A Far Upstream, Cell Type-specific Enhancer of the Mouse Thrombospondin 3 Gene Is Located within Intron 6 of the Adjacent Metaxin Gene

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Thrombospondin 3 (TSP3) is a secreted, pentameric glycoprotein whose regulation of expression and function are not well understood. Mouse Thbs3 is located just downstream from the divergently transcribed metaxin gene (Mtx), which encodes an outer mitochondrial membrane import protein. Although Thbs3 and Mtx share a common promoter region, previous studies showed that Mtx is regulated by proximal elements that had little effect on Thbs3 expression. In this study, transient transfection of rat chondrosarcoma cells and NIH-3T3 fibroblasts demonstrated that Thbs3 is regulated in a cell type-specific manner by a position- and orientation-independent far upstream enhancer located within intron 6 of Mtx. Despite its greater proximity to the transcription start site of Mtx, the Thbs3 enhancer did not have a significant effect on Mtx expression. Two DNA-protein complexes, which were both required for activity, were identified when nuclear extracts were assayed with a probe containing the enhancer sequence. The protein in one of these complexes was identified as Sp1, while the other DNA-protein complex remains uncharacterized. A 6-kilobase pair promoter containing the enhancer was able to direct specific expression of the E. coli lacZ gene in transgenic mice, whereas a 2-kilobase pair promoter that lacked the enhancer was inactive. Thus, despite their close proximity, the genes of the Mtx/Thbs3 gene cluster are regulated independently.

The thrombospondins (TSPs)¹ comprise a family of five secreted matrix glycoproteins that have a characteristic modular structure consisting of a distinct amino-terminal globular domain, three (TSP1 and 2) or four (TSP3 to 5) type II (epidermal growth factor-like) repeats, seven type III (Ca²⁺-binding) repeats, and a homologous carboxyl-terminal globular domain (for reviews, see Refs. 1 and 2). In addition to these common motifs, TSP1 and TSP2 contain a cysteine-rich α1(I) procollagen homology domain and three type I (TSP or properdin) repeats, separating the amino-terminal domain and the type II repeats. Unlike TSP1 and TSP2, which are trimers, TSP3 to TSP5/COMP (cartilage oligomeric matrix protein) are pentamers (3–5). Specific domains in TSP1 and TSP2 have been shown to interact with different components of the extracellular matrix, such as types I and V collagen, fibronectin, and laminin, and amino acid sequences within these domains have been implicated in binding to cell surface receptors, including several integrins, CD36, lipoprotein receptor-related protein, and integrin-associated protein (6–9).

Each member of the TSP protein family is expressed in a different temporal and spatial pattern during murine development and in the adult organism (2, 10–13). In the adult mouse, Thbs3 is expressed predominantly in the lung, in the central nervous system, in cartilage, and in the gastrointestinal tract, with lower levels of expression in other tissues (12, 14). The gene has been mapped to chromosome 3E3-F1 and is located downstream from and transcribed divergently from Mtx, so that 1352 nucleotides separate the translation start sites of the two genes (15, 16). Unlike TSP3, metaxin is a ubiquitously expressed gene that encodes an outer mitochondrial membrane import protein and is essential for embryogenesis (17, 18). The divergently transcribed mouse Mtx and Thbs3 genes are also closely linked to the glucocerebrosidase (Gba) and epispinalin (Muc1) genes (15). The Muc1 gene is located 2.3 kb downstream from the Thbs3 gene and is transcribed in the same direction (15). The Mtx and Gba genes, on the other hand, are transcribed convergently so that their major polyadenylation sites are separated by only 431 nucleotides (17). The human GBA gene is of considerable clinical interest, since mutations in the gene are responsible for the lysosomal storage disorder, Gaucher disease (19).

Although the Thbs3 and Mtx genes share a common promoter region, transient transfection experiments in Thbs3- and Mtx-expressing rat chondrosarcoma (RCS) and NIH-3T3 cells demonstrated that the Mtx gene is regulated by elements within a short, unidirectional, proximal promoter region (16). The ubiquitous transcriptional activator, Sp1, and the transcriptional repressor, Sp3, bind to clustered GC boxes within the proximal promoter, but their role in regulation of Mtx is uncertain (16). In this study, we demonstrate by transient transfection experiments in RCS and NIH-3T3 cells that the Thbs3 gene is regulated in a cell type-specific manner by a far upstream orientation- and position-independent enhancer element located within intron 6 of the divergently transcribed Mtx gene. This upstream enhancer, when included in a 6-kb promoter sequence, was able to direct specific expression of the E. coli lacZ gene in transgenic mice.
**EXPERIMENTAL PROCEDURES**

Cell Culture and Transient Transfection Assays—RCS cells (20, 21), a gift from Dr J. Kimura, and NIH-3T3 cells (ATCC CRL-1658) were cultured and transiently transfected using the calcium phosphate-DNA precipitation method, as described previously (16). The cells were co-transfected with a β-galactosidase gene, driven by the SV40 promoter and enhancer (Promega), to control for variation in transfection efficiency. 5′-fragments and internal deletion fragments of the Thbs3 promoter (see Figs. 1, 3, and 5) were obtained from genomic clones containing the Mtx gene (22) or the 3′-end of the Gba gene (23) and were subcloned into the pGL2-Basic luciferase vector (Promega). The Thbs3 enhancer was also subcloned, in both orientations, into the previously described −53 and −377 bp Mtx promoter-luciferase reporter gene constructs, either upstream from the Mtx promoter fragment or downstream of the luciferase gene (16). Cells lysates were prepared from transfected cells by the freeze-thaw method (24), and the luciferase activity in extracts was measured using the Luciferase Assay System (Promega), as described by the manufacturer. The β-galactosidase activity in RCS and NIH-3T3 cell extracts was measured using the Galacto-Light Chemiluminescent Reporter Assay (Tropix), or with 0.1-nitrophenyl-β-D-galactopyranoside as a substrate (24).

Northern Blot Analysis—10–20 μg of RNA from RCS or NIH-3T3 cell RNA was fractionated on 1.2% agarose gels, transferred to ZetabindTM Transfer Membranes (CUNO), hybridized with cDNA probes for Thbs3 or β-ac- tin, and washed and stripped according to standard procedures. The Thbs3 probe was a 1-kb Xhol fragment from the 5′-region of the cDNA, while a 1.1-kb mouse β-actin DECA probe, spanning nucleotides 762–1837 (Ambion), was used to control for loading and transfer of RNA.

DNA Sequence Analysis—DNA sequence analysis of constructs was performed. The resulting sequences were analyzed with the Genetics Computer Group (GGC) or GENEPRO (Riverside Scientific, Seattle) programs.

Protein-DNA Binding Assays—Nuclear proteins were isolated from RCS and NIH-3T3 cells using the method of Lee and Green (25), and their concentrations were determined by the Bradford method (26), while a 1.1-kb mouse β-actin DECA probe, spanning nucleotides 762–1837 (Ambion), was used to control for loading and transfer of RNA. The samples were analyzed with an Applied Biosystems PRISM™ 377 DNA sequencer, and the resulting sequences were analyzed with the Genet- ics Computer Group (GGC) or GENEPRO (Riverside Scientific, Seattle) programs.

RESULTS

A Far Upstream, Cell Type-specific Enhancer of the Thbs3 Gene Is Located in Intron 6 of the Melxin Gene—Although the mouse Thbs3 and Mtx genes share a common promoter region, we previously showed that Mtx is regulated by a short, unidi- rectional, proximal promoter region containing clustered Sp1-binding motifs (16). The Mtx-Thbs3 intergenic region, on the other hand, was virtually inactive when assayed in the Thbs3 direction in Thbs3-expressing RCS cells. Northern blot analysis showed that the endogenous Thbs3 gene was expressed at relatively high levels in these cells, while the gene was expressed at much lower levels in NIH-3T3 cells (Fig. 1A). These findings suggest that additional element(s), not contained in the intergenic region, contribute to the expression of the Thbs3 gene.

Some eukaryotic genes encoding extracellular matrix components contain enhancers within their first introns. A 2.2-kb BamHI fragment, extending from the 5′-region of intron A into exon D of the Thbs3 gene (22), was therefore cloned upstream from the −2034 bp Thbs3 promoter-luciferase gene in the negative orientation and assayed in both RCS and NIH-3T3 cells. There was no significant difference in luciferase activity when the activity of this construct was compared with that of the promoter construct lacking the intragenic segment (data not shown), suggesting that sequences within the 5′ portion of the Thbs3 gene do not contribute to its expression in these cells.

Similar results were obtained when intron A of the human THBS3 gene was assayed for enhancer activity in THBS3-expressing small cell carcinoma cells.2 To test whether far upstream sequences might contribute to the expression of the mouse Thbs3 gene, a 6.0-kb Thbs3 promoter and several of its 5′-deletion fragments were cloned upstream from the luciferase reporter gene, and each was co-transfected with the β-galactosidase reporter gene into RCS and NIH-3T3 cells. The 6.0-kb Thbs3 promoter includes the 1.4-kb intergenic region and most of the Mtx gene. As shown in Fig. 1B, the 6.0-kb Thbs3 promoter was 10 times more active than the 2.0-kb promoter in RCS cells, but the two constructs were equally active in NIH 3T3 cells. Estimates of the relative expression of ∼6.0 Thbs-LUC in RCS and NIH-3T3 cells also indicated a 10-fold higher expression in RCS cells (data not shown), a finding consistent with the relative expression of the endogenous Thbs3 gene in the two cell lines (Fig. 1A).

There was a 5-fold decrease in luciferase activity in RCS cells when the activity of the 5.4-kb (SacI) Thbs3 promoter construct was compared with that of the 5.5-kb (PstI) construct. In contrast, similar luciferase activities were obtained for all 5′ deletions of the 6.0-kb construct, up to an RsaI site at −1346 bp, when these constructs were transfected into NIH-3T3 cells (Fig. 1B and Table I). A gradual decrease in luciferase activity was noted when additional 5′-deletion constructs of the −1346 bp Thbs3 promoter were assayed in the latter cells (Table I; Ref. 16). Further analysis of the 6.0-kb Thbs3 promoter demonstrated that additional factors bound within other regions of the promoter, but apparently they play only a minor role in the constitutive regulation of the gene. These findings suggest that a cis-acting element(s), located between −5.4 and −5.5 kb in the 3′-region of intron 6 of the Mtx gene, together with its associated transcription factor(s), serves as a cell-specific enhancer of Thbs3 in RCS cells and that its activity correlates with the cellular expression of the endogenous Thbs3 gene (Fig. 1B). Additional evidence that the Thbs3 enhancer functions in

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2 J. Silver and P. Bornstein, unpublished observations.
FIG. 1. Deletion analysis of the TBS3 promoter in RCS and NIH-3T3 cells. A, Northern blot analyses of RCS and NIH-3T3 cell RNA hybridized with cDNA probes for TBS3 or β-actin. Two different preparations of RNA from each cell type were used. The left and right panels were derived from the same gel and were probed with the indicated cDNA. B, activity of TBS3 promoter-luciferase constructs after transfection into RCS and NIH-3T3 cells. A schematic diagram of the mouse Mtx gene, the TBS3-Mtx intergenic region, and exon A of the TBS3 gene is shown. C, activity of extended TBS3 promoter-luciferase constructs in RCS cells. A schematic diagram of the mouse Gba and Mtx genes, the TBS3-Mtx intergenic region, and exon A of the TBS3 gene is shown. In B and C, the translated sequences and untranslated sequences are represented as filled and open boxes, respectively. Transcription start sites and direction of transcription are indicated with arrows. The percentage of luciferase activity, expressed relative to the 6-kb promoter, S.D. values for each promoter construct, and the number of determinations (n) are indicated. A CeII site, situated immediately upstream from the TBS3 translation start site, served as the 3′ boundary of the TBS3 promoter. B, Apal; S, SmaI; P, PstI; Sa, SacI; N, Nhel; H, HindIII; Sn, SnaBI; C, CelII; K, KpnI; n.d., not determined.

Table I

| Construct          | Luciferase activitya (%) | RCS cells | NIH-3T3 cells |
|--------------------|--------------------------|-----------|---------------|
| −2034 TBS3-LUC     | 9.9 ± 1.7 (7)            | 94.9 ± 18.2 (6) |
| −1346 TBS3-LUC     | 13.1 ± 2.6 (4)           | 118.7 ± 23.0 (6) |
| −1150 TBS3-LUC     | 8.7 ± 1.1 (4)            | 69.7 ± 9.6 (4) |
| −976 TBS3-LUC      | 8.3 ± 0.7 (4)            | 57.2 ± 16.4 (6) |
| −463 TBS3-LUC      | 5.6 ± 1.5 (12)           | 24.9 ± 6.7 (5) |
| −311 TBS3-LUC      | 7.1 ± 1.3 (3)            | 27.7 ± 6.8 (3) |
| LUC                | 1.8 ± 1.3 (6)            | 5.2 ± 1.6 (12) |

a Adjusted to a value of 100% for the 6-kb TBS3 promoter (see Fig. 1B). The number of determinations (n) is shown in parentheses.

b These data are from Collins and Bornstein (16) and are also adjusted to a value of 100% for the 6-kb promoter.

A cell-specific manner was provided by the finding that the −5.7 (−5.1/0.5)TBS3-LUC construct containing the TBS3 enhancer (see Fig. 3) was unable to activate the TBS3 basal promoter when transfected into NIH-3T3 cells (data not shown).

The major polyadenylation sites of the convergently transcribed Mtx and glucocerebrosidase (Gba) genes are only 431 bp apart (17). Because of the close proximity of the TBS3 enhancer to the Gba gene, it was possible that elements within the body of the Gba gene could also participate in regulation of the TBS3 gene. To test this hypothesis, a KpnI site within exon 7 of the Gba gene was used to clone a 9.0-kb TBS3-promoter luciferase construct (Fig. 1C). As shown in Fig. 1C, there was a 35% decrease in luciferase activity when the 9-kb promoter was assayed in RCS cells and compared with the −6.0 kb promoter construct. This finding indicates that no net positive regulatory elements for the TBS3 gene are located within the 3′ two-thirds of the Gba gene.

All of the elements responsible for TBS3 gene expression in NIH-3T3 fibroblasts were located within the Mttx-TBS3 intergenic region (Table I; Ref. 16). Three positive regulatory regions, each of which contributed equally to the luciferase activity in the fibroblasts, were identified. The first region contains the TATA-less 311-bp proximal TBS3 promoter. Since a unique transcription start site for the TBS3 gene has not been determined and several sites may exist, the promoter is numbered from the translation start site. Two INR sequences, 5′-YYANWYY-3′, were identified upstream from the translation start site, generating 5′-untranslated regions of 57 and 96 nucleotides, respectively (Fig. 2B; 30). The lengths of both potential 5′-untranslated regions are within the average length of between 20 and 100 nucleotides for most eukaryotic mRNAs (31). As shown in Fig. 2B, the 311-bp promoter also contains an inverted CCAAT box and two inverted GC boxes. A 513-bp sequence between nucleotides −976 and −463 formed the second potential 5′-untranslated regions of the TBS3 gene, while the third region, extending from −1346 to −1150, contained the 196-bp GC-rich Mtx minimal promoter. These data confirm our initial findings that the Mtx minimal promoter, which is located upstream from −1150, does not play a major role in the regulation of the TBS3 gene.

The Far Upstream TBS3 Enhancer Functions in an Orientation- and Position-independent Manner—To test whether the
enhancer element located in intron 6 of the metaxin gene could function in a position-independent manner, a 344-bp SmaI–SacI fragment, which was expected to contain the enhancer based on the experiments described in Fig. 1B, was cloned upstream of the SnaBI site at −5590 was obtained from accession number L36962 (17). The Mtx gene stop codon and polyadenylation recognition sites are in boldface type and underlined. The 4-bp internal deletion within the SacI site is indicated with an asterisk. The 102-bp DNase I footprint is indicated by square brackets. The sequence has been deposited in the DDBJ/EMBL/GenBank™ Data Libraries under accession number AF059277. B, the sequence of the Thbs3 proximal promoter is the reverse complement of a sequence in accession number U68257 (16). The translation start site of Thbs3 is indicated in boldface type, while the putative INR sequences are underlined. Putative cis-acting elements were identified by searching transcription factor data bases.

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**A**

| Nucleotide Sequence | Function |
|---------------------|----------|
| −648 TCTCTTCG CAGGAGACGG CCCCCCTTTT GGTGCGTCCA ACCATGGGA CAGCAGGAC | ApaI |
| −609 GCTTGTGAT CAGCGCTGAA AAGGCTGATC CTGGGAAACG GAACCCCAAC TC ATCTCTCC | |
| −630 TCTCTTTTC GAGCACCGCC AGGCGGGG CTGGTCCGGA CUGGCCAAGC CGGGCCCG | |
| −597 GATGCAAGA ACCGACCTGA CAGGAGACGA CCCACCAACC GCTCCCAAGC CCCTCAGCAC | |
| −591 ACCGACCTGA TCGCGCGCGG CGCCCTGGGC CTCTCTCCGC GTCGCTCCGG CTCCCGGTTT | |
| −587 GCTCAGGGTA GTGCAGGGGT CTCAAGGAGA GGAGAGAGG GAAGGAGGGG GAGGAGAG | |
| −555 GAGGACACTG CAGAGGACCA ACAGAGGAGA AGAGGAGGAA AGCTGCTCAA GGAGGCGG | |
| −570 AAGTCACTGT TCGGACGTGG CGGCCACAGG CCGGACACTT GGGACATGCC CCCAGGGG | |
| −570 GCCGCGCGCG CGCCGCGCGG CGCCGCGCGG CGCCGCGCGG CGCCGCGCGG CGCCGCGCGG | |
| −561 AAGCAGGACCC GAGCGAGGGA CGTCAAGGAGA GGAGAGAGG GAAGGAGGGG GAGGAGAG | |
| −555 GAGGACACTG CAGAGGACCA ACAGAGGAGA AGAGGAGGAA AGCTGCTCAA GGAGGCGG | |

| Nucleotide Sequence | Function |
|---------------------|----------|
| −540 GAAGGCCGCA AGGAGACCG CAGAGGACCA ACAGAGGAGA AGAGGAGGAA AGCTGCTCAA GGAGGCGG | |
| −530 TGGCAGCGCC AGGAGACCG CAGAGGACCA ACAGAGGAGA AGAGGAGGAA AGCTGCTCAA GGAGGCGG | |

**B**

| Nucleotide Sequence | Function |
|---------------------|----------|
| −860 TACCCCTGCT CTCCTGCTGA CTTCCCTAG A CACTAGTACCA AAGATGTGC CACTGACATC | SnaBI |
| −220 CACCCTCCAC ACCGCGAAGA TACGGCTGCT ACCGCTGATT CACCAGAGGG GCAGAGACAT | |
| −360 GCTCTTTTCCA ACAGCGGCTGA GAGCAGGAC GAACCTGGGT ATCGCTCTGA TAAAGGGAAA | EcoRI |
| −300 GCTCCAGAA ACCAGAGTCG AGCGCTAAGG GAGCGATCTT TACGGAAAAT CATTAGGGG | |
| −240 GACAGCAGAG CAGCGAGGGA ATCTGACGTC CTGGGAAACG GAACCCCAAC TC ATCTCTCC | |
| −180 TACCTCGCTG AAGGATTG C ACCTCGGTTT GTTCACAGCC CCCGGCGTTT CCCGGCGGTC | |

| Nucleotide Sequence | Function |
|---------------------|----------|
| −120 CCCCCGGGCGC CCCGACCGTC CTGCCTGCTG ACCGGCGTAC GCAGAGACG | CCAAT box |
| −60 CTTCTCTCT CTTGCCTCCA ATGCTCTGCC AGAGCTACCG CTCAACCG | |

**C**

| Nucleotide Sequence | Function |
|---------------------|----------|
| −1 AGT | INR |

**D**

| Nucleotide Sequence | Function |
|---------------------|----------|
| −60 CTTCTCTCT CTTGCCTCCA ATGCTCTGCC AGAGCTACCG CTCAACCG | GC Box |

**E**

| Nucleotide Sequence | Function |
|---------------------|----------|
| −1 AGT | INR |

**F**

| Nucleotide Sequence | Function |
|---------------------|----------|
| −60 CTTCTCTCT CTTGCCTCCA ATGCTCTGCC AGAGCTACCG CTCAACCG | GC Box |

To test whether the sequences flanking the SacI site could activate the Thbs3 promoter, several fragments containing these sequences were cloned upstream from the minimal Thbs3 promoter (Fig. 3). As shown in Fig. 3, a 607-bp SnaI–XmnI fragment, containing intron 6, most of exon 7, and the 3′-end of exon 6 of the Mtx gene (−5.7 (−5.1/−0.5) Thbs3 LUC), was able to activate the −463 bp Thbs3 proximal promoter in an orientation-independent manner. Removal of most of exon 7 by deletion of the SnaI/PstI sequence (Fig. 2A) to create −5.5
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**FIG. 3.** Analysis of the Thbs3 far upstream enhancer element. The upper part of the figure shows a schematic diagram of the mouse Thbs3 promoter with the terminal exons of the Gba gene, the Mtx gene, the Thbs3-Mtx intergenic region, and exon A of the Thbs3 gene. Translated and untranslated sequences are represented as filled and open boxes, respectively. Transcription start sites and the direction of transcription are indicated by arrows. Thbs3 promoter-LUC constructs are represented in relation to the above genetic map. Large deletions in constructs are represented in relation to the 3′-boundary of the Thbs3 promoter. The percentage of luciferase activity is expressed relative to the activity of the 6.0-kb construct, and S.D. values for each promoter construct and the number of determinations (n) are indicated. 2.4x (n=31). 2.4x (n=12), 1.7 ± 0.5 (n=6), 60.1 ± 22.2 (n=7), 75.9 ± 12.8 (n=8), 91.2 ± 14.7 (n=6), 23.9 ± 1.4 (n=6), 5.9 ± 1.0 (n=6), 100.0 (n=53). The lower part of the figure shows a table of relative luciferase activity.

(−5.1/−0.5)Thbs3-LUC did not significantly affect the luciferase activity of the construct (Fig. 3). Further analysis of the 413-bp Pet1–Xmn1 I fragment (Fig. 2A) mapped the Thbs3 enhancer to within a 177-bp Sty1–Sty1 I fragment (Fig. 2A), as demonstrated by the activity of a −5.45 (−5.3/−0.5)Thbs3-LUC construct (Fig. 3). There was also a drastic (>80%) decrease in the ability of a 607-bp Smal–Xmn1 I promoter fragment, containing a 4-bp internal deletion within the SacI site, to activate the −463 bp proximal Thbs3 promoter (Fig. 3; −5.7 (−5.1/−0.5)△SacI, 4bp). However, the 4-bp deletion did not totally abolish the activity of the enhancer, since the activity of the latter construct, 13.2 ± 2.5%, was still about twice as great as that of the proximal promoter alone, 5.6 ± 1.5% (p < 0.001; Fig. 3).

**Binding of Nuclear Proteins to the Thbs3 Enhancer in Vitro**—The 177-bp Sty1–Sty1 I enhancer fragment was used as a probe in EMSA. As shown in Fig. 4B (lanes 2 and 6), two major complexes were identified when crude nuclear extracts from either RCS or NIH-3T3 cells were assayed with the Sty1 probe. Since a number of putative Sp1-binding motifs (GC motifs) were identified within the Thbs3 minimal enhancer (Figs. 2A and 4A), we tested the ability of increasing molar excesses of double-stranded GC box consensus and mutated oligonucleotides to compete with the labeled Sty1 probe for complex formation with RCS nuclear extracts. Fig. 4B shows that the slower migrating complex was specifically competed by an oligonucleotide containing a GC box consensus sequence (lane 3) but not by a mutated oligonucleotide (lane 4). This complex was also supershifted when an anti-Sp1 polyclonal antibody was used (lane 5). There was, however, no detectable shift in any of the complexes when anti-Sp2, -Sp3, or -Sp4 was assayed (data not shown), suggesting that these Sp1-related proteins did not bind to these GC-rich sequences. Additional support for the binding of a transcriptional activator, Sp1, to the Thbs3 enhancer was obtained with the use of the purified protein. Sp1 was shown to bind to the Sty1 probe and comigrated with the upper complex generated with extracts from RCS and NIH-3T3 cells (lane 7). Similar results were obtained when extracts of NIH-3T3 cells were assayed in competition and supershift assays (data not shown).

Since a 4-bp internal deletion within the Thbs3 enhancer drastically reduced its activity (Fig. 3), the 177-bp Sty1 fragment, containing the 4-bp deletion within the SacI site (△SacI), was used as a probe in EMSA to determine whether the deletion affected protein-DNA complex formation. Surprisingly, both complexes formed with the △SacI probe when RCS nuclear extracts were used (Fig. 4B, lane 8). As expected, the slower migrating complex was reduced by competition with an oligonucleotide containing the GC box consensus sequence (lane 9) but not by the mutated oligonucleotide (data not shown). Thus, the 4-bp deletion did not appear to abolish or significantly affect the binding of transcription factors to their cognate elements. To test whether the cis-acting elements responsible for the observed complex formation were situated on either side of the SacI site, RCS nuclear extracts were assayed with either a 76-bp Sty1–SacI or a 101-bp Sac1–Sty1 probe (Fig. 4C). The slower migrating Sp1 complex formed with the upstream Sty1–Sac1 probe when nuclear extracts or purified Sp1 was assayed in EMSA and supershift assays (lanes 10–13), while only the faster migrating complex formed with the downstream Sac1–Sty1 probe (lane 14). Although the Sp1 complex was not detected when nuclear extracts were assayed with this probe, trace amounts of purified Sp1 (1 footprinting unit) were able to bind weakly with this probe (lane 15). Only the faster migrating complex formed with the 60-bp Nar1–Nar1 probe (Fig. 4C, lanes 18 and 19), and purified Sp1 was unable to bind to this probe (lane 17). Oligonucleotides corresponding to sequences between the upstream Nar1 site and the SacI site were unable to compete for the faster migrating complex (data not shown).

Taken together, the data presented in Fig. 4, B and C, show that Sp1 binds to an element upstream from the SacI site, within the 54-bp Sty1–Nar1 I fragment, while the faster migrating complex formed with an element downstream from the SacI site, within the 38-bp Sac1–Nar1 I fragment (see Fig. 4A). The ability of a 4-bp deletion in the SacI site to reduce markedly the activity of this composite enhancer element presumably results from the reduction in spacing between the two components of the element. Although the downstream GC box most closely resembles the Sp1 decanucleotide consensus sequence, 5′-KGGGCGGRRY-3′ (32), it was only able to bind very weakly to purified Sp1. The upstream element is less homologous to the published Sp1 consensus motifs and was only identified during searches of data bases using less stringent criteria. A large
102-bp DNase I footprint, showing partial protection and extending from −5441 to −5339 (Fig. 2A), was generated when RCS nuclear extracts were assayed with the 177-bp StyI probe (data not shown). Although it was not possible to define precisely the cis-acting elements within the Thbs3 enhancer from the footprinting data, the footprint nevertheless confirmed the EMSA binding data presented in Fig. 4.

The functional studies in Fig. 1 and the binding data in Fig. 4 clearly suggest that cooperative interactions between Sp1 and the factor(s) responsible for the formation of the faster migrating DNA-protein complex are involved in the regulation of the Thbs3 gene. To test whether the Sp1-binding and the adjacent downstream element(s) were both involved in regulation of the Thbs3 gene, internal deletion mutations of each of these elements, within the context of the 6.0-kb Thbs3 promoter, were generated and assayed (Fig. 5). As shown in Fig. 5, there was a drastic reduction in luciferase activity when either the upstream element, extending from a StyI to a NarI site, or the downstream element, extending from a SacI to the downstream NarI site, was deleted. These findings indicate that both elements in the composite enhancer are required for activity.

The Far Upstream Thbs3 Enhancer Does Not Regulate the Metaxin Gene—Since the far upstream Thbs3 enhancer was mapped to intron 6 of the Mtx gene and is therefore closer to the transcription start site of Mtx than it is to the transcription start site of Thbs3, it was possible that the enhancer also regulated Mtx gene expression. To test this hypothesis, the 607-bp SmaI–XmnI fragment containing the upstream enhancer (Fig. 2A) was cloned into Mtx promoter-luciferase reporter gene constructs and assayed in RCS and NIH-3T3 cells. A 377-bp Mtx proximal promoter sequence was used, since this promoter was previously shown to be maximally active in both cell types (16). When the enhancer fragment was cloned 5′ to the Mtx promoter in either orientation and transfected in either RCS or NIH-3T3 cells, a less than 2-fold increase in luciferase activity was observed (data not shown). Introduction of
the same enhancer fragment in either orientation 3’ to the luciferase gene in the Mtx-luciferase construct and transfection into RCS cells also showed no significant increase in luciferase activity (data not shown). We therefore conclude that the upstream Thbs3 enhancer is specific for the Thbs3 gene and does not function as a locus control region for the Mtx/Thbs3 gene cluster.

The 6-kb, but Not the 2.0-kb, Thbs3 Promoter Is Active in Transgenic Mice—To test whether the −6.0 kb promoter is capable of specific expression of the Thbs3 gene in vivo, −6.0 kb and −2.0 kb Thbs3 promoter-β-galactosidase reporter gene constructs were generated and used to produce transgenic mice. 11 of 78 (14.1%) positive −2.0 kb Thbs3-β-Gal founder mice and 8 of 68 (11.8%) positive −6.0 kb Thbs3-β-Gal founders were identified by slot blot analysis of tail DNA (data not shown). Two of the −2.0 kb and five of the −6.0 kb Thbs3-β-Gal founders were inbred with control siblings to generate transgenic lines. Careful histological analysis of both whole embryos and of multiple tissues from −2.0 kb Thbs3-β-Gal animals of different ages, stained with X-Gal, failed to show any significant staining above the background seen in nontransgenic animals. In particular, the brains of these animals were negative. In contrast, the brains of −6.0 kb Thbs3-β-Gal transgenic mice were consistently positive after X-Gal staining. The appearance of the olfactory bulbs from −2.0 and −6.0 kb Thbs3-β-Gal animals at 5 weeks of age is shown in Fig. 6. A similar distribution of stain was seen in the olfactory bulbs of all three −6.0 kb Thbs3-β-Gal transgenic lines that were examined. In general, the distribution of the stain in other parts of the brain also supported the previous localization of Thbs3 mRNA (10) and protein (12), although there were minor differences among the transgenic lines, and expression of the transgene was age-dependent. Transgenic animals older than 4 months showed little or no expression of the transgene. Since β-galactosidase activity was not detected in the lungs of −6.0 kb Thbs3-β-gal transgenic mice and its presence was uncertain in cartilage and gut because of the high background in these tissues in postnatal animals, it seems likely that elements outside of the −6.0 kb promoter region are responsible for expression of the Thbs3 gene in these tissues. We conclude, on the basis of the transfection analyses shown in Figs. 1 and 3, which localize the enhancing activity of the −6.0 kb promoter to between −5.5 and −5.4 kb, that this far upstream enhancer is also responsible for expression of β-galactosidase in transgenic mice.
A Far Upstream Enhancer of the Thbs3 Gene

The mouse Thbs3, Mtix, and Gba genes have been mapped to chromosome 3E3-F1 in close proximity to each other (15). Although the Thbs3 and Mtix genes are transcribed divergently and share a common 1.4-kb intergenic region, the ubiquitously expressed Mtix gene is regulated by a strong, unidirectional, 377-bp proximal promoter (16). The promoter lacks a CCAAT and a TATA box; contains clustered GC-rich motifs, which bind the ubiquitous transcriptional activator, Sp1, and the Sp1-related transcriptional repressor, Sp3, and has essentially no effect on the expression of the divergently transcribed Thbs3 gene (16). In this study, we demonstrate that the Thbs3 gene is regulated by an orientation- and position-independent far upstream enhancer element, located within intron 6 of the Mtix gene. The upstream enhancer element appears to function in a cell type-specific manner, since it was inactive when transiently transfected into NIH-3T3 fibroblasts. Northern blot analysis showed that these fibroblasts only produced small amounts of Thbs3 mRNA. Furthermore, the enhancer functions in a promoter-specific fashion, since it stimulates the Thbs3 basal promoter from a distance of 5.5 kb but has little or no effect on the more proximal Mtix basal promoter. Two DNA-protein complexes were identified by electrophoretic mobility shift assays when RCS and NIH-3T3 nuclear extracts were assayed with a probe containing the minimal enhancer element. The protein in one of these complexes has been identified as the ubiquitous transcriptional activator, Sp1, by use of competition and supershift assays. The second DNA-protein complex remains to be characterized. The binding of both complexes to their cognate cis-acting elements was important for enhancer activity in RCS cells, suggesting that cooperative interactions between Sp1 and the unknown factor(s) are responsible for activating the Thbs3 gene.

Many cellular and viral genes are activated by Sp1, which recognizes the decanucleotide consensus sequence, 5′-KGGGCGGRRY-3′ (32). Many ubiquitously expressed genes, like metaxin, contain clustered Sp1-binding sites within their proximal promoters and are synergistically activated by Sp1-Sp1 interactions (16, 33, 34). Other genes are regulated by cooperative interactions between Sp1 and a non-Sp1 transcription factor, which binds to an adjacent cis-acting element (35, 36). A highly specific cooperative interaction between NF-κB and Sp1 is required for induction of the HIV-1 long terminal repeat expression (35). A cooperative interaction between Sp1 and CCAAT/enhancer-binding protein β in controlling the expression of the rat CYP2D5 gene has also been reported (37), and cooperation between Sp1 and a sterol regulatory element-binding protein has been implicated in the regulation of the low density lipoprotein receptor gene (36). Since both the Sp1-binding element and the adjacent downstream element are required for Thbs3 enhancer activity in RCS cells, a similar cooperative mechanism may serve to regulate the Thbs3 gene.

Although the Thbs3 enhancer appears to function in a cell type-specific manner, both complexes were nevertheless identified by electrophoretic mobility shift assays when NIH-3T3 nuclear extracts were assayed with a probe containing the minimal enhancer element. Transient transfection experiments demonstrated that the enhancer was inactive in these cells. It is possible that, in vivo, the enhancer complex forms only in cells that actively express the gene. Chen et al. (38) have previously shown, by in vivo DNase I footprinting, that although many cell types contained trans-acting factors that can bind to the mouse proximal Col1A1 and Col1A2 promoters in vitro, these factors bind to their cognate sites only in fibroblasts, which synthesize collagen. Alternatively, there could be a difference between Thbs3-expressing and -nonexpressing cells in the post-translational modification of one or both of these factors. A cell type-specific bridging factor could also be responsible for the difference in activity of the Thbs3 enhancer in different cells.

The Thbs3 gene is expressed predominantly in lung, brain, cartilage, and gastrointestinal tract, with high levels of expression seen in lungs of 4-week-old mice (10, 12, 14). Low levels of Thbs3 expression have also been detected in other tissues (14). Thbs3 gene expression is also tightly regulated in a temporal and spatial manner during murine embryogenesis (10, 12). Iruela-Arispe et al. (10) were only able to detect Thbs3 gene expression by in situ hybridization from day 15 onwards in cartilage and from day 17 onwards in lung and brain during mouse development. In the brain Thbs3, mRNA was detected in the hippocampus and in the diencephalon, and a strong signal was localized to the accessory olfactory bulb. Although Qabar et al. (12) were able to detect TSP3 by immunocytochemistry prior to day 15 of gestation, they also reported that Thbs3 gene expression peaked on days 17–19 of gestation. During late gestation, Thbs3 gene expression was also found in the lung, in various cartilaginous tissues, in intestines, and in various regions of the brain, with high levels in the olfactory bulb (12).

The cell specificity of the far upstream Thbs3 gene enhancer, suggested by its activity in RCS cells and lack of activity in NIH-3T3 cells, was supported by the results of transgenic experiments. β-Galactosidase activity was readily detected in the olfactory bulbs and in other parts of the brain of transgenic mice containing the −6.0 kb Thbs3 promoter-lacZ reporter gene construct but not in the brain or in any other tissue of transgenic mice containing the corresponding −2.0 kb Thbs3 promoter construct. There was, however, variability in the precise location and intensity of β-galactosidase expression in the brain among different 6-kb Thbs3 promoter transgenic lines, a finding that most likely reflects a position effect of insertion of the transgene. These findings confirm the location of an enhancer between −5.5 and −5.4 kb in the 5′-flanking region of Thbs3, as determined by cell transfection analyses of a series of constructs containing 5′-deletions and internal mutations in the Thbs3 promoter. Since there was a high background of β-galactosidase activity in cartilage and intestine, it was difficult to determine whether the −6.0 kb Thbs3 promoter-lacZ reporter gene construct was expressed in these tissues. However, no β-galactosidase activity was detected in the lungs of transgenic mice containing either the 6.0- or 2.0-kb promoter construct. It is possible that regions outside of the −6.0 kb promoter are responsible for the lung-specific expression of the Thbs3 gene. It is also possible that the β-galactosidase reporter gene system is not sensitive enough to detect promoter activity in some TSP3-producing tissues such as lung.

In its sequence, and in its association with the far upstream enhancer described in this paper, the promoter of the Thbs3 gene differs substantially from the two thrombospondin promoters that have been described to date, Thbs1 and Thbs2. The latter two genes also differ from each other in their promoter sequences, in their responses to cytokines and growth factors, and in their spatial and temporal patterns of expression during mouse development (10, 39–41). Structurally, TSP3 resembles TSP4 and TSP5 more than it does either TSP1 or TSP2, and its functions might therefore be allied more closely to those of the former two proteins in the TSP gene family than to those of the latter two (1, 2). Unfortunately, other than the patterns of expression of TSP3, -4, and -5/COMP in tissues (10–13, 42–45), little is known of their function. Recently, mutations in the type III Ca2+-binding motifs in TSP5/COMP have been described in the human skeletal disorders pseudochondrodysplasia and multiple epiphyseal dysplasia (46, 47). Our laboratory has also
recently performed a targeted disruption of the Thbs3 gene in mice, but a limited preliminary examination of the knockout mice has detected no obvious structural or functional defects.\(^3\) The promoter sequences and mode of regulation of Thbs4 and Thbs5 have not been reported. It would be of interest to compare the regulatory control of these three genes as a means of elucidating certain aspects of their function.

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REFERENCES

1. Bornstein, P. and Sage, E. H. (1994) Methods Enzymol. 245, 62–85
2. Adams, J., Tucker, R. P., and Lawler, J. (1995) The Thrombospondin Gene Family, Springer-Verlag, Heidelberg
3. Malashkevich, V. N., Kammerer, R. A., Efimov, V. P., Schulthess, T., and Engel, J. (1996) Science 274, 761–765
4. Lawler, J., McHenry, K., Duquette, M., and Derick, L. (1995) J. Biol. Chem. 270, 2809–2814
5. Qabar, A., Derick, L., Lawler, J., and Dixit, V. (1995) J. Biol. Chem. 270, 12725–12729
6. Bornstein, P. (1995) J. Cell Biol. 130, 503–506
7. Gao, A. G., Lindberg, F. P., Finn, M. B., Blystone, S. D., Brown, E. J., and Frazier, W. A. (1996) J. Biol. Chem. 271, 21–24
8. Mikhailenko, I., Krylov, D., Argraves, K. M., Roberts, D. D., Liau, G., and Strickland, D. K. (1997) J. Biol. Chem. 272, 6784–6791
9. Lawler, J., and Hynes, R. O. (1989) Blood 74, 2022–2027
10. Iruela-Arispe, M. L., Lisaka, D., Sage, E. H., and Bornstein, P. (1993) Dev. Dyn. 197, 40–56
11. Lawler, J., Duquette, M., Whittaker, C. A., Adams, J. C., McHenry, K., and DeSimone, D. W. (1993) J. Biol. Chem. 268, 12192–12196
12. Qabar, A. N., Lin, Z., Wolf, F. W., O'Shea, K. S., Lawler, J., and Dixit, V. M. (1994) J. Biol. Chem. 269, 1262–1269
13. DiCesare, P., Hauser, N., Lehman, D., Pasumarti, S., and Paulsson, M. (1994) FEBS Lett. 354, 217–240
14. Vos, H. L., Devarayalu, S., de Vries, Y., and Bornstein, P. (1992) J. Biol. Chem. 267, 12192–12196
15. Vos, H. L., Mockensturm-Wilson, M., Rood, P. M. L., Maas, A. M. C. E., Dubig, T., Gendler, S. J., and Bornstein, P. (1995) Mamm. Genome 6, 820–822
16. Collins, M., and Bornstein, P. (1996) Nucleic Acids Res. 24, 3661–3669
17. Bornstein, P., McKinney, C. E., LaMarca, M. E., Winfield, S., Shingu, T., Devarayalu, S., Vos, H. L., and Ginns, E. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4547–4551
18. Armstrong, L. C., Komiyi, T., Bergman, B. E., Mihara, K., and Bornstein, P. (1997) J. Biol. Chem. 272, 6510–6518
19. Beutler, E. (1995) Adv. Genet. 32, 17–49
20. Choi, H. U., Meyer, K., and Swann, R. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 877–879
21. Mukhopadhyay, K., Lefebvre, V., Zhou, G., Garrels, S., Kimura, J. H., and de Crombrugghe, B. (1995) J. Biol. Chem. 270, 27711–27719
22. Bornstein, P., Devarayalu, S., Edelhoff, S., and Distech, C. M. (1993) Genomics 15, 607–613
23. Tybulewicz, V. L., Tremblay, M. L., LaMarca, M. E., Willemesen, R., Stubblefield, B. K., Winfield, S., Zablocka, B., Westphal, H., Mulligan, R. C., and Ginns, E. I. (1992) Nature 357, 497–410
24. Rosenthal, N. (1987) Methods Enzymol. 132, 704–720
25. Lee, K. A. W., and Green, M. R. (1990) Methods Enzymol. 181, 20–30
26. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
27. Mercier, E. H., Hoyle, G. W., Kapur, R. P., Brinster, R. L., and Palmiter, R. D. (1991) Neuron 7, 703–716
28. Hogan, B., Constantini, F., and Lacy, E. (1986) Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Laird, P. W., Zijlstra, A., Linders, K., Rudnicki, M. A., Jaenisch, R., and Berns, A. (1991) Nucleic Acids Res. 19, 4293
30. Javahery, R., Khachi, A., Lo, K., Zennie-Gregory, B., and Smale, S. T. (1994) Mol. Cell. Biol. 14, 116–127
31. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
32. Kadenaga, J. T., Jones, K. A., and Tjian, R. (1986) Trends Biochem. Sci. 11, 5–9
33. Courey, A. J., Holtzman, D. A., Jackson, S. P., and Tjian, R. (1989) Cell 59, 827–836
34. Anderson, G. M., and Freytag, S. O. (1991) Mol. Cell. Biol. 11, 1935–1943
35. Perkins, N. D., Edwards, N. L., Maity, S. N., Agranoff, A. B., Schmid, R. M., and Nabel, G. J. (1993) EMBO J. 12, 3551–3558
36. Sanchez, H. B., Yieh, L., and Osborne, T. F. (1995) J. Biol. Chem. 270, 1161–1169
37. Lee, Y., Yano, M., Liu, S., Matsunaga, E., Johnson, P. F., and Gonzalez, F. J. (1994) J. Biol. Chem. 269, 1262–1268
38. Bali, M. A., Lefebvre, V., Zhou, G., Garrels, S., Kimura, J. H., and de Crombrugghe, B. (1995) Methods Enzymol. 237, 245–265
39. Hecht, J. T., Nelson, L. D., Crowder, E., Wang, Y., Elder, F. F. B., Harrison, W. R., Franscomano, C. A., Prange, C. K., Lennon, G. G., Deere, M., and Lawler, J. (1995) Nat. Genet. 10, 325–329
40. Briggs, M. D., Hoffman, S. M. G., King, L. M., Olsen, A. S., Mohnensiefer, H., Leroy, J. G., Mortier, G. R., Rimon, D. L., Lachman, R. S., Gaines, E. C., Cekleniak, J. A., Knowlton, R. G., and Cohn, D. H. (1995) Nat. Genet. 10, 330–336

\(^3\) S. Hormuzi and P. Bornstein, manuscript in preparation.