properly. Nonetheless, the screen did show a limited expression of TIMP-4 in cultured cells, and 2 cell types, MRC5 and PC3 express the gene, which would make them suitable for expression analysis.

Both MRC5 and PC3 cells were used for transfection and reporter analysis of the Timp-4 deletion series, however, no reporter activity was seen in the lines for any of the promoter regions transfected into them. There are two possible reasons for the absence of expression. First, the short promoter regions used for the analysis might lack important regulatory regions located further upstream. Alternatively, the human cells might lack the transcription factors necessary to activate transcription from the murine promoter. When murine 10T1/2 fibroblasts were used for the transfections, the -161/-28 construct showed increased activity relative to the -87/-28 construct, indicating a positive regulatory element.

There is also evidence of a other positive regulatory region within the large, upstream fragment of the Timp-4 promoter. Two regions, -3488/-1028, and –1028/-74 were able to enhance expression from the active Timp-1 promoter, -223/+190 Timp-1. However, -4000/-74, and –3488/-74 within pBLCAT3 were unable to activate expression of the reporter alone. This might be a consequence of only 2 of the 3 identified tsp sites being present in these constructs, or alternatively, it might be due a unique interaction between the large Timp-4 fragment with something in the Timp-1 promoter region. In any case, a 5'-deletion series of constructs starting at 5'-3488, with a downstream endpoint of –22 is presently under construction.

In summary, Timp-4 has multiple tsp's, however, there are few transcription start points. The few predicted cis-regulatory elements are not located in the regions identified by reporter assays as positive regulatory regions. However, several regions are important in positively regulating expression of the gene, by deletion analysis. These results are consistent with the low, and restricted expression of the Timp-4 gene.

4.6 Future Considerations

This thesis has focused on the mechanisms which regulate the expression levels of the TIMP genes, which has relevance on the net TIMP/MMP balance. This is necessary because of the observation that the TIMP/MMP balance is disrupted in many different types of pathologies. The approach taken was the analysis of the transcriptional mechanisms regulating TIMP levels. However, an important consideration is that gene expression is dependent upon many different signalling pathways, and is also dependent upon the cell type. Therefore, while I have identified several regions which are important

in the overall expression of the murine Timp-1 and Timp-4 genes, there are still additional positive regulatory regions to be identified.

In the Timp-1 promoter, I have identified protein-DNA interactions at both -115/-100 and -63/-49 by a factor described as ssT1. A critical next step is the identification of the ssT1. Although many unsuccessful attempts were made already to clone or purify the DNA-binding protein, all methods have not been exhausted. As described previously, I believe that a C3H10T1/2 expression library should be used for screening. Furthermore, it would be useful to confirm that bacterially expressed ssT1 will still interact with the –115/-100 top sequence. Experiments into phosphorylation of ssT1 would clarify this point, and it would be interesting to demonstrate whether calf intestinal phosphatase treated ssT1 would still interact with the -115/-100 top DNA sequence.

I believe from Southwestern blotting that the ssT1 factor is quite abundant within the nuclear extracts. However, I was unable to isolate a single band by SDS-PAGE which migrates at 54 kDa, because a cluster of bands is present within this size range. One method of resolving ssT1 would be to run a 2D gel. Identification of ssT1 could then be performed by Southwestern blotting the 2D-gel, and the resulting band could then be microsequenced. Alternatively, perhaps sufficient quantities of ssT1 could be obtained and purified using *in vitro* translation, followed by separation of the product on SDS-PAGE gel, and characterization with SW-blot. The identification of this protein would enable further experiments which would demonstrate its role in Timp regulation. The factor could be overexpressed in transfection systems to demonstrate directly the effect on Timp-1 transcription. In addition, localization during development, and in adult

tissues could be performed. Coexpression with Timp-1 during development would indicate a mechanism of Timp-1 developmental regulation.

While the identification and characterization of the ssT1 protein is imperative to the continuation of this project, there are still experiments which could characterize ssT1 without the cloning of the protein. Three different nuclear extracts have been screened by Southwestern blot for ssT1-like binding activity, liver, testes, and C3H10T1/2 extracts, which revealed a different distribution of proteins which interact with the –115/-100 top oligonucleotide. This might indicate a family of single-stranded DNA-binding transcription factors which have specific tissue distributions. Cloning ssT1 would be the most useful for identifying other family members. However, using just the oligonucleotide, additional information could be gained by screening nuclear extracts from many more different tissue and cell types. Perhaps Timp-1 promoter construct transfections into cell lines which express high ssT1 compared to low ssT1, and comparing basal reporter activity would demonstrate the effect on basal activity.

Another approach would be to identify potential single-stranded regions within the promoter. One method of studying this would be to treat nuclei with S1 nuclease, which will cleave any single-stranded regions. PCR primers which bracket the -63/-49 region, or -115/-100 region could then be used to amplify the regions. If there is a potential to form single-strands, the regions will not amplify as effectively. While I have demonstrated a basal transcriptional effect, and DNA binding, the methods have used Timp-1 promoter constructs, which might not accurately represent what happens in situ. Presumably, sites of ssT1 interaction (at –115 and –63) would be single-stranded,

because I have demonstrated that ssT1 does not effectively interact with double-stranded DNA.

I have explored other regions of the Timp-1 gene which are important in directing its expression levels. The first intron has multiple regulatory elements which are presently unidentified. While I have confirmed a positive element within the +150/+190 region, I was unable to identify it as an AP-1 (at least c-Fos and c-Jun) component. Still, evidence the Werb lab (Logan et al., 1996) that there is a direct AP1 binding site is convincing, and future experiments should be targeted towards the expression of different Fos and Jun family members which form the AP1 binding factor, and examining the effect on reporter expression. The Werb lab (Logan et al., 1996) showed that –50/+190 constructs (the –63/-49 AP-1 binding site is deleted) were still responsive to overexpression of c-Jun and c-Fos. While I was unable to reproduce this result, I was studying a different cell type, which might suggest a cell-specific response.

There are also the putative silencer regions which have been roughly mapped to the +47/+870 region of the intron. The data from our transgenic mice lines would support the notion that a silencer is operating in this region, and a more detailed search for the specific mechanism is warranted. This could be done with additional deletion constructs spanning the promoter region. One difficulty with this approach is that the intron appears to contain several positive and negative regulatory regions in close proximity (Clark et al., 1997). These sites could then have an additive, or subtractive effect, making interpretation of reporter activity difficult. A more appropriate method of investigation would be an *in vivo* analysis. The expression of reporter constructs in transgenic mice is a valuable tool for examining gene regulation during development.

This is a step up from basic *in vitro* expression analysis because the transgene is exposed to the full gamut of stimuli within the developing mouse. I have already constructed a promoter spanning from -2700/+785 driving the β-Gal gene. This can be directly compared to the -2700/+47 β-gal construct we already have. In addition, several other 3'-intron deletions could be made. A comparison of in vivo expression should localize the intron regions necessary for appropriate Timp-1 expression during development.

I have identified multiple putative transcription start points for the *Timp-4* promoter. Also, I have been involved in sequencing and analyzing the promoter region. My RACE-PCR data has been confirmed by primer extension (Ian Clark, personal communication), however, both techniques rely upon reverse transcriptase. The best confirmation of tsp sites is by RNAse protection of the suspected tsp.

While I believe that I have correctly identified the Timp-4 promoter, a comparison of the *Timp-4* promoter with a transcription factor database revealed that the region upstream from the tsp has only a few sequences with identity to transcription factor binding sites. I have identified some moderate positive regulatory regions in close proximity to the tsp by reporter analysis, and further studies will identify specifically the regions conveying the response, using finer deletion endpoints and EMSA binding assays. In addition, EMSA analysis with oligonucleotides corresponding to the active promoter fragments highlighted in the deletion analysis will give evidence of transcription factor interactions, and may even lead to the identification of transcription factors involved in *Timp-4* regulation.

In addition to the promoter proximal region, regions further upstream of the tsp are presently being examined. By reporter analysis, large upstream promoter fragments

are able to enhance activity from an active fragment of the *Timp-1* promoter, however, this effect cannot be attributed to any specific cis elements yet. A particularly interesting upstream region of the Timp-4 promoter is the CA repeats. A deletion which eliminates the repeat, as well as reducing the number of CA repeats should be tested in reporter analysis. Furthermore, the poly C and poly T regions might be involved in the transcriptional regulation of the gene. Given my experience with *Timp-1*, an analysis of the *Timp-4* first intron may be useful to understand the regulation of *Timp-4*.

This thesis has examined transcriptional regulatory regions in two members of the TIMP gene family, Timp-1 and Timp-4. A summary of the cis-acting elements I have identified (with respect to previously identified regions) is shown in figure 36. There are not many similarities between the regulatory elements of all four TIMPs. However, this is consistent with the differences in both the induction, and tissue-specific expression patterns that has been observed for these genes. I have identified several regulatory elements within the Timp-1 and Timp-4 promoters, which has contributed to our overall understanding of TIMP and ECM regulation. In addition, the notion that a single-stranded binding protein interacts at two regions of the Timp-1 promoter, and increases basal activity, has contributed towards our knowledge of the mechanisms of transcription regulation.

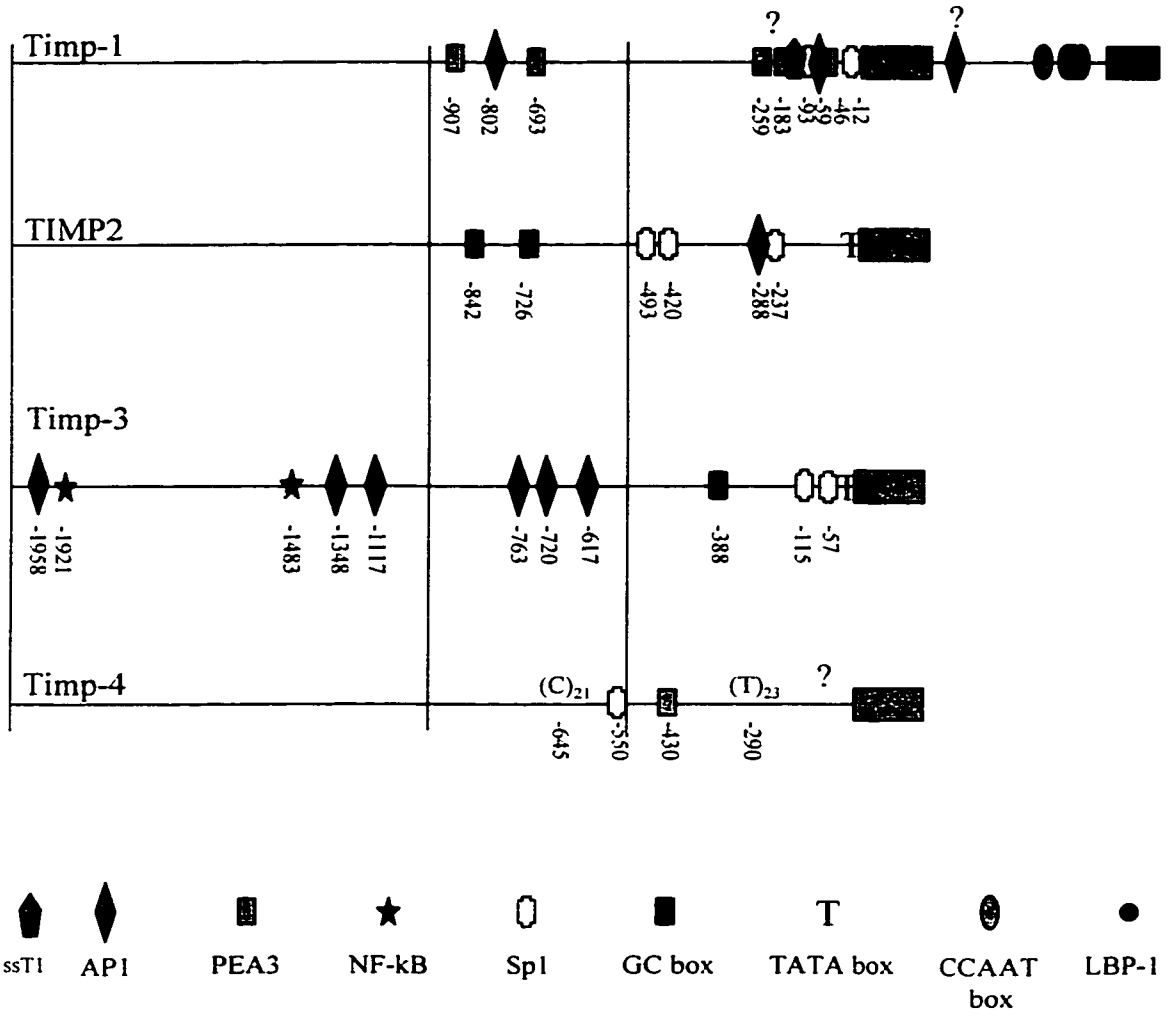


Figure 36. Summary of TIMP promoter regulatory elements. Depicted are the regulatory elements within the TIMP promoter, including identified elements, as well as several which are regulatory, but unidentified (?).

Publications

Yeow, K., Phillips, B., Beaudry, P., Leco, K., Murphy, G., and Edwards, D. (in press). The expression of MMPs and TIMPs in mammalian cells. Matrix Metalloproteinases Protocols. Edited by I.M. Clark. (Totowa, New Jersey); Humana Press.

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