Overlapping Egr-1 and Sp1 Sites Function in the Regulation of Transcription of the Mouse Thrombospondin 1 Gene*

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We have evaluated the basis for the constitutive and serum-regulated expression of the mouse thrombospondin (TSP) gene in both transiently and stably transfected NIH-3T3 cells. Experiments with deleted and mutated mouse promoter/CAT constructs and gel mobility assays demonstrated that an Egr-1 binding site in the proximal promoter, flanked by overlapping GC boxes and an adjacent GC-rich region, functioned to positively regulate the constitutive activity of the gene. These motifs, and their cognate transcription factors, appear to act in concert, with partial redundancy, so that discrete mutations were only partially effective in reducing transcriptional activity. The Egr-1 site corresponds to an NF-Y binding site in a domain as a result of several serum-response elements and their cognate transcription factors, which bind synergistically with a distal serum-response element (SRE) and a proximal NF-Y binding site (8). When we examined comparable regions of the mouse TSP1 promoter (13, 14), we found that the distal SRE was conserved, but that, in place of the proximal NF-Y binding site, the mouse gene contained a consensus Egr-1 motif. egr-1 is an early growth response gene that shows c-fos-like induction kinetics in fibroblasts, epithelial cells, and lymphocytes in response to mitogenic stimulation (15-19). mRNA levels for Egr-1 are also rapidly up-regulated by serum, epidermal growth factor, and PDGF in the presence of cycloheximide; thus induction of the egr-1 gene does not require de novo protein synthesis. It therefore seemed possible that, in mouse TSP1, an Egr-1 cis-acting element could serve in place of the SRE to mediate the serum response.

The structure and functions of platelet thrombospondin 1 (TSP1) have been studied intensively during the past 15 years (see Refs. 1 and 2 for reviews). It is now recognized that most mammalian cells have the capability to synthesize and secrete TSP1 (3) and that the wide range of functions ascribed to the protein, effects that include roles in platelet aggregation, stimulation, and inhibition of cell growth, adhesion and cell shape, chemotaxis, and angiogenesis, are likely to result from the ability of TSP1 to interact with a multiplicity of cell surface receptors (1, 4). A distinguishing feature of the cellular synthesis of TSP1 is its rapid induction by mitogens such as serum and PDGF (5-8), a response characteristic of a group of genes termed immediate early genes (9). In this respect TSP1 differs from its close relative, TSP2, which responds poorly if at all to mitogen stimulation (10, 11), unless cells are subject to extreme deprivation of growth factors (12).

We have analyzed the transcriptional regulation of human TSP1 by serum in some detail (8) in the expectation that such a study would shed light on the role of TSP1 in cellular growth responses and, ultimately, on the mechanisms by which the protein influences cellular behavior. The response of stably transfected human TSP1 promoter/CAT constructs in NIH-3T3 cells is mediated by the synergistic effect of a distal serum-response element (SRE) and a proximal NF-Y binding site (8). When we examined comparable regions of the mouse TSP1 gene (13, 14), we found that the distal SRE was conserved but that, in place of the proximal NF-Y binding site, the mouse gene contained a consensus Egr-1 motif. egr-1 is an early growth response gene that shows c-fos-like induction kinetics in fibroblasts, epithelial cells, and lymphocytes in response to mitogenic stimulation (15-19). mRNA levels for Egr-1 are also rapidly up-regulated by serum, epidermal growth factor, and PDGF in the presence of cycloheximide; thus induction of the egr-1 gene does not require de novo protein synthesis. It therefore seemed possible that, in mouse TSP1, an Egr-1 cis-acting element could serve in place of the SRE to mediate the serum response.

In this study we have used mouse TSP1 promoter/CAT constructs, in both transiently and stably transfected NIH-3T3 cells, to evaluate the basis for the constitutive expression of mouse TSP1, and we have also compared the serum-regulated expression of the mouse and human genes. We find that Egr-1, Sp1, and, possibly, related factors interact with a GC-rich region in the proximal promoter to mediate the constitutive expression of the mouse gene.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The parental construct, -2800 CAT, contains 2800 bp of promoter and 5'-flanking sequence and part of exon 1 of mouse TSP1 (from -2800 to +48) in pBS-CAT (13). The latter plasmid was described previously (8). Two of the 5'-deletion constructs were generated by religation of vectors derived from -2800 CAT after the following treatment: 1) -480 CAT, BamHI digestion, 2) -135 CAT, KpnI digestion, and 3) deletion of -135 to -84, PCR product derived from -84 to +48. Internal deletion constructs were generated by insertion of PCR fragments into either parental vector (-2800 CAT or -480 CAT) digested by Aval and SphI. The deletion end points and inserted PCR products are as follows: 1) deletion of -135 to -41, PCR product derived from -41 to +48; 2) deletion of -84 to -58, PCR product derived from -155 to -84 and from -58 to +48; and 3) deletion of -135 to -84, PCR product derived from -84 to +48. Four sets of mutant constructs were produced by insertion of PCR products into either parental vector digested by Aval and SphI. The inserted fragments, including the mutations, were generated by a PCR protocol according to a method described by Higuchi et al. (20). The mutated sequences, located between -84 and -48 are described in Table I. All constructs were confirmed by DNA sequencing using the dideoxy method.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) J05605 and M62449.

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The abbreviations used are: TSP, thrombospondin; PDGF, platelet-derived growth factor; CAT, chloramphenicol acetyltransferase; SRE, serum-response element; bp, base pair(s); PCR, polymerase chain reaction.

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chain termination method in conjunction with an Applied Biosystems model 373A sequencer and with a dye terminator cycle sequencing kit.

Cell Culture, Transfection, and Assays of Gene Expression—NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum, penicillin, streptomycin, and butyl-p-hydroxybenzoate. To generate transiently transfected cells, 1 μg of the plasmid of interest and 1 μg of pKNeo, linearized by KpnI and EcoRI, respectively, were transfected into NIH-3T3 cells by the calcium-phosphate procedure, and the transfected cells were selected in medium supplemented with 400 μg/ml (activity) Geneticin (Life Technologies, Inc.).

For basal expression assays of transiently transfected cells, 3.6 × 10^6 cells were plated per 6-cm dish. Approximately 18 h later the medium was changed and 1 μg of the plasmid of interest, 0.5 μg of cytomegalovirus β-galactosidase as an internal control and 3.5 pg of pBSM13(+) as a carrier DNA, were transfected by the calcium-phosphate method, and the transfected cells were selected in medium supplemented with 400 μg/ml (activity) Geneticin. Dynamic model 400s PhosphorImager. Each experiment was performed three times, and each blot was exposed twice to maximize the accuracy of the analysis. The average of six values for each construct was used for the normalization of CAT activities. Differences in copy number among the constructs tested were normalized for copy number by slot hybridization of cellular RNase protection assay using the riboprobe, T7ARsaCAT. Quantitation of the analysis. The average of six values for each construct was used for the normalization of CAT activities. Differences in copy number among the constructs tested were normalized for copy number by slot hybridization of cellular RNase protection assay using the riboprobe, T7ARsaCAT. Quantitation of the analysis. The average of six values for each construct was used for the normalization of CAT activities. Differences in copy number among the constructs tested were normalized for copy number by slot hybridization of cellular RNase protection assay using the riboprobe, T7ARsaCAT.

For basal expression assays using stably transfected cells, cells were plated at 3.6 × 10^6 cells/6-cm dish. Approximately 72 h later the cells were harvested and cell extracts were assayed as above. Since stable cell populations were multilocal, >300 clones were picked for each construct and CAT activities were normalized for copy number by slot hybridization of cellular DNA with a EcoRIHindIII -1.2-kilobase pair fragment of CAT cDNA. Quantitation was performed by imaging gels with a Molecular Dynamics model 400s PhosphorImager. Each experiment was performed three times, and each blot was exposed twice to maximize the accuracy of the analysis. The average of six values for each construct was used for the normalization of CAT activities. Differences in copy number among the constructs tested were usually less than 20%.

A serum stimulation assay was performed as described previously (8). Gene activities in stably transfected cells were analyzed by an RNase protection assay using the riboprobe, T7ARsaCAT. Quantitation was performed by imaging gels with a PhosphorImager. Serum responsiveness is presented as a ±fold increase, calculated as the ratio between the CAT activity before and 3 h after serum stimulation.

Analysis of DNA-Nuclear Factor Interactions—Nuclear extracts were prepared from NIH-3T3 cells, with or without stimulation by 15% fetal calf serum for 30 min, as described elsewhere (21). The dialysis buffer consisted of 20 mm HEPES, pH 7.5, 70 mm KCl, 5 mm MgCl₂, 0.05% Nonidet P-40, 12% glycerol, 1 mg/ml bovine serum albumin, 50 mm dithiothreitol, and 0.1 mM ZnCl₂. For gel mobility shift assays, 5 μg of nuclear extract was incubated with 2 μg of poly(dI-dC) as nonspecific competitor, then, specific probe and competitors were added and the sample was incubated for an additional 25 min at room temperature. The reaction mixture was electrophoresed on 4% polyacrylamide gels with 0.5 × TBE (0.045 X Tris borate, 0.01 X EDTA, pH 8.0) at room temperature.

The following wild type and mutant oligonucleotides, corresponding to the sequence from -64 to -58 in mouse TSP1, were synthesized.

-84
WT: 5'-CCCGTCCCCCGCCCCCGCCCCCAGAA-3'
M1: 5'-CCCGTCCCCTTATCCCCCCCCCAAGAA-3'
M2: 5'-CCCGTCTATCCCCCCCCCCCAAGAA-3'
M3: 5'-CCCCGTCCTCCCCCCCCCCCCCAAGAA-3'
M4: 5'-CCCCGTCCTCCCCCCCCCCCCCAAGAA-3'

The mutated bases are underlined. A wild type oligonucleotide extending from -60 to -36 (see Fig. 1 for sequence) was also synthesized. A 25-mer corresponding to nucleotides -907 to -883 in the promoter region of the mouse zif/268 gene served as an Egr-1 binding site (16). Sp1 binding was assessed with either a 32-mer or a 29-mer corresponding to bases +913 to +944 (A1) or +983 to +1011 (A3), respectively, in the first intron of human α1(I) collagen gene (22). All oligonucleotides were purified on polyacrylamide gels before use. To generate probes for assays, the oligonucleotides were labeled with T4-polyuridine kinase and then purified on polyacrylamide gels.

RESULTS

Serum Responsiveness of the Mouse TSP1 Gene—We had demonstrated previously that the human and mouse TSP1 genes were rapidly responsive to serum and PDGF, and that two transcriptional elements in the human TSP1 promoter, a distal element at -1280 (a serum response element or SRE) and a proximal element at -65 (an NF-Y binding site), were required for the response of the human TSP1 gene to serum (8). A similar distal element is found in the mouse TSP1 gene at -1210 and is homologous to the SRE in the human TSP1 gene; 18 bases out of 22 are identical to the human sequence and the core CA motif is completely conserved between the two genes (Fig. 1A). Both elements are homologous to the SRE in the c-fos gene which has been shown to bind the serum response factor, SRF (23). On the other hand, the sequence of the proximal region is quite different between the two genes. Instead of an NF-Y binding site, an Egr-1 binding site is centered at -70 in the mouse TSP1 gene (Fig. 1B). We considered the possibility that the Egr-1 binding site might contribute to the serum response of the mouse TSP1 gene, since cooperative binding of a SRE and another element has been demonstrated in a number of genes (see Ref. 8 for a discussion). Therefore, we determined whether the proximal region of the mouse TSP1 promoter was required for the serum response of the gene. For this purpose we constructed the promoter-CAT plasmids, -2800 CAT, -2800(A-135/-41) CAT, -480 CAT, and -480(A-135/-41) CAT, and introduced these plasmids into NIH-3T3 cells by stable transfection. Fig. 2 shows a representative time course of changes in the levels of CAT mRNA for each of the four constructs during the course of serum stimulation, as determined by a ribonuclease protection assay. Four independent analyses gave similar results. The expression of -2800 CAT was induced within 1 h after serum stimulation and was maximal after about 2 h. The -fold induction (about 20-30-fold) is comparable with the stimulation of CAT mRNA levels in NIH-3T3 cells stably transfected with the human TSP1 promoter-CAT construct, -2033 CAT (8). The somewhat lower and earlier peak responses of the mouse and human transfected CAT genes, compared with the endogenous mouse TSP1 gene (8), can be explained by the presumed reduced stability of CAT mRNA in comparison with TSP1 mRNA. Since -2800 CAT lacks the first intron of the mouse TSP1 gene, we can conclude that, as for the human TSP1 gene, the first intron is not required for serum stimulation. However, in contrast to the human TSP1 gene, deletion of a sequence upstream from the TATA box did not compromise the serum responsiveness of the mouse gene. Thus, the stably transfected -2800(A-135/-41) CAT gene, which lacks the Egr-1 binding site, is induced by serum in a manner similar to that of -2800 CAT (Fig. 2). The apparent difference in the kinetics of the responses for the two constructs was not reproducible. The average response of -2800 (Δ-135/-41) CAT, at 1 h after serum stimulation, was 87% of the response of -2800 CAT; at 2 h the response of the deleted construct was 180% that of the parent construct. As expected, the constructs -480 CAT and -480(A-135/-41) CAT, which lack the SRE centered at -1210, are unresponsive to serum (Fig. 2). Thus, in the mouse TSP1 gene, the SRE and its associated transcription factors interact directly with the TATA box transcriptional complex or, possibly, another sequence located between -135 and -2800 is required for serum responsiveness.

Regulation of the Constitutive Expression of the Mouse TSP1 Gene—Since a number of consensus sequences for known transcription factors are located within the first 2800 bp of the 5′-flanking region in mouse TSP1 (Fig. 1B; Refs. 13 and 14), we first tested the basal expression of a series of promoter deletion constructs. Accordingly, the CAT activity of -2800 CAT, -480 CAT, -135 CAT, and -41 CAT was tested, in both transiently and stably transfected NIH-3T3 cells (Fig. 3A). There was no significant difference among the CAT activities of the four constructs tested in transient transfection. On the other hand, in

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A

mouse TSP 1  -1223 CAAGA?CCCTATTGCTGATGC -1202
human TSP 1  -1289 TGGAGATCCTTATTTGGTCAAGC -1267

B

-482 ATCCCCACC CACCAAGATT GACCTAGGAG GCAGCTTCTT TTAAGGGCT CAACCTTTTA TTTTIAACC
-400 AGACTGTCTC GATCCCCAAG CCGCTCTGTC ATGGAATTAT TCAAGGAGAT GTGCTTTAAT GAAAGGCTCC CTAAAGGGTC
-320 TTAGCTGGCC CCCAAGAAAG CATCCGCTCT GGGGACCTTC ATCCGAGACC TTCTCCGATT ACATGGCCQA AGATCTTAAG
-240 CGCTAACGAC TGACTACGCC AAGGCTGCGT GGGGACGAG ACCTATTATTC TGACAGAGTC CAGGGGCTCC TGTCGGGAT
-160 CGAGCTTCCC CCTCTCCTT TCAGCCCGAG AGCTCTGCGC CAAGCAGCAG GGGGCGGAGA AATGAACCG TCCGTCCCAG

Fig. 1. A, comparison of the sequences of the SREs in mouse TSP1 and human TSP1. The dots indicate identical nucleotides and the consensus CArG sequences are shown in bold. B, sequence of the mouse TSP1 from -482 to +48. The sequences of AP-1 and Egr-1 binding motifs, GC boxes and the TATA box are indicated in bold. The Egr-1 binding motif is also identified with a bracket.

stable transfection, the CAT-activity of -41 CAT was significantly decreased to 13% of that for -2800 CAT. We believe the results of stable transfections might be more reliable for the reasons discussed below.

A number of internal deletions were made in the TSP1 promoter, both in the context of a 2800-bp and of a 480-bp sequence, and the resulting plasmids were tested in transient and in stable transfections (Fig. 3B). The expression of TSP1 CAT constructs which lacked the region between -135 and -41 was reduced by a factor of about five, to a level that approximated that for -41 CAT in stably transfected cells (Fig. 3A). Thus, the majority of constitutive transcriptional activity of the TSP1 promoter is mediated by a 95-bp sequence just upstream from the TATA box (Fig. 1B). However, attempts to define a functional sequence by creating smaller deletions within this 95-bp sequence were not very informative. A deletion between
The distinction between bands 1 and 2 can be made more readily in less intense autoradiograms. Bands 1, 2, and 4 were competed by an Sp1 oligonucleotide but not by an Egr-1 binding site. In contrast, band 3 was competed by an Egr-1 binding site but not by an Sp1 sequence. To verify that band 3 was formed by binding of proteins to an Egr-1 binding site, an anti-Egr-1 antibody (a kind gift of Dr. E. Adamson, La Jolla, CA) was incubated with the nuclear extract before addition of the probe. Although a supershifted band was not observed, the intensity of band 3 was reduced by the antibody, but not by preimmune serum from the same animal (Fig. 4B). The results from these gel mobility shift assays therefore suggest that transcription factors Egr-1 and Sp1, or related factors, bind to the -70 region.

To investigate further the function of the -70 region, we created mutations in the Egr-1 motif and in the flanking GC-rich region. We reasoned that discrete mutations might reveal functional effects that would be obscured by deletions. These mutations are described in Table I. The intent was to limit the mutations, created by PCR, to 3-bp changes. However, as shown in Table I, in only one case (M1), was this possible. In mutations M1A, M2A, and M3A additional unanticipated substitutions of one or two Ts for Cs occurred in the GC-rich region downstream from the -70 region. These mutations were nevertheless used and proved to be informative.

As shown in Table I, constructs -2800(M1) CAT and -480(M1) CAT, with mutations in the Egr-1 motif, were expressed less actively than the parent construct, by a factor of about 2. This result was observed in both transiently and stably transfected cells (Fig. 4B).

Despite the lack of a functional effect of deletion of the -84 to -58 region, we decided to investigate this region further because the sequence contained two overlapping GC boxes straddled by a 9-bp consensus Egr-1 motif (Fig. 1B). It seemed possible that the 27-bp deletion had removed both positively and negatively acting elements with a net effect that was neutral. Alternatively, the juxtaposition of sequences flanking the deletion could have compensated for the removal of a net positively acting element.

Accordingly, we synthesized a 27-mer oligonucleotide extending from -84 to -58 (designated the -70 region) to assess DNA-protein interactions with nuclear extracts from NIH-3T3 cells. The -70 region, when used as a probe, produced four gel-shifted bands all of which were competed by the unlabeled -70 region (Fig. 4A). The distinction between bands 1 and 2 is made more readily in less intense autoradiograms. Bands 1, 2, and 4 were competed by an Sp1 oligonucleotide but not by an Egr-1 binding site. In contrast, band 3 was competed by an Egr-1 binding site but not by an Sp1 sequence. To verify that band 3 was formed by binding of proteins to an Egr-1 binding site, an anti-Egr-1 antibody (a kind gift of Dr. E. Adamson, La Jolla, CA) was incubated with the nuclear extract before addition of the probe. Although a supershifted band was not observed, the intensity of band 3 was reduced by the antibody, but not by preimmune serum from the same animal (Fig. 4B). The results from these gel mobility shift assays therefore suggest that transcription factors Egr-1 and Sp1, or related factors, bind to the -70 region.

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Fig. 4. A, gel mobility shift assays with a 27-mer oligonucleotide from -84 to -58 (-70 region) as a probe and nuclear extracts from NIH-3T3 cells. B, gel mobility shift assay of the -70 region with Egr-1 antibody and Sp1 as competitors. The arrow designates the gel-shifted band formed by binding of Egr-1. PS, preimmune serum. Bands are numbered 1-4.

Table I
Basal expression of mutant mouse TSP1 CAT constructs with mutations assayed in both transient and stable transfections

<table>
<thead>
<tr>
<th>Construct</th>
<th>Transient</th>
<th>Stable</th>
</tr>
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<tbody>
<tr>
<td>-2800(M1) CAT</td>
<td>0.56 ± 0.07</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>-2800(M1A) CAT</td>
<td>0.28 ± 0.03</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>-2800(M2A) CAT</td>
<td>1.30 ± 0.25</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>-2800(M3A) CAT</td>
<td>1.18 ± 0.34</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>-480(M1) CAT</td>
<td>0.68 ± 0.11</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>-480(M1A) CAT</td>
<td>0.36 ± 0.04</td>
<td>0.40 ± 0.06</td>
</tr>
<tr>
<td>-480(M2A) CAT</td>
<td>1.45 ± 0.15</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>-480(M3A) CAT</td>
<td>1.42 ± 0.12</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
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WT: 5′-CCCGTCCCCGCCCCCCCCAGAACCTCCCTCCCCCTCC-3′
M1: 5′-CCCGTCCCCGCTCTCCCTCCCCCCCCGCCCCCTCC-3′
M2: 5′-CCCGTCCCCGCCCCAGAAGGCCTCCCCCTCCCCCTCC-3′
M3: 5′-CCCGTCCCCGCCCCGCCCCAAGAACCTCCCCCTCCCCCTCC-3′
M4: 5′-CCCGTCCCCGCCCCGCCCCGCCCCCTCCCTCCCCCTCC-3′

About two or three, in both transient and stable transfection. The substitution of T for C at positions -51 and -52 in constructs -2800(M1A) CAT and -480(M1A) CAT reduced CAT activity even further and suggests that the GC-rich region downstream from the -70 region, termed the -50 region, also plays a role in regulation of constitutive transcription. Mutations M2A and M3A, in the context of either -2800 CAT or -480 CAT, were ineffective in transiently transfected cells but reduced CAT activity in stably transfected cells by a factor of about two. In the case of -480(M3A) CAT, the reduction of CAT activity was even greater, about 10-fold. We currently have no good explanation for the apparent discrepancy between the activity of -480(M3A) CAT and -2800(M3A) CAT in stably transfected cells. It is possible that an element between -480 and -2800 can partially compensate for the M3A mutation.

To investigate the basis for the functional effects observed with mutant -2800 CAT and -480 CAT constructs, we synthesized four mutant oligonucleotides representing the -70 region (designated M1, M2, M3, and M4; see “Experimental Procedures”), and assessed DNA-protein interactions by gel mobility shift assay. When M1, with a 3-bp substitution in the center of the Egr-1 motif, was used as a probe (Fig. 5A, lane M1), no shifted bands were produced; this finding suggests that the core sequence of the Egr-1 motif is required for the binding of both Egr-1 and Sp1. Oligonucleotide M2, in which the GC box upstream from the Egr-1 site had been mutated, yielded the three shifted bands that were attributed to the binding of Sp1 but not the band caused by the binding of Egr-1 (see Fig. 4).

Conversely, when oligonucleotide M3 was used as a probe, the shifted band attributed to binding of Egr-1, but not those caused by Sp1, was observed. Oligonucleotide M4, which combines the mutations in M2 and M3, yielded no shifted bands. The experiments in Fig. 5B, in which the mutated oligonucleo-
tides were used as competitors for nuclear protein binding to a wild type -70 region probe, yielded consistent data. The ability of high concentrations of M3 to partially compete for binding to Sp1 motifs is in keeping with the overlapping arrangement of the binding sites for Egr-1 and Sp1. We can conclude from these experiments that the Egr-1 consensus motif and the upstream GC box are required for the complete binding of Egr-1 and that the Egr-1 motif and the downstream GC box are required for the complete binding of Sp1.

In the functional study described in Table I construct M1A, containing additional substitutions of two Ts for Cs at -51 and -52, showed lower expression than construct M1, and the expression of constructs M2A and M3A, containing an additional substitution of one T at -52 or one T at -46, respectively, was decreased in stably transfected cells. It seemed possible that the sequence of the GC-rich region downstream from the -70 region (-58 to -41) might include positively acting element(s). We therefore synthesized a 25-mer oligonucleotide extending from -60 to -36 (designated the -50 region) for use in gel mobility shift assays (Fig. 6). When the -50 region was used as a probe, four bands were produced, all of which were competed by an excess of unlabeled oligonucleotide. It should be stressed that the similarity of the gel retardation pattern in Fig. 6 to those in Figs. 4 and 5 does not necessarily indicate the binding of identical proteins, since different DNA sequences were used as probes. Nevertheless, when the Sp1 binding site was used as a competitor, the retardation of bands 1, 2, and 4 was decreased in stably transfected cells. It seemed possible that the -50 region, and factors that bind to GC-rich sequences, including Sp1 and factor(s) related to Egr-1, may contribute to regulation of constitutive transcription of the mouse TSP1 gene.

**Stable Versus Transient Transfection**—In our studies, the discrepancy between the results of transient and stable transfection was particularly apparent in analyses of expression of -41 CAT (Fig. 3) and -480(M3A)CAT (Table I). We believe that the results of stable transfections are more reliable for several reasons. Functional analyses in stably transfected cells were consistent with the results of promoter deletions and gel mobility shift assays. It seems likely that the proximity of vector sequences to a minimally active promoter in transiently transfected genes can lead to the participation of cryptic vector enhancer motifs in expression or to the use of transcription start sites within the vector. The latter possibility was documented by Berkowitz et al. (24) in their study of the mouse c-fos promoter. This complication was avoided in our analyses in stably transfected cells since constructs were linearized at a site between the vector and basal promoter prior to electroporation. Furthermore, the chromosomal integration and replication of stably transfected genes may substantially influence the interaction of transcription factors, a consequence that is not seen with transiently transfected DNA (25). Guertin et al. (26), in comparing stable and transient transfections, similarly concluded that the expression of stably transfected α1-fetoprotein genes more accurately reflected the tissue specificity of the endogenous gene.

**DISCUSSION**

TSP1, in both the human and the mouse, is an immediate-early response gene that is rapidly induced by serum, PDGF, and basic fibroblast growth factor (5-7, 12, 27). There is a possibility that the concomitant induction of TSP1 by growth factors is essential to their stimulation of proliferation and migration of smooth muscle cells, since monoclonal antibodies to TSP1 inhibit both responses (28, 29). The manner in which TSP1 facilitates these processes in smooth muscle cells is not understood, nor are the signaling pathways that enable growth factors to induce the gene. In an effort to elucidate these processes, we have begun to examine the regulation of expression of TSP1 in the expectation that a better definition of the events that occur at the level of the gene can be used to trace, in a retrograde fashion, a signaling path to the cell surface.

**The cis-Acting Elements That Mediate the Serum Response of the TSP1 Gene Differ in Human and Mouse**—Two transcriptional elements, an SRE at -1280 and an NF-Y binding site at -65, function synergistically to mediate the serum response of the human TSP1 gene (8). Cooperativity between an SRE and another cis-acting element has also been observed in the β-actin, c-fos, and Krox 20 and Krox 24 genes (30-32). The mouse gene contains a highly conserved SRE centered at -1210, but lacks a consensus NF-Y site (33) in the proximal promoter region or in the first 3 kilobase pair of the 5'-flanking region (13, 14). However, an Egr-1 motif is present at -70 in the mouse, in a position corresponding to that of NF-Y in the human. Thus, NF-Y and Egr-1 are equidistant from the TTTAAA boxes in their respective genes. Since egr-1 is an immediate-early gene that is induced by serum, epidermal growth factor, and PDGF (16), we considered the possibility that Egr-1 might play a role analogous to that of NF-Y in the serum response of the mouse TSP1 gene.

As shown in Fig. 2, the region upstream from -480, which includes the SRE at -1210, is essential to the serum response of the mouse TSP1 gene, since the stably transfected -2800 CAT transgene was responsive to serum whereas the -480 CAT transgene was not. However, the -2800 CAT and -2800Δ-135/ -41 CAT transgenes responded equally well to serum. We can therefore exclude the region upstream from the TTTAAA box, including the Egr-1 motif, in the serum response of the mouse TSP1 gene. It is still formally possible that another element, that is neither NF-Y nor Egr-1 and is located between -135 and -2800, contributes to the serum response of the mouse gene. However, in that case, the mouse TSP1 gene would represent an exception to the general rule that distal SREs interact with more
proximal elements to mediate serum responsiveness (8, 30).

Transcription Factors That Bind to GC-rich Regions, Including Egr-1 and Sp1, Mediate Constitutive Expression of the Mouse TSP1 Gene—A number of observations indicate that overlapping Egr-1 and Sp1 binding sites, present in a stretch of the proximal promoter termed the -70 region (Fig. 1), function in concert to positively regulate constitutive expression of mouse TSP1. 1) Internal deletions between -135 and -41 in -2800 CAT and in -480 CAT reduced expression of CAT in both transient and stable transfections by 4-8-fold (Fig. 3). 2) Mutations of the Egr-1 binding site and of the Sp1 binding site reduced expression by 2-3-fold in stably transfected cells (Table I). 3) Egr-1 and Sp1 binding sites competed specifically for shifted bands in gel mobility shift assays (Fig. 4). 4) An antibody to Egr-1 disrupted the formation of bands attributed, through competition, to Egr-1.

egr-1 is an immediate early gene that encodes three repeated zinc finger motifs (15) and is induced by serum and growth factors (15, 16, 19). The expression of the egr-1 gene has been observed in a variety of tissues, including brain, skeletal and cardiac muscle, and bone (15, 34, 35). Cotransfection studies of cultured cardiac myocytes with Egr-1 expression vectors demonstrated that Egr-1 up-regulates the α-myosin heavy chain gene (35), and up-regulation of egr-1 expression is associated through competition, to Egr-1. Thus, it is observed in a variety of tissues, including brain, skeletal and cardiac muscle, and bone (15, 34, 35). Cotransfection studies of Spl, on the other hand, is ubiquitous and also binds to similar sequences. The repression of transcription by 2-3-fold in stably transfected cells (Table I) demonstrated that Egr-1 up-regulates the a-myosin heavy chain gene (35), and up-regulation of egr-1 expression is associated through competition, to Egr-1. Thus, it is observed in a variety of tissues. Spl, on the other hand, is an ubiquitous transcription factor in the regulation of mouse TSP1 will therefore require further study.

REFERENCES