A Sequence-selective Single-strand DNA-binding Protein Regulates Basal Transcription of the Murine Tissue Inhibitor of Metalloproteinases-1 (Timp-1) Gene*

(Received for publication, May 12, 1999)

Blaine W. Phillips‡§, Renuka Sharma‡, Pamela A. Leco‡, and Dylan R. Edwards‡¶

From the ‡Department of Medical Biochemistry, University of Calgary, Calgary T2N 4N1, Canada

Tissue inhibitor of metalloproteinases-1 (TIMP-1) is important in maintaining the extracellular proteolytic balance during tissue remodeling processes. To allow homeostatic tissue turnover, the murine Timp-1 gene is expressed by most cells at a low basal level, and during acute remodeling its transcription is activated by a variety of stimuli. A non-consensus AP-1-binding site (5'-TGAGTAA-3') that is conserved in mammalian timp-1 genes is a critical element in basal and serum-stimulated transcription. We show here that each strand of this unusual AP-1 site binds a distinct single-stranded DNA-binding protein, although neither strand from a perfect consensus AP-1 site from the human collagenase gene shows similar binding. One of the single-strand binding factors, which we term ssT1, binds to a second upstream Timp-1 region between nucleotides –115 and –100. Deletion analysis demonstrated that this region is important in basal but not serum-inducible transcription. The ssT1 factor was 52–54 kDa by UV cross-linking of electrophoretic mobility shift assays and Southern blot analysis. Its binding to DNA shows sequence selectivity rather than specificity, with 5'-CTATTN4-3ATC-3' as a favored motif. Multiple ssT1-like activities were found in nuclear extracts from mouse fibroblasts and rat liver and testis, suggesting that these factors may regulate basal Timp-1 transcription in a tissue-specific fashion.

Extracellular matrix turnover is highly regulated at multiple levels, a critical one being the balance between the levels of matrix metalloproteinases (MMPs) and their specific inhibitors, the TIMPs (tissue inhibitors of metalloproteinases (1, 2)).

The TIMP family includes four members, designated TIMP-1, -2, -3, and -4, each of which can inactivate the active forms of MMPs by formation of a tight 1:1 non-covalent complex (3). Although the TIMPs are largely interchangeable in their MMP-inhibitory abilities, it has become clear that they have certain properties that are specific to particular family members, supporting the idea that they have unique as well as shared physiological roles. For instance, compared with TIMP-2, -3, and -4, TIMP-1 is a poor inhibitor of the membrane-type MMP group of MMPs, several of which function as cell surface activators of progelatinase-A (MMP-2 (4–7)). TIMP-1 also stands out in its ability to form a complex with pro-gelatinase-B (MMP-9) (8), through which it regulates the rate of pro-MMP-9 activation (9, 10). Finally, TIMPs show highly individual patterns of expression in vivo and in vitro (2, 11), indicating that the use of a particular TIMP may be advantageous in certain tissue remodeling situations.

Of the four timp genes, timp-1 and -3 are both highly inducible in diverse cell types in vitro, in response to stimuli such as serum, phorbol ester, and transforming growth factor-β (12–14), whereas timp-2 expression remains largely constitutive under most stimulatory conditions (15). In the developing mouse embryo, strong expression of Timp-1 is restricted to sites where extensive tissue remodeling is occurring, particularly at sites of osteogenesis in the limbs, digits, ribs, vertebræ, and skull (16, 17). Tight spatio-temporal control of expression is also seen in the adult ovary and uterus during pregnancy under the influence of paracrine and endocrine factors (18). However, many tissues express Timp-1 at a low basal level that can only be detected by sensitive techniques such as ribonuclease protection assay, possible reflecting a tissue maintenance function (16).

Previous work from several laboratories has shown that expression of Timp-1 is controlled principally at the level of transcription (19, 20) which therefore dictates that a detailed analysis of its transcriptional regulation be performed. The Timp-1 genes from mouse, rat, and human have been cloned and their promoter and regulatory regions analyzed (19, 21–24). In several characteristics Timp-1 resembles a housekeeping gene, including the absence of a canonical TATA box, the presence of Sp1 sites, and multiple transcription start sites (25–28). Additionally, the promoter proximal region contains several cis-acting regulatory elements that have been shown to be important for conveying transcriptional induction in response to stimuli. Most notable is a highly conserved region that has an API-(activator protein-1) binding motif in close proximity to a PEA3/Ets domain, an arrangement found in many other API-responsive genes (29). Several laboratories have demonstrated the importance of both of these regions, but clearly, the API element is the principal site for conferring stimulatory responses following serum stimulation in vitro (27–29). Interestingly, the API-binding site is a non-consensus
form (5′-TGAGTAA-3′) that differs by a single base from the consensus AP1-binding motif (5′-TGAGTCA-3′) found in most AP-1-responsive genes, including inducible MMPs such as human collagenase (mmp-1), stromelysin-1 (mmp-3), and gelatinase-B (mmp-9) (30, 31). We previously showed by electrophoretic mobility shift assay that in addition to the ability of the Timp-1 –59/–53 AP-1 site to bind complexes containing Fos and Jun proteins, other proteins could bind that showed no affinity for the consensus collagenase AP-1 sequence (otherwise known as the 12-O-tetradecanoylphorbol-13-acetate-responsive element) (28).

We therefore undertook a more detailed investigation of the characteristics of the Timp-1 AP-1 site, in conjunction with a search for further important regulatory elements in the promoter proximal zone of the gene. We report here that single-strand versions of the Timp-1 AP-1 site bind distinct nuclear factors and that the consensus AP-1-binding site does not share this ability. Furthermore, similar single-strand DNA binding activity is also seen at a site further upstream, at –115/–100. These interactions affect the basal expression of Timp-1, rather than its induction in response to serum stimulation. We speculate that these promoter-interacting single-stranded DNA-binding proteins may be significant factors in the dual housekeeping/inducible behavior of Timp-1 and other genes.

**MATERIALS AND METHODS**

**Cell Culture—**Marine fibroblast C3H10T1/2 cells were cultured in Dulbecco’s modified Eagle’s 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.

**Transient Transfection Analysis of mTIMP-1 Luciferase and CAT Reporter Constructs—**All transient transfections were performed by the Chen and Okayama (32) procedure essentially as described previously (28). Briefly, cells were plated at a density of 5 × 10^4 cells/ml and grown overnight in DMEM/F12 containing 10% FBS. To transfect the cells, 20 μl of plasmid was brought up to 900 μl of final volume in TE, followed by the addition of 100 μl of CaCl2 (2.5 M). The mixture was then added dropwise to 1 ml of 2 BES-buffered saline (50 mM BES, 280 mM NaCl, 1.5 mM MgCl2, pH 7.0, 100 μM CaCl2), which enabled the formation of DNA-calcium phosphate precipitates. Precipitation proceeded for exactly 20 min, and 1 ml of the mixture was added dropwise to each of two plates of cells. The cells were incubated for 18 h at 37 °C in an atmosphere of 97% (v/v) air, 3% (v/v) CO2. After the transfection, the samples were fixed by the addition of 117 μl of neutralization solution (0.9 M NaOH, 2.25 M NaCl) for 15 min. The volume was increased to 510 μl with buffer (1.4 M Tris-HCl (pH 7.0), 1.5 M 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 cross-linking, hybridized to specific probes (pBLCAT3 or pGL2-basic), and exposed to x-ray film (Kodak). Intensity of signal (measured densitometrically) was then used to standardize the reporter expression to amount of input plasmid.

**Constructs—**Inserts for a deletion series of the Timp-1 promoter were generated by the polymerase chain reaction (PCR) using standard protocols with the primers shown in Table I. The constructs, corresponding to amount of input plasmid.

**Luciferase reporter assays were performed using the luciferase assay system according to manufacturer’s instructions (Promega, Madison, WI).** Briefly, 20 μl of cell lysate was mixed with 100 μl of luciferase lysis buffer as per manufacturer’s instructions. Following stimulation, cell extracts were collected by harvesting with reporter lysis buffer as per manufacturer’s instructions.

**Luciferase reporter assays were performed using the luciferase assay system according to manufacturer’s instructions.**

**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 the 5′-3′ orientation. The Sac primers are all sense, and the Xho 47 primer is antisense for production of the deletion constructs. Directionality is mentioned for all other primers.

| Name | Sequence |
|------|----------|
| Sac 223 | GGAGGCCTCAAGTTTGGCCCTTCCTCTCT |
| Sac 195 | GGAGGCTCTCAAGGCGCCCGAGACAGAC |
| Sac 180 | GGAGGCTCCAGGTGCTGACCTGGAG |
| Sac 165 | GGAGGCTCGTGGAGCTGGGGGAAGGG |
| Sac 150 | GGAGGCTCGGGGGCAGTGGGCTGCT |
| Sac 125 | GGAGGCTCAGAGGAGTAACCCTTCCC |
| Xho 97 | TTGGGTGAGTAATGCGTCAGGAGAG |
| coll.AP1 top | CTTCTTGGACGACGATCTACATCA |
| coll.AP1 bot | CACGGGCTGGTGGAGTATCGGTCAGGAG |
| mutAP1 top | CTTCTTGGACGACGATCTACATCA |
| mutAP1 bot | CACGGGCTGGTGGAGTATCGGTCAGGAG |
| mutPEA3 top | CTTCTTGGACGACGATCTACATCA |
| mutPEA3 bot | CACGGGCTGGTGGAGTATCGGTCAGGAG |
| Hind –115mut1 | CAAAGGCTCGGAGGTGTTTATCGGGGGG |
| Hind –115 | CAAAGGCTCGGAGGTGTTTATCGGGGGG |
| Bam +47 | AAGGATCCCTCGGAGATGCGGCG |

**Probes—**Single-stranded probes were generated by annealing 60 ng of

K (10 mg/ml), which was then incubated for 3 h at 37 °C. After the protease digestion, the mixture was centrifuged for 5 min at 12,000 rpm, and the supernatant was retained and extracted with 1:1 phenol:chloroform. The samples were then prepared for slot-blot transfer by mixing 50 μl of supernatant with 3.7 μl of 1 M HCl for 5 min to denature, followed by the addition of 117 μl of neutralization solution (0.9 M NaOH, 2.25 M NaCl) for 15 min. The volume was increased to 510 μl with buffer (1.4 M Tris-HCl (pH 7.0), 1.5 M 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 cross-linking, hybridized to specific probes (pBLCAT3 or pGL2-basic), and exposed to x-ray film (Kodak). Intensity of signal (measured densitometrically) was then used to standardize the reporter expression to amount of input plasmid.
oligonucleotide to a modified T4-poly nucleotide kinase (PNK) buffer (0.5 m Tris-HCl (pH 7.6), 0.1 m MgCl₂, 10 mm DTT, 10 mm EDTA, 25% polyethylene glycol, w/v) with 1 μl of [γ-³²P]ATP, and 5 units of T4-PNK. Reactions were incubated at 23 °C for 30 min and then stopped with 50 μl of 25 mm EDTA. The probes were purified by phenol/ chloroform extraction, and unincorporated dNTPs were removed using G-50 spin columns. Duplex fill-in probes were generated by adding 50 ng of annealed oligonucleotide to 0.5 mM dATP, dGTP, dTT, 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM EDTA, 1 mM DTT). The next day, the samples were soaked in binding buffer with single-stranded, end-labeled probe (100,000 cpm/ml) for 6 h to overnight at 4 °C. Following the probing, the blots were rinsed 4 times in binding buffer for 8 min at 4 °C and air-dried. The washed blots were then exposed to Biomax x-ray film (Kodak).

RESULTS

The Unusual −59/−53 AP1-binding Site Is the Key Regulatory Element in the Timp-1 Promoter Proximal Region—We showed previously that the 5′-TGAGTAA-3′ motif at −59/−53 in the Timp-1 promoter was able to bind AP-1 and activate transcription from a linked promoter (28). Subsequent studies have confirmed the importance of this site in both the human and mouse promoters (29, 34, 35). However, we found that this sequence was able to associate with additional proteins besides Fos/Jun AP-1, which were unable to bind to a consensus colla genase AP-1 site (5′-TGAGTAA-3′), and we speculated that this unusual AP1-binding site might convey additional properties to the Timp-1 promoter (28). To test the involvement of the −59/−53 AP-1 site in promoter activity, we incorporated point mutations that either convert the site to a consensus AP1 motif (coll-AP1), or render it non-functional (mut AP1). In addition we introduced an inactivating mutation into the neighboring PEA3/Ests-binding site (Fig. 1). Transient transfection assays were carried out using CAT reporter plasmids carrying these alternative versions of the −223/+47 region of the Timp-1 promoter. The mutAP1 construct confirmed that a functional AP1-binding motif is essential for maximal basal and serum-inducible gene expression. In contrast, mutation of the PEA3/Ests site led to a more modest reduction of activity, to about 60% of the maximum observed with the wild-type promoter. Fig. 1 also shows that the unusual Timp-1 AP1 and the consensus AP1 sites have equivalent abilities to drive both basal and serum-stimulated expression of CAT in the context of the −223/+47 region.

A Single-stranded Binding Protein Interacts with the Timp-1 Promoter AP-1 Region—We examined in more detail the nature of the nuclear factors that are able to interact with the −59/−53 AP1-binding motif is essential for maximal basal and serum-inducible gene expression. In contrast, mutation of the PEA3/Ests site led to a more modest reduction of activity, to about 60% of the maximum observed with the wild-type promoter. Fig. 1 also shows that the unusual Timp-1 AP1 and the consensus AP1 sites have equivalent abilities to drive both basal and serum-stimulated expression of CAT in the context of the −223/+47 region.
beled A was competed by both unlabeled −63/−49 Timp-1 and collagenase AP1 sequences (lanes 3 and 4). Band A was also supershifted when anti-Fos or anti-Jun antibodies were added to the binding reactions (data not shown). These data identify shift A as AP1 bound to the 

\[ \begin{align*} &\text{GGTGCGTGGGA} \\
&\text{TGAAT} \\
&\text{G} \times \text{mutAP1} \\
&\text{CTGGAGGCA} \\
\end{align*} \]

probe, confirming our previous data and other reports (28, 29). However, in addition to AP1 binding activity, −63/−49 also demonstrated additional specific binding properties (band B) that were not competed by the collagenase AP1 (lane 4). Unlabeled single-stranded −63/−49 sequences were effective competitors of complex B formation (lanes 7 and 8). The AP1 shift was not competed by single-stranded −63/−49 oligonucleotides, and single-stranded collagenase AP1 cold competitors (top or bottom strands) were unable to compete complex A or B. These data demonstrate that complex B represents the interaction of a nuclear factor that is not AP1 with the individual strands of the −63/−49 region and that this interaction is specific since it could not be competed by a closely related perfect consensus AP1 motif.

To examine whether the complex B shift was attributable to one of the oligonucleotides that comprise the −63/−49 probe (i.e. the top or bottom strands), we carried out EMSAs using each labeled oligonucleotide separately, as well as corresponding sequences from the collagenase AP1 (Fig. 2B). The samples were electrophoresed longer for greater resolution of the bands. Both Timp-1 −63/−49 strands were able to complex with nuclear factors, generating bands with slightly different mobilities that are labeled as B and B' for the top and bottom strand probes, respectively. The corresponding single strands of the collagenase AP1 sequence did not interact with anything in the nuclear extracts. We were unable to carry out competition assays to determine whether both top and bottom −69/−43 strands interact with the same protein, since an excess of the complementary strand generated double-stranded DNA, which does not bind the single strand-specific nuclear factor, as will be shown later in Fig. 5. However, other data to be discussed argue that the B and B' complexes involve different nuclear factors.

A Positive Regulatory Element That Affects Basal Timp-1 Transcription Is Located between −125/−95—Whereas the above studies were ongoing, we also aimed to refine the analysis of regulatory elements in the promoter proximal region, since our previous studies had shown that additional positive acting elements must reside within the −223/−47 promoter used in Fig. 1 (28). Constructs with alternative 5′-end points were generated by PCR and subcloned into the luciferase reporter plasmid, pGL2-basic, and then used in transient trans-
were resolved more compared with single- or double-stranded competitors. In from 10T1/2 cells, showing two distinct bands, AP1-binding site were labeled and used for EMSA with nuclear extracts without added competitor DNA; seen, labeled Timp-1 AP1-binding site. End labeling in this way generates both double-stranded and single-stranded forms of both 63/49 and the corresponding strands from the collagenase consensus AP1-binding site for a nuclear factor, but flanking sequences may also influence the nature of the complex that is formed. With this preparation of PNK-labeled −63/−49 probe, we observed three bands migrating in the position of the B/B’ complexes seen with individual top and bottom −63/−49 single-stranded probes in Fig. 2B. The number and intensities of these bands labeled B/B’ were somewhat variable with different experiments and nuclear extracts (see also Fig. 5A). The data shown in Fig. 4 were obtained with a nuclear extract from C3H 10T1/2 cells stimulated for 30 min with serum, but there were no qualitative or quantitative changes in band patterns with extracts from unstimulated or 3-h serum-stimulated cells.

Since the experiment in Fig. 4A was performed with annealed, PNK-labeled, oligonucleotides that may contain both double-stranded and single-stranded forms (as in Fig. 2), we also carried out an analysis with Klenow-labeled double-stranded probes. The Klenow-labeled, double-stranded −115/−100 and −125/−95 probes were greatly impaired in their ability to interact with DNA-binding proteins compared with the PNK-labeled probes (Fig. 4B; compare lanes 2 with 7, and 4 with 8). This reduction paralleled the diminished representation of the B/B’ shifts seen when the Klenow-labeled −63/49 Timp-1 AP1 probe (Fig. 4B, lane 9) was compared with the same probe labeled with PNK (Fig. 4A, lane 1). These data suggested that as is the case for the B/B’ shifts with the −63/−49 probes, a single-stranded DNA-binding protein may interact with the −115/−100 mTIMP-1 promoter region.

Both −115/−100 and −63/−49 Promoter Regions Interact with the Same Single-stranded DNA-binding Protein—We carried out EMSA with single-stranded PNK-labeled probes from the −125/−95 region (Fig. 5). Single-stranded probes corresponding to −125/−95, −115/−100, and −63/−49 all show complex patterns of protein interactions (Fig. 5A). Strong shifts were obtained with both the −125/−95-top and −115/−100-top oligonucleotides but not with corresponding bottom strands (lanes 1–4). No shifts were seen with either top or bottom strands from the −125/−110 and −108/−92 sequences (data not shown). In contrast, both top and bottom −63/−49 strands were able to interact with nuclear proteins, giving B/B’ shifts as in Fig. 2. Since all of the probes used had the same additional nucleotides added at the 5’-ends to generate restriction enzyme cloning sites (5’-AGCTT- for the “top” strand probes and 5’-GATCC- for the “bottom”), we also generated and tested additional single-stranded probes lacking these sequences. These probes gave the same results as those shown in Fig. 5 (data not shown), confirming that the sequence-specific DNA binding that we had observed was attributable to the indicated Timp-1 sequences alone.

In order to determine the specificity of the protein-DNA interactions the −115/−100-top probe was used for EMSA in competition with various unlabeled oligonucleotides (Fig. 5B). The top strand of −115/−100 was competed effectively by −115/−100-top and −125/−95-top (lanes 10 and 12), demonstrating specificity of the complex. Surprisingly, competition between the −63/−49-bottom strand and the −115/−100-top strand was also seen (lane 15), although the top strand −63/−49 did not compete (lane 14). This competition from the
The bottom strand of either \([-125/-95]\) or \([-115/-100]\) were also effective "competitors" of the \([-115/-100]\)-top shift, but this is misleading. The bottom strand would anneal to the probe to form duplex DNA, which we had established in Fig. 4 has relatively poor binding to the single-stranded DNA binding factor. Such annealing is seen in a slower migration of the free probe at the bottom of the EMSA gel in Fig. 5B in the lanes with either \([-125/-95]\)-bottom or \([-115/-100]\)-bottom as cold competitor. Neither the \([-63/-49]\)-top nor either of the coll.AP1 strands competed for the same nuclear factors that bind to the \([-115/-100]\)-top probe (lanes 14, 16, and 17). These data establish that a nuclear single-stranded DNA binding factor, hereafter called ssT1, that is unrelated to AP1 interacts with both the \([-63/-49]\)-AP1-bottom and the \([-115/-100]\)-top sequences.

The Single-stranded DNA-binding Protein Shows Relaxed Specificity—The sequence of \([-115/-100]\)-top is 5'-agcttATCT-TTGGGTTTATC-3' and \([-63/-49]\)-bottom is 5'-gatecGCAT-TACTCATCCA3'. There is minimal similarity between these sequences, the two commonalities being 5'-C(T/A)TT motif and a 5'-ATC sequence toward the 3'-side. This prompted us to analyze further the specificity of the sequences in which the protein binds. Mutations were produced in which the first 8 bases of the \([-115/-100]\)-top sequence were replaced with the first 8 bases of the consensus coll.AP1-top sequence that was shown earlier to not bind to ssT1 (2B) (mutant 1). Similarly, the last 8 bases of the \([-115/-100]\) were replaced with the last bases of the coll.AP1-top (mutant 2). Both single-stranded mutants were labeled and used as probes for EMSA (6B-left) and also used as cold competitors against the wild-type \([-115/-100]\)-top probe (6B-right). When end-labeled and used to probe nuclear extracts, both mutants were impaired in their interaction with ssT1 compared with the wild-type sequence. However, disruption of the upstream 7 bases (mut1) had a far greater effect, greatly reducing complex formation compared with mut2. This observation is mimicked by cold competition experiments, where both mut1 and mut2 were not as effective a competitor as wild-type sequences, with mut2 being a slightly better competitor compared with mut1. These results show that both halves of the \([-115/-100]\) sequence contribute to protein-DNA interaction, with a greater contribution of the upstream half.

We also tested competitor oligonucleotides in which 3 bases at a time in the \([-115/-100]\)-top sequence were varied. Quantification of the abilities of these DNAs to compete with the wild-type sequence in EMSA is shown in Fig. 6C. None of these mutations completely eliminated competition ability, although disruption of the 5'-CTT at \([-113/-111]\) as well as the 5'-ATC at \([-102/-100]\) were the most deleterious. These data suggest that both of these sequences may contribute to the ssT1 showing sequence selectivity for a consensus 5'-CA/T/TTN_{4-6}ATC motif.

**Fig. 3.** Deletion analysis of the Timp-1 promoter region. Deletions spanning \([-223/-95]\) were produced by the polymerase chain reaction, and the resulting fragments were cloned into pGL2-basic luciferase reporter plasmid (Promega). Following transfection of the deletion constructs, nuclear extracts were prepared from C3H10T1/2 cells cultured in serum-free media (unstimulated) and cells that were serum-stimulated for 24 h. Luciferase reporter activity for each trial was standardized for sample-sample variation by measuring the input DNA using the Hirt's assay (as described under "Methods and Materials"). The graph shows relative activity, with the largest value displayed by the \([-123/-47]\) construct in serum-stimulated cells, designated as I.
The data also indicate that there are likely many sequences that will interact with the ssT1 nuclear factor.

The ssT1 Nuclear Factor Is a Positive Regulator of the −63/−49 Region of the Timp-1 Promoter.—To analyze further the role of ssT1 in basal promoter activity, we 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 as in Fig. 1. Since both the mutant AP1 and the coll.AP1 sequences do not interact with ssT1, this allowed us to discriminate the contributions of the −115/+100 and −63/+49 ssT1 sites in basal promoter activity. Incorporation of the mut1 sequence used in Fig. 6 into the −115/+47 reporter caused a 15–20% reduction in promoter activity (Fig. 7). However, this mutation had greater impact in the reporter carrying the coll.AP1 site, reducing expression by 40%. Elimination of AP1 binding ability had a dramatic effect on promoter activity, as we had seen previously with the −223/47 constructs used in Fig. 1, with the mutant AP1 construct yielding only 20% of the expression seen with the −115/+47 wild-type Timp-1 promoter region. However, this low basal level could be further reduced to approximately 10% of the wild-type promoter by inclusion of the mut1 sequence at −115/−100. These data argue that both ssT1-binding sites at −63/+49 and −115/+100 contribute to the basal activity of the Timp-1 promoter. We suspect that the effect of mutating the ssT1 site is not as severe as deleting it (compare Fig. 7 with Fig. 3) because the mut1 mutation still retains some ability to interact with ssT1 (Fig. 6).

Both Regions, −115/−100 Top and −63/−49 Bottom. Interact with a Protein of Approximate Molecular Mass of 50–55 kDa—To characterize further the ssT1 factor, EMSA binding reactions with the −115/−100-top probe were UV cross-linked and then separated on a 10% SDS-polyacrylamide electrophoresis gel (Fig. 8A). A complex pattern of bands was observed migrating between 40 and 65 kDa; however, two major bands at approximately 50 and 55 kDa were detected. Specificity of these interactions is demonstrated by competition from excess unlabeled −115/−100-top oligonucleotides. The complexes were not competed by cold coll.AP1 single strands, but they were competed by −63/−49-bottom as expected (data not shown).

As further characterization of the ssT1 factor, Southwestern blots were performed (Fig. 8B). 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. All of the bands labeled 1–4 were specific since they were eliminated by adding excess unlabeled oligonucleotide competitor to the binding solution (data not shown). 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 (Fig. 8B). There are, however, additional weaker interactions with bands 1 and 3 at 90 and 32 kDa, respectively. When using the −63/−49-bottom probe with 10T1/2 nuclear extracts, we observed a similar banding pattern; however, there is an approximately equal distribution of signal between bands 1 and 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-competitions we have observed. We suggest that the band-2 54-kDa nuclear binding activity identified by this Southwestern analysis is the ssT1 factor. At this time, we do not know the identity of any of the bands. It is possible that the lower molecular weight bands (labeled 3 and 4 in Fig. 8B) are breakdown products of the higher molecular weight species. The band 2 signal is the only significant binding activity detected in liver nuclear extracts with either the −115/−100-top or the −63/−49-bottom probes; in the case of the weak signal with the −63/−49-bottom this resolved into a doublet. Both probes interacted with proteins of approximately 54 kDa in liver nuclear extracts; however, −115/−100-top gave a much stronger response. Alternatively, only −115/−100-top interacted with nuclear factors from rat testes, giving a series of 4–5 bands of approximately 58, 54, 48, 40, and 36 kDa, which may represent a distinct family of testis-specific single-stranded binding proteins or different isoforms of the ssT1 factor.

DISCUSSION

The mechanisms involved in the tissue-specific and stimulus-responsive transcription of Timp-1 are not fully understood. 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 (27–29). We show here that this site, which differs from a consensus AP1-binding motif by a single base (5′-TGAG-TAA-3′), confers additional protein binding properties on single-stranded versions of the sequence covering −63/−49 of Timp-1. Our 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 54-kDa protein that we have 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 prefer-
ences. 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 54-kDa
protein by Southwestern 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 a family of factors, each of which may 
prefer particular sequence motifs. The distinction between 
ssT1 and the factor that binds the −63/−49 AP1-top strand 
was shown by both competition data and UV cross-linking
studies analogous to those of Fig. 7A, which revealed bands at
approximately 35, 41, and 50 kDa (data not shown).
Comparison of the −63/49 AP1-bottom and the −115/−100-
top sequences suggests a possible consensus motif for ssT1
binding as 5'-CA/TTTN4–6 ATC-3'. Within this motif the 5'- 
sequences may be the most critical for several reasons. First,
the underlined T residue indicates the distinguishing differ-
ence 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 collagen-
ase AP1 site indicated the loss of the first half containing the
5'-CTTT was somewhat more deleterious (Fig. 6B), and this
was supported by additional mutations involving triplet re-
placements through the sequence (Fig. 6C). Third, this se-
quence is most conserved between mouse and rat (5'-CTTT-
GGTATATC-3' versus 5'-CTTTGGGTACCGC, respectively
(24)). However, these mutational studies also show that mul-
tiple 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 bind-
ing, as shown by the failure of the −123/−110-top and the
−109/−92-top sequences to bind. 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 comple-
ment the functional analysis of promoter activity from tran-
sient 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 induc-
tion 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 to maintain the
housekeeping level of Timp-1 promoter activity.

The involvement of sequence-selective DNA-binding proteins
in transcriptional regulation has been documented for other
genes. The muscle factor 3 (MF3) single-stranded binding ac-
tivity (36) binds to three individual sequences that show few
significant regions of identity as follows: the CARG motif of
muscle regulatory element (CC(A/T)6GG), the E box of creatine
kinase (TCAGGCACTTGTTGG), and MCAT (CAT-
TCCT), which is found in many muscle gene promoters. How-
ever, 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
adipin 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 double-stranded DNA binding activ-
ity (37). 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 40-base pair regulatory region upstream
of the gelatinase-A (MMP-2) promoter is involved in high level
expression of the gene in glomerular mesangial cells (38).
It has recently been shown that this site binds transcription factors
AP2 and YB-1 (39), with YB-1 showing preferential binding to
the isolated single strands of the response element (38).

Other identified single-stranded DNA-binding proteins pro-
vide additional possible modes of action. The ssDNA-BP, DNA
binding stimulatory factor interacts with purified estrogen re-
ceptor, enabling it to bind to its response element (40), which
implies transcription factor recruitment as a mechanism of
transcriptional activation. Such recruitment is also seen for the
Aa core protein, which is involved in regulation of the Aa
fibrinogen gene. The Aa core protein, which is related to the
mitochondrial ssDNA-BP, P16 (41), has been shown in overexpression studies to be involved in interleukin-6-induced transcription, possibly through recruitment of STAT signaling molecules (42). 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 2125/2145) as an oncostatin-M/interleukin-6-responsive element that binds STAT3 (24). 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 NssSB, which activates σ70, and does not bind to double-stranded DNA (43). 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 (44, 45). Alternatively, subsequent steps of RNAP function could be targeted (43). Another mechanism of action, as demonstrated by the EcoSSB single-stranded binding protein is the enhancement of transcription by establishing adequate DNA secondary structure (46). 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 (47, 48). 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

![Fig. 6. The single-stranded DNA-binding protein displays sequence selectivity.](image-url)
In conclusion, we have identified a 54-kDa nuclear single-stranded binding protein that 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. Therefore, efforts at purifying the protein are presently under way.

Acknowledgments—We thank Dr. Ian Clark for critically reviewing the manuscript. D. R. E. is grateful to the Norwich and Norfolk Big C Appeal.

REFERENCES

1. Woessner, J. F. (1991) FASEB J. 5, 2145–2154
2. Denhardt, D. T., Feng, B., Edwards, D. R., Cocucci, E. T., and Malyankar, U. M. (1993) PharmacoL Ther. 59, 329–341
3. Matsurita, I. M. (1990) Trends Genet. 6, 121–125
4. Cowell, R. S., Knauper, V., Stewert, M. L., Detho, M. P., Stanton, W., Hembry, R. M., LopezOtín, C., Reynolds, J. J., and Murphy, G. (1998) Biochem. J. 331, 453–458
5. Knauper, V., Will, H., LopezOtín, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M., and Murphy, G. (1996) J. Biol. Chem. 271, 17124–17131
6. Butler, G. H., Will, H., Atkinson, S. J., and Murphy, G. (1997) Eur. J. Biochem. 244, 653–657
7. Kinoshita, G. S., Satoh, T., Takino, T., Itoh, M., Akizawa, T., and Seiki, M. (1996) Cancer Res. 56, 2535–2538
8. Goldberg, G. I., Strongin, A., Collier, I. E., Genrich, L. T., and Marmer, B. L. (1999) J. Biol. Chem. 274, 4583–4591
9. Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, A. G., and Goldberg, G. G. (1998) J. Biol. Chem. 273, 17213–17221
10. Itoh, Y., and Nagase, H. (1995) J. Biol. Chem. 270, 16518–16521
11. Edwards, D. R., Leco, K. J., Lim, M. S., Phillips, B. W., Raja, J., and Sharma, R. (1999) Inhibitors of Metalloproteinases in Development and Disease (Hawkes, S. P., Edwards, D. R., and Khokha, R., eds) Harwood Academic Publishers, Lausanne, Switzerland, in press
12. Edwards, D. R., Murphy, G., Reynolds, J. J., Whitman, S. E., Doherty, A. J. P., Angel, P., and Heath, J. K. (1987) EMBO J. 6, 1899–1904
13. Overall, C. M., Wrana, J. L., and Sodek, J. (1981)  J. Biol. Chem. 266, 14066–14071
14. Overall, C. M., Wrana, J. L., and Sodek, J. (1991) J. Biol. Chem. 266, 14061–14071
15. De Clerck, Y. A., Darville, M. l., Eackhout, Y., and Rousseau, G. G. (1994) Gene (Amst.) 139, 185–191
16. Nomura, S., Hogan, B., Wills, A. J., Heath, J. K., and Edwards, D. R. (1989)

2 P. A. Leco and D. R. Edwards, unpublished observations.
17. Flenniken, A. M., and Williams, B. R. G. (1990) *Genes Dev.* 4, 1094–1106
18. Edwards, D. R., Parfett, C. L. J., and Denhardt, D. T. (1985) *Mol. Cell. Biol.* 5, 3280–3288
19. Ponton, A., Coulombe, B., Steyaert, A., Williams, B. R. G., and Skup, D. (1992) *Gene (Amst.)* 116, 187–194
20. Edwards, D. R., Waterhouse, P., Holman, M. L., and Denhardt, D. T. (1986) *Nucleic Acids Res.* 14, 8863–8878
21. Ponton, A., Coulombe, B., Steyaert, A., Williams, B. R. G., and Skup, D. (1992) *Gene (Amst.)* 116, 187–194
22. Edwards, D. R., Rocheleau, H., Sharma, R., Wills, A. J., Cowie, A., Hassell, J. A., and Heath, J. K. (1992) *Biochim. Biophys. Acta* 1171, 41–55
23. Logan, S. K., Garbedian, M. J., Campbell, C. E., and Werb, Z. (1996) *J. Biol. Chem.* 271, 774–782
24. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Herrlich, P., and Chambard, J. (1987) *Cell* 52, 2256–2266
25. Chen, C., and Okayama, H. (1987) *Proc. Natl. Acad. Sci. USA* 84, 12691–12695
26. Wu, S.-Y., and Chiang, C.-M. (1998) *J. Biol. Chem.* 273, 12492–12498
27. Ge, H., and Roeder, R. G. (1994) *Cell* 77, 513–523
28. Glucksmann-Kuis, M. A., Dai, X., Markiewicz, P., and Rothman-Denes, L. B. (1996) *Genes Dev.* 10, 12691–12695
29. Ge, H., Zhao, Y., Chait, B. T., and Roeder, R. G. (1994) *Proc. Natl. Acad. Sci. USA* 91, 12691–12695
30. Werten, S., Langen, F. W. M., van Schaik, R., Timmers, H. T. M., Meisterernst, M., and van der Vliet, P. (1998) *J. Biol. Chem.* 273, 367–377