Multiple and Essential Sp1 Binding Sites in the Promoter for Transforming Growth Factor-β Type I Receptor*

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Maximal gene expression driven by the promoter for the transforming growth factor β type I receptor (TGF-βRI) occurs with a 1.0-kilobase pair fragment immediately upstream of exon 1. This region lacks a typical TATA box but contains CCAAT boxes, multiple Sp1, and PEBP2/CFβα binding sites among other possible cis-acting elements. Alterations within two CCAAT box sequences do not mitigate reporter gene expression driven by the basal promoter, and no nuclear factor binding to consensus elements, are maintained in bone cells at different stages of differentiation. Finally, nuclear factor binding to Sp1 sites, whereas other elements may account for the variations in TGF-βRII levels that parallel changes in bone cell differentiation or activity.

Transforming growth factor-β (TGF-β) receptors occur on most cells, and a functional TGF-β type I receptor (TGF-βRI) is required for all known TGF-β-dependent effects. In some situations its activity is controlled by complex interactions with other cell surface components (1–3). However, in contrast to TGF-βRII and the cell surface proteoglycan also termed TGF-βRIII or betaglycan, expression of TGF-βRI is maintained on differentiated bone cells (4). For these reasons, and because little is known about the molecular control of TGF-βRII expression, we cloned the rat TGF-βRI promoter and characterized several of its functional aspects in cultures of primary and continuous skeletal and nonskeletal cells derived from fetal rats. The rat TGF-βRI promoter lacks a typical TATA box, but initiates transcription at multiple sites within a 220-bp span upstream of the initial methionine codon in differentiated bone cells. The 3′-terminal 300-bp sequence encompassing this region contains a GC-rich CpG island, seven consensus Sp1 binding sites, and two CCAAT boxes. Transfection studies using different fragments of TGF-βRI promoter cloned upstream of the reporter gene luciferase demonstrated maximal activity by a 1.0-kb fragment that encompassed these and other possible cis-acting elements. Importantly, several dispersed elements appeared to cooperate for maximal reporter gene expression in osteoblast-enriched cultures (5). Coincident with this work, the human TGF-βRII promoter was cloned, and its sequence reveals a similar organization with identically spaced CCAAT box motifs (6).

These features suggested that the TGF-βRII gene is driven by a constitutively active promoter that maintains expression of TGF-βRII in many cells. Nevertheless, this promoter is partly unusual to the extent that other promoters organized in a similar way tend to lack CCAAT box sequences. Imposed on this are our previous observations that the proportions of TGF-βRII mRNA and protein may vary with the osteoblast phenotype and that its levels are rapidly controlled by certain stimulatory and inhibitory bone growth regulators (4, 7). Initial TGF-βRI promoter activity studies substantiate that osteoblast-related variations in steady state mRNA levels are controlled at least in part at the level of gene transcription (4, 5). Therefore, the widespread expression of TGF-βRII, driven by a constitutively active promoter, may in some instances be regulated by other cis-acting regulatory elements.

In the present study we investigated in more detail sequences within the TGF-βRII promoter that are required for maximal and basal activity. We examined the importance of two CCAAT boxes and various consensus and putative binding sites for Sp1 transcription factor family members that occur in differentiated bone cells (4). For these reasons, and because little is known about the molecular control of TGF-βRII expression, we cloned the rat TGF-βRII promoter and characterized several of its functional aspects in cultures of primary and continuous skeletal and nonskeletal cells derived from fetal rats. The rat TGF-βRII promoter lacks a typical TATA box, but

EXPERIMENTAL PROCEDURES

Cell Cultures—Using procedures approved by Yale Animal Care and Use Committee, parietal bones from 22-day-old Harlan Sprague Dawley rat fetuses (Charles River Breeding Laboratories) were dissected free of sutures and digested for five 20-min intervals with collagenase. The first digestion releases less differentiated periosteal cells, and the last three digestions are enriched with cells with differentiated osteoblast characteristics. Primary cultures were plated at 5 × 10⁵ cells/cm²

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§ The abbreviations used are: TGF, transforming growth factor; TGF-βRI, transforming growth factor β receptor; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction.

2 D. J. Chang, C. Ji, T. L. McCarthy, and M. Centrella, unpublished results.

3 C. Ji, D. J. Chang, T. L. McCarthy, and M. Centrella, unpublished results.
Active Sp1 Elements in TGF-β Type I Receptor Promoter

Table I

| Designation | 5'-sequence' | Position | Shift | Factor |
|-------------|-------------|----------|-------|--------|
| SA1         | GTCCGGAGGCTGGTTAGAG | -260 to -241 | +     | Sp1    |
| SX1         | CGCTATGGCAGGCCCCAGGCCCCAGGCCGCCGC | -225 to -193 | -     |        |
| AX5         | AGGCCCCGAGGGGCGCTCCGCCTGCCCTAGGCGGCT | -209 to -166 | +     |        |
| AX2         | CCGCCGGGGGCGGCCCCGCCGCCC | -163 to -141 | +     |        |
| AX6         | GGGCCGGGGCCGGGGCACCCACTTCGC | -140 to -111 | -     |        |
| AX3         | CGCCGAGCACTTGCTGCG | -131 to -111 | -     |        |
| XN1         | TCAGCAGTTACAAGGGCGAGGCCCCAGGCGGCGG | -108 to -71 | +     | PEPP2/CBFa |
| AX1N        | TCAGCAGTTACAAGGGCGAGGCCCCAGGCGGCGG | -108 to -71 | +     | Sp1     |
| XSN1.2      | GGGCCGGGGCCGGGGGGGCGGGGGGGGGGGG | -91 to -58  | PEPP2/CBFa |
| XN2         | GCAGGCTGGTTAGAGAGAGAG | -70 to 16   | +     |        |
| XN3         | GAGGCGGCTTGGAGGAGGACCTGAG | -48 to -20  | -     |        |
| SP1         | ATTCGATGGCCGCCCAGGCC | none     | +     | Sp1     |
| PEPP2/CBF   | GCTATACCGACCATCTGCGGCG | none     | +     | PEPP2/CBFa |

Positions of oligonucleotides are by reference to rat TGF-βRI promoter sequence (5). Shift refers to slower migration through polyacrylamide gel; +, presence; -, absence. Factor refers to gel shift reactions identified with transcription factor specific antisera. Nucleotide substitutions in pXN1 that differ from the wild type sequence of region XN1 the rat TGF-βRI promoter are shown in boldface and underlined.

in Dulbecco’s modified Eagle’s medium containing 20 mM HEPES (pH 7.2), 100 μg/ml ascorbic acid, penicillin and streptomycin, and 10% fetal bovine serum. Cultures reach confluence (5–6 x 10^5 cells/cm^2) within 6–7 days. Proliferating cultures were collected at 75% confluence. Every 3–4 days, confluent cultures were re-seeded the same medium except that ascorbic acid and serum were reduced by half. Differentiated cultures were collected 1 week after confluence. Mineralizing cultures were supplemented with 3 mM β-glycerol phosphate and collected 2 weeks after confluence. Mineralized nodules were only observed in Fig. 9, were prepared with pAN0.4 DNA as template. Insert for isolate bone cells were cultured and treated by similar procedures (4).

Plasmids—Construc pE1N.0, pEXH.9, pSN0.8, pAN0.4, pAX0.2, pAX0.2, pSN0.1, and pSN0.1 containing fragments of the rat TGF-βRI promoter cloned upstream of the rat PE24 transcription factor was released with Xho I and inserted into pSN0.8 (5) previously digested with the same restriction enzymes. Plasmid constructs were purified with the Wizard Maxiprep Kit (Promega). DNA of all constructs was verified by sequencing.

Transfections— Cultures at 50–60% confluence were rinsed in serum-free medium and exposed to 1–1.5 μg of plasmid construct per 4.5 cm^2 culture with 0.5% Lipofectin (Life Technologies, Inc.) for 3 h. Cells were re-fed medium supplemented with 5% fetal bovine serum and cultured 10 days to reach confluence. Cultures were rinsed with phosphate-buffered saline and extracted with cell lysis buffer (Promega). Nuclei were cleared by centrifugation at 12,000 x g for 5 min. A commercial kit was used to measure luciferase activity (Promega) in supernatants and corrected for protein by the Bradford method (10).

Nuclear Extracts— Cultures were rinsed with phosphate-buffered saline containing the phosphatase inhibitors sodium orthovanadate (1 mM) and sodium fluoride (10 mM) on ice. Cells were scraped into the buffer and centrifuged by nuclear. Nuclear extracts were prepared by the method of Lee et al. (11, 12) with minor modifications. Briefly, cells were lysed in hypotonic buffer (10 mM HEPES (pH 7.4), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol) supplemented with phosphatase inhibitors, protease inhibitors, phenylmethylsulfonyl fluoride (0.5 mM), pepstatin A (1 μg/ml), leupeptin (2 μg/ml), and aprotinin (2 μg/ml), and 1% Triton X-100. Nuclei were pelleted and resuspended in hypotonic buffer containing 0.42 mM NaCl, 0.2 mM Na2EDTA, 25% glycerol, and the phosphatase and protease inhibitors described above. Soluble proteins released by 30-min incubations on ice were collected by centrifugation at 12,000 x g for 5 min, and the supernatant was aliquoted, and corrected for protein content (10), and stored at -75°C.

Electrophoretic Mobility Shift Assays—Double-stranded oligonucleotide probes were annealed by heating to 95°C and cooling to 25°C in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 5 mM MgCl2 over a period of 1 h. Nuclei were cleared by centrifugation at 12,000 x g for 5 min. A commercial kit was used to measure luciferase activity (Promega) in supernatants and corrected for protein by the Bradford method (10).
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EDTA) by electrophoresis for 2.5 h at 20 °C with 130 V. Gels were dried and analyzed by autoradiography.

Immunoblots—Forty μg of nuclear protein was fractionated by electrophoresis through an 8% denaturing polyacrylamide gel (12). Proteins were blotted to the gels onto Immobilon P membranes (Millipore) by electrophoresis (Ideal Scientific Company, Inc.). Membranes were washed in TBST buffer (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.05% Tween 20), and blocked in 5% defatted milk dissolved in TBST. Blots were incubated with a 1:2000 dilution of anti-Sp1 antibody (Santa Cruz) for 1 h, washed, incubated with a 1:3000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), washed in TBST, incubated with enhanced chemiluminescence (Amersham Corp.) reagents, and exposed to x-ray film.

Statistical Analysis—Data were analyzed in multiple samples after multiple determinations and where appropriate are expressed as means ± S.E. In experiments comparing more than one group, statistical differences were assessed by analysis of variance with limits set by Dunnet. In experiments where a single group was compared, analysis defaulted to Student's t test. Comparisons were performed with a commercial statistical software package (SigmaStat®). Differences among groups were considered significant when p values were <0.05.

RESULTS

Multiple Sp1 Binding Sites within Active Elements of the TGF-βRI Promoter—By sequence analysis, many transcription factor binding sites occur within a CpG island at the 3'-terminal 300-bp region of the rat TGF-βRI promoter. Consistent with other similarly organized promoters, transcription initiation at several sites within a ~200-bp span immediately upstream of exon 1 (5). There are seven consensus Sp1 binding sites, including three GC boxes, and at least nine other potential Sp1 binding sites with 80–90% sequence homology to consensus GC boxes within the 0.9-kb region upstream of the initiator methionine codon at position +22 to +25 (Ref. 5; see Table II). As shown in Fig. 1, deleting various spans that include Sp1 binding sites from the maximally active 1.0-kb region termed EN (flanked by EcoRI and NcoI restriction sites) significantly limited reporter expression. As in earlier studies, pSN0.7, pAN0.4, pXN0.1, and pSN0.1, derived from pEN1.0 by truncation from the 5’ end, caused incremental decreases in reporter gene expression. However, even the short fragments contained in pXN0.1 and pSN0.1 maintained a low but significant level of promoter activity by comparison to the promoter-less pGL3-Basic vector. Deletions from the 3’ end (pEXH0.8, pXH0.2, and pAS0.2) also limited reporter expression. With pXH0.2 and pAS0.2, reporter gene expression was consistently below the activity driven by pGL3-Basic. These findings indicated that sequences in region XN (flanked by XhoI and NcoI restriction sites) in the TGF-βRI promoter are essential for basal promoter function, although other sequences upstream of the XhoI site appear necessary for maximal promoter activity. Many cis-acting Sp1 binding elements occur within regions EN, AN (flanked by ApaI and NcoI restriction sites), and XN of the promoter (see Fig. 1 and Table II). Region AX (flanked by ApaI and XhoI restriction sites) contains four consensus Sp1 binding sites, including one GC box. When the Sp1 binding sites in this region were deleted internally in construct pSX3, promoter activity was severely limited. Therefore, sequences encompassing various Sp1 sites appear to be important components of the TGF-βRI promoter, and several may be required for maximal activity.

Two CCAAT Boxes in the Rat TGF-βRI Promoter Are Inactive in Bone Cells—Because promoter construct pAN0.4 maintains strong activity, we used oligonucleotide probes spanning each of the several clusters of cis-acting elements in this area in

| TABLE II |
| Locations of GC boxes, Sp1 binding sites, and potential Sp1 binding sites in the rat TGF-βRI promoter sequence |
| Positions of oligonucleotides are by reference to rat TGF-βRI promoter sequence; values were b 0.05. Positions of oligonucleotides are by reference to rat TGF-βRI promoter sequence; values were b 0.05. |
| 5’-Sequence-3’ | Position | Homology to GC box/Sp1 sites |
| GGGCGGAGGG | -838 to -848 | 90 |
| GGGCGGAGGG | -767 to -758 | 80 |
| GGGCGGAGGG | -694 to -703 | 100 (GC box) |
| GGGCGG | -631 to -626 | 100 (Sp1 site) |
| GGGCGG | -344 to -353 | 90 |
| GGGCGG | -255 to -246 | 100 (GC box) |
| GGGCGG | -210 to -215 | 100 (Sp1 site) |
| GGGCGG | -200 to -195 | 100 (Sp1 site) |
| GGGCGG | -162 to -157 | 100 (Sp1 site) |
| GGGCGG | -151 to -142 | 100 (GC box) |
| GGGCGG | -142 to -133 | 80 |
| GGGCGG | -93 to -84 | 90 |
| GGGCGG | -71 to -62 | 80 |
| GGGCGG | -63 to -54 | 90 |
| GGGCGG | -58 to -49 | 80 |

Fig. 1. Cooperative effect among Sp1 binding sites in the rat TGF-βRI promoter. A, positions of various restriction endonuclease cleavage sites used to generate fragments of the rat TGF-β promoter. B, putative nuclear factor binding sites identified by sequence analysis. The black arrow at position +1 indicates the most downstream transcription initiation site (5). C, DNA encoding the portions of the TGF-βRI promoter indicated by gray bars was ligated upstream of the reporter gene luciferase in transfection vector pGL3-Basic and co-transfected with a reporter construct encoding β-galactosidase (5) in primary osteoblast-enriched cultures from fetal rat bone. Data are shown as relative luciferase activity (by comparison to pGL3-Basic vector), corrected for protein content. β-Galactosidase activity never varied by more than 6% (S.E.) within an experiment. Data are results from 2 to 14 separate overlapping studies with 6–44 replicate cultures per condition. By analysis of variance, all other constructs are significantly lower in activity than pEN1.0.
Electrophoretic mobility shift assays. As shown in Fig. 2 and Table I, probes SA1, SX1, AX2, AX3, AX5, and AX6 possess several putative Sp1 binding sites. While most growth factor receptor gene promoters so far identified lack TATA and CCAAT boxes, two CCAAT boxes occur in this GC-rich region of the TGF-βRI promoter. One is at −216 to −220 in the forward orientation within SX1, and the other is at −124 to −120 in the forward orientation within AX3. When oligonucleotide probes were 32P-labeled and combined with nuclear extract from primary osteoblast-enriched bone cell cultures, no DNA-protein complexes occurred with probes SX1 or AX3 (Fig. 3A). Probe AX6, encompassing but slightly larger than AX3, was also examined with nuclear extract from these cells, from dental fibroblasts, from undifferentiated periosteal bone cells, and from the highly differentiated osteosarcoma-derived osteoblast-like cell line, ROS17/2.8. Analogous to results with 32P-A3, 32P-AX6 never bound nuclear factor from osteoblast-enriched cultures (Fig. 3B) or any other cell type examined so far (data not shown). PCR primers with substitutions in the CCAAT box regions were used to create mutated reporter plasmid constructs. As shown in Fig. 4, pCATT21 (with alterations in the forward CCAAT box) and pCATT22 (with alterations in the backward CCAAT box) each promoted reporter gene expression equivalent to the parental constructs. Although less overall TGF-βRI promoter activity occurs with undifferentiated bone cells (5), similar results occurred in these cells and the osteoblast-enriched bone cell cultures transfected with pCATT21. Consequently, neither CCAAT box motif binds nuclear factor nor are they essential for basal promoter activity in bone cells.

Functional Sp1 Binding Sites in the TGF-βRI Promoter—Primary osteoblast-enriched cell cultures are derived from normal tissue and appear to be controlled in appropriate physiological ways. Because they express high levels of TGF-βRI mRNA, protein, and promoter activity (4, 5, 7, 9), and protein that associates with Sp1 binding sites, many subsequent studies were performed with extracts from this culture model. Probes SA1, AX2, and AX5 all formed slowly migrating radio-labeled complexes identical to those with 32P-SP1, containing a consensus Sp1 binding site, and were effectively reduced by unlabeled consensus oligonucleotide SP1 (Fig. 3B). With oligonucleotide AX5 as a representative site to characterize Sp1 binding further, a portion of band S1 supershifted to an even more slowly migrating complex with anti-Sp1 antiserum, while the remainder of band S1 and all of band S2 were insensitive to any amount of anti-Sp1 antiserum that we examined (see below). Parallel results occurred with 32P-labeled oligonucleotide probes SA1, AX2, AX5, and SP1 (data not shown). Since unlabeled oligonucleotide SP1 displaced bands S1 and S2 completely, inefficient binding by anti-Sp1 antibody may account in part for this difference. Alternatively, other Sp1-like nuclear proteins might also bind these probes. Two Sp1 family members, Sp2 and Sp3, bind analogous DNA elements with similar affinities (13). Sp3 is comparable to M, to Sp1 and could account for complexes in bands S1 and S2 that are resistant to anti-Sp1 antibody. Addition of 0.1 or 1 μg of anti-Sp1 antibody each supershifted band S1 to a similar extent, and 0.1 or 1.0 μg of anti-Sp3 antibody depleted band S2 and in part band S1. Simultaneous use of both antibodies eliminated nearly all 32P-probe from bands S1 and S2. The more obvious effect at band S1 with both antibodies suggests that loss of Sp3 (band S2) when only anti-Sp3 antibody is used may make more 32P-probe available for binding by Sp1. No gel shift, supershift, or depletion occurred with normal rabbit IgG (Fig. 5). Band S1 therefore appears to contain Sp1 (by supershift) and Sp3 (by antibody depletion), whereas band S2 contains predominantly Sp3. The fractional amount of nucleotide binding to Sp1 and Sp3 was similar with each 32P-labeled probe examined. Partitioning of Sp1 and Sp3 in these ways has been noted previously (14–17) and may relate in part to multiple Sp3 isoforms arising from different initiation codon utilization.4

Binding to Sp1-like sites occurred with oligonucleotide probes XN1 (from −108 to −71) and XN2 (from −70 to −46) of region XN and nuclear extract from osteoblast-enriched cultures (Fig. 6A). 32P-XN2 formed nuclear factor complexes similar to those with probes SA1, AX2, AX5, or SP1, whereas binding to 32P-XN1 occurred in several complexes. As shown in Fig. 6B, after a longer exposure to film, band S1, while minimal, was detected with 32P-XN1, was specifically reduced by excess unlabeled probe S1P (Fig. 6B), and supershifted with anti-Sp1 antibody (data not shown). Band S2 was not detected with 32P-XN1, consistent with the faint binding in band S1 that normally accounts for the majority of nuclear factor binding by Sp1-like sites. Also, unlabeled XN1.2 in which the Sp1 binding site was eliminated did not reduce 32P-XN1 in band S1 (Fig.

4 J. M. Horowitz, Duke University, personal communication.
No nuclear factor complexes formed with \[^{32}\text{P}]\text{XN3}.

The various Sp1 sites in regions SA, AX, and XN were also compared by competitive binding studies where 25- and 50-fold excess unlabelled oligonucleotides inhibited nuclear factor binding to \[^{32}\text{P}]\text{Sp1} to different extents. Consistent with direct binding studies (Fig. 3), oligonucleotides SA1, AX5, and XN2 competed with very high affinity, and SA1 and XN1 did not. Because the same Sp1 sites occur in SX1 and AX5, upstream sequences may restrict Sp1 binding to this region in some instances. Probe AX2, which contains both an Sp1 binding site and a GC box, inhibited Sp1 binding to \[^{32}\text{P}]\text{Sp1} less efficiently than SA1, AX5, and XN2 (Fig. 7). Although AX2 clearly binds Sp1 (Fig. 3), the proximity of the two Sp1 sites or flanking sequences may cause less avid binding to Sp1 than other sequence configurations. Analogous to the low to negligible amounts of Sp1 binding to \[^{32}\text{P}]\text{XN1} and \[^{32}\text{P}]\text{XN3} seen in Fig. 6, oligonucleotide XN1 inhibited \[^{32}\text{P}]\text{Sp1} binding only weakly (Fig. 7), and XN3 had no effect (data not shown).

Oligonucleotide XN2 contains four potential, overlapping Sp1 binding sites (designated as Sp1 binding domains 2 to 5 in Fig. 8) and effectively competed with \[^{32}\text{P}]\text{Sp1}. Because Sp1-related complexes were not competed by and did not form with oligonucleotide SXN1.2 (Fig. 6B, and other data not shown), binding domains 2 and 3 in oligonucleotide XN2 are unlikely to be active, whereas domain 4 may require more 3' sequence. To assess whether domain 4 or 5 or both are functional, probes XN2-\mu, XN2b\mu, and XN2\mu3, derived from XN2 by specific nucleotide substitutions shown in Fig. 8, were examined. Although small decreases in binding occurred with XN2\mu and XN2b\mu, only XN2\mu could not form nuclear factor complex (Fig. 9A), indicating site 4 as the most active site in XN2. Consistent with this, when the nucleotide substitutions found in XN2\mu were introduced into the reporter construct pAN0.4 to create p\text{AN0.4x}2, reporter activity fell to the level of pGL3Basic (Fig. 9B), revealing that this specific Sp1 binding site is essential for basal gene expression from the TGF-\betaRI promoter.

**Fig. 4.** Effects of CCAAT boxes within the rat TGF-\betaRI promoter on reporter gene expression. Wild type sequences contained in constructs pAN0.4 and pCAAT\mu2 and the substitutions introduced into pCAAT\mu1 and pCAAT\mu2 are shown on the left. **Arrows** indicate the positions and orientation of each CCAAT box sequence. Plasmid constructs were inserted upstream of the reporter gene luciferase and transfected into less differentiated periosteal cells and osteoblast-enriched cell cultures from fetal rat bone. Data are shown as relative luciferase activity, corrected for protein content, and are results from three separate studies and nine replicate cultures per condition. By Student's t test, there was no significant difference between constructs with wild type and modified sequences.

**Fig. 5.** Binding of Sp1 and Sp3 to rat TGF-\betaRI promoter DNA. Nuclear protein (5 \(\mu\text{g}\)) from fetal rat osteoblast-enriched cultures was incubated for 1 h on ice with antibody or control solutions and then incubated for 30 min with \[^{32}\text{P}]\text{AX5} plus no addition (−), 0.1 or 1 \(\mu\text{g}\) of anti-Sp1 or anti-Sp3 IgG, or 1 \(\mu\text{g}\) of nonimmune rabbit IgG, as indicated. Oligonucleotide sequences are shown in Table I, and positions are shown in Fig. 2. Protein-DNA complexes were analyzed by 5% native polyacrylamide gel electrophoresis and autoradiography. **S1** and **S2** refer to complexes that are distinguished by anti-Sp1 and anti-Sp3 specific IgGs.

**Fig. 6.** Nuclear factor binding to regions downstream of \(-0.1\) kb in the rat TGF-\betaRI promoter. **A**, nuclear protein (7 \(\mu\text{g}\)) from fetal rat osteoblast-enriched cultures was combined with oligonucleotides \[^{32}\text{P}]\text{oligonucleotides XN1, XN2, or XN3 from the TGF-\betaRI promoter. B**, nuclear protein was combined with \[^{32}\text{P}]\text{XN1} and the following additions: no addition (−); 100-fold molar excess unlabeled oligonucleotides XN1, XN1.2, PEBP2/CBF, or Sp1 as indicated. Oligonucleotide sequences are shown in Table I and positions are shown in Fig. 2. Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. **S1** and **S2** refer to a complexes reactive with anti-Sp1-specific IgG, **C** refers to a complex consistent with binding to PEBP2/CBF\alpha transcription factor(s), and **U** refers to complexes containing presently uncharacterized nuclear protein.
type and differentiation status (1, 4). Using several fetal rat skin- and bone-derived cultures, we found consistent increases in the relative amounts of cell surface TGF-βRI protein, mRNA, and promoter activity in parallel with expression of the osteoblast phenotype (4, 5). These variations could not be accounted for by the amounts of Sp1 or Sp3 in nuclear extracts from these cultures. In contrast to TGF-βRI protein, mRNA and promoter activity profiles, Sp1 and Sp3, were more abundant in fetal rat fibroblasts and osteoblast-like ROS 17/2.8 osteosarcoma-derived cells relative to primary bone-derived cell cultures, by both gel mobility shift and immunoblot analyses (Figs. 3B and 10). Furthermore, the ratio of band S1 (containing Sp1 and Sp3) to band S2 (containing Sp3) was similar in each cell type when the nuclear extracts were assessed by gel mobility shift assay without or with anti-Sp1 antibody (Fig. 10A). Nuclear extracts from proliferating, differentiating, or mineralizing osteoblast-enriched cell cultures (18) also showed analogous ratios between bands S1 and S2 and isofrom distribution patterns with anti-Sp1 and anti-Sp3 antisera, although at later stages of culturing the nuclear factor profiles became more complex (Fig. 11).

**Other Possible Transcription Factor Binding Sites in the TGF-βRI Promoter**—Sequence analysis also revealed other possible cis-acting elements within region AX. As described earlier, upstream oligonucleotides SA1, AX5, and AX2 bound nuclear protein from fetal rat bone cells and dermal fibroblasts essentially in complexes consistent with binding to Sp1 sites. Several possible cis-acting sequences reside downstream of these sites. Of these, a binding site for hepatocyte nuclear factor (HNF)-5 occurs downstream of nucleotide −21 and has not yet been investigated. No nuclear factor complexes formed with oligonucleotide XN3, which spans nucleotides −48 to −20 (Fig. 6A). In addition to the Sp1 binding sites in XN1 and XN2, XN1 contains a potential cis-acting element for transcription factors of the PEBP2/CBFα family (19–21). As shown in Fig. 6B, 32P-XN1 binding within complexes designated as band C was inhibited by unlabeled oligonucleotides XN1 (intact) and SXN1.2 (where the Sp1 binding site in XN1 was eliminated). Binding to band C was also reduced by a probe containing a PEBP2/CBFα consensus sequence (19), but not by μXN1, where the PEBP2/CBFα binding site in XN1 contained three nucleotide substitutions. Complexes designated as band U also formed with 32P-XN1. They were essentially eliminated by unlabeled XN1 but varied slightly in intensity in the presence of the other site specific, truncated, or mutated XN1-derived probes that we tested. Formulation of these complexes may in some instances depend on the presence of other nuclear factors. In other cases they may become more evident when other binding sites found in 32P-XN1 are eliminated and fewer nuclear factors are therefore competing for probe. We have not yet identified the proteins that elicit the U bands.

**DISCUSSION**

The rat TGF-βRI promoter contains a variety of cis-acting elements that could contribute to constitutive or conditional expression. By transfecting reporter gene constructs into osteoblast-enriched cultures, we previously defined regions within the TGF-βRI promoter that are associated with maximal and basal activity. To understand TGF-βRI gene expression in more detail, we have now defined certain important transcription factor binding sites that may control basal promoter activity in many cells.

Similar to various growth factor receptor promoters, the TGF-βRI promoter lacks TATA box sequence but contains a GC-enriched so-called CpG island (22, 23) with many transcription factor Sp1 binding sites. Analogous to the human TGF-βRI promoter (6), CCAAT box-like sequences that occur in this region make them unlike promoters for many other growth factors or growth factor receptors (24–32). Nonetheless, oligonucleotides spanning the CCAAT box sites do not bind detectable levels of nuclear protein, and reporter gene expression was not reduced when these sites were disrupted. Therefore, flanking sequences or the association of other transcription elements in nearby areas may limit the contribution of the CCAAT boxes to TGF-βRI expression under the conditions that we have examined so far.

Unlike genes controlled by CCAAT box and TATA box elements, rat TGF-βRI mRNA transcription initiates from multiple locations, characteristic of a constitutively expressed gene controlled by an Sp1-dependent promoter (5, 33). Although
from osteoblast-enriched cultures was combined with 32P-labeled oligonucleotides XN2, XN21, XN2p2, or XN2q2. Oligonucleotide sequences are shown in Table I, and positions are shown in Fig. 2. Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. B, pGL3-Basic (vector), pAN0.4, or pAN0.42, containing the substitutions found in XN2p2, were assessed for reporter gene expression in fetal rat-derived osteoblast-enriched cultures. Data are shown as relative luciferase activity, corrected for protein content. Data are results from three separate studies with nine replicate cultures per condition. By analysis of variance, only pAN0.4 was significantly different from pGL3 basic.

Deletions that included various Sp1 binding sites invariably limited TGF-βRI promoter activity, some of these elements, clustered within a 0.3-kb sequence at the 3’ end of the promoter, appeared more essential than others. By gel shift and immunodetection assays, we determined that these regions associated to equivalent extents with either Sp1 or with the closely related transcription factor Sp3, also present in the rat cell nuclear extracts that we examined. This result is consistent with the similar structural features, conservation of DNA binding domains, and similar abilities of both Sp1 family members factors to recognize specific cis-acting elements with identical affinities (13–17, 34, 35). Unlike Sp1, Sp3 may reduce Sp1-dependent gene expression (34). We detected two complexes reactive with antibody specific for Sp3 by gel shift analysis consistent with earlier reports (14–16). Furthermore, whereas TGF-βRI and its mRNA levels vary by relation to other TGF-β receptors on various bone- and skin-derived cells, we did not find changes in the amounts of Sp1 or the ratio of Sp1 to Sp3 that could account for those differences in the various cell types, or in osteoblast-enriched cultures at various stages of differentiation. Consequently, in the basal state, constitutively low levels of TGF-βRI expression may be tempered by the presence of both Sp1 and Sp3.

In other situations the proportions of Sp1 and Sp3 may change, or other cellular proteins may modify their function. For example, Sp1 forms heteromeric complexes with several cellular proteins, p107, a member of the retinoblastoma family of proteins, binds Sp1 and represses Sp1-dependent transcription, whereas retinoblastoma itself has been reported to interact with Sp1 and Sp3. Furthermore, p107 and retinoblastoma may complex with E2F, with cyclins, and cyclin-dependent kinases. Sp1 also interacts with the RelA subunit of transcription factor NF-κB, and the cellular protein YY1 (36–40). Therefore, several conditions may arise that could account for variations in Sp1 activity, and its ability to drive TGF-βRI gene expression during the cell cycle, in various cell lineages, or in cell phenotype development.

Overall, our studies support a crucial role for several sequences throughout the TGF-βRI promoter that contain Sp1 binding sites. Deletion of 5′ upstream sequences, reducing the promoter region to 0.7 kb, decreases its activity by 40–60%. Elimination of either another 0.4 kb from the 5′ end or an internal downstream sequence further suppresses promoter function. However, elimination of 0.1 kb of sequence from the 3′ end, a region that itself directs only moderate reporter gene expression, potently suppresses the activity of longer promoter fragments that still retain multiple Sp1 binding sites. Therefore, several regions can contribute to optimal TGF-βRI gene expression, although sequence information within the 0.1-kb 3′ span is essential for basal promoter activity. Using several overlapping oligonucleotides spanning this region, we located a specific sequence where substitution by two nucleotides completely eliminated Sp1 binding and suppressed reporter gene expression from a minimal promoter fragment. These results...
confirm the importance of multiple Sp1 sites throughout the TGF-βRI promoter and establish that one downstream site at position −63 to −54, −90% homologous to consensus Sp1 binding sites, contributes heavily to basal promoter activity. This finding is analogous results with the TGF-α promoter, where several related but nonconsensus Sp1 binding sites are also required for optimal promoter activity (41). It is difficult to compare our results directly with those for the human TGF-βRI promoter. Even the longest construct used to assess the human promoter region reached upstream only as far as 0.7 kb and, most importantly, did not contain the 3′ 109-bp sequence where we detect an essential Sp1 site (6). Thus, at least two regulatory sites, including the important downstream Sp1 binding site (numbered −80 to −71 in the human promoter), have not yet been assessed for their effect on gene expression driven by the human TGF-βRI promoter.

Within the 3′-terminal 0.1-kb region of the TGF-βRI promoter, we also found a related binding site for members of the PEBP2/CBF family. PEBP2/CBF family members (also termed polypoma virus enhancer binding protein 2, or PEBP2α; and acute myelogenous leukemia factors; Refs. 19–21) were previously identified in nuclear extracts from differentiated osteoblasts (4), expression and promoter activity by differentiated osteoblasts (4), and several other even more potent PEBP2/CBF members (also termed polyoma virus enhancer binding protein 43). Our studies with the TGF-βRI promoter have now identified a new target for PEBP2/CBF activity beyond the virus-infected or immunological tissues where their identity was first established (19). The presence of a PEBP2/CBF-related element in this downstream region of the TGF-βRI promoter may account in part for the high level of TGF-βRI mRNA and protein expression and promoter activity by differentiated osteoblasts (4), imposed upon the constitutive levels regulated by Sp1 and other basal elements. We are continuing to characterize this and several other even more potent PEBP2/CBF binding sites further upstream (see Fig. 1) to identify the osteoblast-enriched PEBP2/CBF family members that bind to these sequences and to examine variations in PEBP2/CBF expression during osteoblast differentiation.

In addition to the Sp1 and PEBP2/CBF-related complexes, others designated as band U also form with an oligonucleotide from this important 3′-terminal control region. This sequence contains elements for two other transcriptional regulators, HNF-5 and heat shock protein 70 (Hsp70). By relative migration, the slower migrating band presently seems inconsistent with a complex containing the Mₛ of transcription factor HNF-5. It also seems unlikely to be accounted for by Hsp70 because of the basal growth conditions of our studies and the presence of another possible Hsp70 site in oligonucleotide SX1 that does not exhibit the same complex. However, it may represent a complex containing a basal transcription factor of the TFII family. TFII-related proteins are commonly involved in the expression of many genes transcribed by polymerase II, although the TGF-βRI promoter lacks a TATA box where these agents customarily bind (5, 6). Nevertheless, our studies demonstrate that basal gene expression from the TGF-βRI promoter relies heavily on several Sp1 binding sites. One of these cis-acting elements, which occurs far downstream, appears essential for optimal TGF-βRI promoter activity. However, the effectiveness of these sites may be modified by other negative or positive transcription regulators whose expression may vary with cell phenotype or with other extracellular circumstances.

These and other differences may account for changes in TGF-βRI levels and therefore sensitivity to this important growth regulator during development, differentiation, or hormonal control in skeletal tissue (4, 44).