Cloning the Promoter for Transforming Growth Factor-β
Type III Receptor

BASAL AND CONDITIONAL EXPRESSION IN FETAL RAT OSTEOBLASTS*

(Received for publication, February 1, 1999, and in revised form, August 6, 1999)

Changhua Ji‡, Yun Chen‡, Thomas L. McCarthy, and Michael Centrella§

From the Plastic Surgery Section, Department of Surgery, Yale University School of Medicine,
New Haven, Connecticut 06520

Transforming growth factor-β binds to three high affinity cell surface molecules that directly or indirectly regulate its biological effects. The type III receptor (TRIII) is a proteoglycan that lacks significant intracellular signaling or enzymatic motifs but may facilitate transforming growth factor-β binding to other receptors, stabilize multimeric receptor complexes, or segregate growth factor from activating receptors. Because various agents or events that regulate osteoblast function rapidly modulate TRIII expression, we cloned the 5′ region of the rat TRIII gene to assess possible control elements. DNA fragments from this region directed high reporter gene expression in osteoblasts. Sequencing showed no consensus TATA or CCAAT boxes, whereas several nuclear factors binding sequences within the 3′ region of the promoter co-mapped with multiple transcription initiation sites, DNase I footprints, gel mobility shift analysis, or loss of activity by deletion or mutation. An upstream enhancer was evident 5′ proximal to nucleotide −979, and a silencer region occurred between nucleotides −2014 and −2194. Glucocorticoid sensitivity matched between nucleotides −687 and −253, whereas bone morphogenetic protein sensitivity co-mapped within the silencer region. Thus, the TRIII promoter contains cooperative basal elements and dispersed growth factor- and hormone-sensitive regulatory regions that can control TRIII expression by osteoblasts.

Several cell surface receptors for transforming growth factor-β (TGF-β) are now known. Type I and type II receptors (TRI and TRII) have intracellular kinase domains responsible for heterologous receptor activation or downstream signal transduction (1–4). The type III receptor (TRIII), a membrane-anchored proteoglycan also termed betaglycan, is thought to have a biological function distinct from TRI and TRII (5–7). The rat TRIII gene encodes a 91.6-kDa protein core that is modified by approximately 10 kDa of N-linked glycosyl residues and 150–200 kDa of heparan and chondroitin sulfate side chains.

TRIII has a relatively short, 43-amino acid cytoplasmic domain that lacks commonly recognized protein docking or kinase like motifs but is enriched with serines and threonines to approximately 42% (5, 6).

TRIII is prevalent on many fetal cells, where it can be the most abundant TGF-β binding site. All TGF-β isoforms bind to TRIII with comparably high affinity, although this is about 3–5-fold less than that for TRI and TRII (7). Certain cells lack TRIII but maintain TGF-β sensitivity. Nonetheless, TRIII may attract and enhance TGF-β binding to TRII and form a more stable ligand-receptor complex (6). On certain cell types this appears more pronounced or limited to the TGF-β2 isoform (8, 9). Moreover, disproportionate levels of TRIII may sequester and possibly limit its binding to signaling receptor complexes (10–12).

The relative amount of TRIII is thought to vary with development, with differentiation, or in a tissue-specific manner. For example, TRIII levels are prevalent on less differentiated bone, endothelial, adrenocortical, prostate, and muscle cells but are rapidly regulated by agents or events that control cell differentiation. On bone cells, TRIII levels decrease when differentiation is enhanced by bone morphogenetic protein (BMP)-2 but rise in response to glucocorticoid or agents that increase intracellular cAMP (10, 12, 13). TRIII levels also decrease with endochondral cell differentiation in three-dimensional culture and during the transition from myoblasts to myotubes. In the ventral prostate, TRIII levels rise after castration and are resuppressed by androgen administration (14–16). Analogous to the effect of parathyroid hormone (PTH) on osteoblasts (10), corticotropin produces a cAMP-dependent increase in TRIII on adrenal cells (17).

Based on these findings, we predicted that complex changes in TRIII are regulated in part by constitutive, developmental, and hormone-dependent genomic elements and in this way control how the effects of TGF-β are perceived within various tissues. To define how these events might occur at the molecular level, we cloned the promoter for rat TRIII. Because we previously defined situations and agents that regulate TRIII levels on bone cells, we have also begun to assess regions within the promoter that may account for constitutive and hormone-dependent changes in its expression by osteoblasts.

EXPERIMENTAL PROCEDURES

Rat Genomic DNA Library Screening—A rat liver genomic DNA library was partially digested with Sau3A1 and cloned into the BamHI site of the EMBL3 Sp6/T7 phage vector (CLONTECH). Approximately 6 × 10⁶ plaques were transferred onto nitrocellulose membranes and screened by hybridization with a 0.4-kb rat TRIII cDNA probe containing 334 bp of 5′-untranslated region and 61 bp of coding sequence (5, 6). The probe was labeled with [α-³²P]dCTP with a random primer labeling kit (New England Biolabs). Hybridization was for 20 h at 42 °C in 50% formamide, 5× Denhardt’s (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% SDS, 5× SSPE (0.9 M NaCl, 5 mM
EDTA, 50 mM sodium phosphate (pH 8.3), and 100 μg/ml denatured salmon sperm DNA. Positive clones were screened twice, recombinant bacteriophage were plaque purified, and phage DNA was isolated by cell lysis (18).

**DNA Sequencing**—Phage inserts were cloned into pBlueScript-KSII vector (Stratagene, La Jolla, CA) and mapped with an assortment of restriction endonucleases, and subclones were produced by restriction site cleavage (see Fig. 1 and Table I). Double-stranded plasmid DNA was denatured with 0.2 N sodium hydroxide and sequenced by the dideoxy chain termination method (19) with a T7 Sequenase sequencing kit (U. S. Biochemical Corp.) and specific synthesized oligonucleotide primers. DNA sequence analysis was performed with the T7 RNA polymerase. Probes were labeled with [γ-32P]UTP using the MaxiScrip kit (Ambion Corp.). 10 μg of total cell RNA and 1 × 10^6 cpm of probe RNA were combined in 30 μl of hybridization buffer (80% formamide, 1 × MDTA, 100 mM sodium citrate, 300 mM sodium acetate, pH 6.4) for 16 h at 45 °C. The samples were then digested at 37 °C for 30 min by adding 300 μl of a solution containing 5 μl EDTA, 308 μM NaCl, 10 μM Tris-Cl (pH 7.5), 1 unit/ml RNase A, and 40 units/ml RNase T1. RNase was inactivated with 17 μl of 10% SDS and 3 μl of proteinase K at 20 mg/ml. Protected transcript fragments were precipitated with isopropanol and resolved on a denaturing 6% polyacrylamide gel alongside sequencing ladders. Bound or protected RNA probes were visualized by autoradiography. RNA standards for Northern analysis were stained with ethidium (12, 25).

**Nuclear Protein Extracts**—Cells were rinsed twice with phosphate-buffered saline at 4 °C, harvested by scraping, gently pelleted, washed, and lysed in hypotonic buffer containing 10 mM HEPES (pH 7.4), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, phosphate inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride), protease inhibitors (0.5 mM phenylmethyl sulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin; Sigma), and 1% Triton X-100. Nuclei were pelleted at 3,500 × g for 5 min, and cytoplasmic supernatants were collected. Nuclei were resuspended in hypertonic buffer containing 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, and the phosphate and protease inhibitors indicated above. Soluble nuclear proteins were released by 60 min of incubation at 4 °C and separated from insoluble material by centrifugation at 12,000 × g for 5 min, and aliquots were stored at 75 °C (24, 25).

**DNase I Footprinting**—The TRIII promoter DNA fragment corresponding to nucleotides −641 to −350 was generated by PCR from rat osteoblast nuclei (see below) and cloned as an HpaI fragment into pGEM3 vector. This fragment was used as a probe in Northern analysis. 

**Transfections**—Promoter/reporter plasmids were co-transfected with a vector carrying the β-galactosidase gene under control of the SV40 promoter using LipofectAMINE (Life Technologies, Inc.). Briefly, cultures at 50–75% confluent density were rinsed and exposed to plasmids in serum free medium, and the solutions were then replaced with fresh medium supplemented with 10% fetal bovine serum. Cultures were expanded for 48 h, rinsed, and treated as indicated in the figure legends. After treatment, cultures were rinsed with phosphate-buffered saline and lysed in 100 μl of a solution containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100. lysates were collected, nuclei were cleared by centrifugation at 15,000 × g for 5 min, and supernatants were analyzed for reporter gene activity and corrected for protein content (24).
TGF-β Receptor III: Basal and Conditional Promoter Elements

and [α-32P]dATP with the Klenow fragment of DNA polymerase I. 5–10 μg of nuclear extract protein was preincubated for 20 min on ice with 2 μg of poly(dI·dC), without or with unlabeled specific or nonspecific competitor DNA, in 60 mM KCl, 25 mM HEPES (pH 7.6), 7.5% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.025% bovine serum albumin. After adding 0.1–0.2 ng of DNA probe (5 × 10^6 cpm) for 30 min on ice, samples were fractionated by electrophoresis on a 5% nondenaturing polyacrylamide gel that was prerun for 30 min at 12.5 V/cm at 25 °C in 45 mM Tris, 45 mM boric acid, 1 mM EDTA. To assess nuclear factors by antibody reactivity, nuclear extract was incubated with 0.2–1.0 μl of antisera for 30 min at 4 °C before adding [32P]-labeled probe. Electrophoresis was performed for 2.5 h under identical conditions. Radioactive DNA bond protein complexes were visualized by autoradiography.

Radioligand Binding—TGF-β1 was radioiodinated with chloramine T to specific activity of 4500 Ci/mmol and isolated by gel filtration in 0.1 N acetic acid, and neutralized with NaOH. [3H]Proline incorporation into collagen and noncollagen protein was measured by differential autoradiography and densitometry, as described previously (12).

Protein Synthesis—Cells were transfected to overexpress native rat TRIII using DNA subcloned from plasmid pBG7 (a gift from Dr. T. Massague, Memorial Sloan-Kettering Cancer Center, New York; Ref. 6) into a rat genomic library. Of these, clone clone 22 contained a 15-kb fragment encoding AP-2 were generously provided by Dr. Trevor Williams (Yale University). Antiserum to AP-4 was generously provided by Dr. J. V. Wilkinson (University of Chicago). These antisera were incubated with [3H]Proline and analyzed by gel filtration in 60 mM KCl, 25 mM HEPES (pH 7.6), 7.5% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.025% bovine serum albumin. [3H]Proline incorporation into collagen and noncollagen protein was measured by differential autoradiography and densitometry, as described previously (12).

Reagents—Transfection vectors pGL2-Basic and pGL2-Control were obtained from Promega Corp. (Madison, WI). Horseradish peroxidase (cortisol) was obtained from Promega Corp. (Madison, WI). Hydrocortisone (cortisol) was obtained from Caltag Laboratories (Burlington, CA). All other chemicals were obtained from Sigma.

RESULTS

Isolation of Rat TRIII Receptor Genomic Clones—Using a 0.4-kb rat TRIII probe containing 334 bp of 5′ untranslated region and 61 bp of coding sequence, four positive clones were selected out of approximately 6 × 10^5 recombinant phages from a rat genomic library. Of these, clone X22 contained a 15-kb insert that produced only two fragments of 5 and 10 kb by digestion with NotI. Both fragments were subcloned into pBluescript and analyzed by restriction enzyme cleavage and sequencing. The 5-kb DNA fragment, which we designate as pTR3Bs/5XN, was located directly 5′ to coding sequence of the rat TRIII gene.

Sequence Analysis—All 5136 nucleotides of pTR3Bs/5XN were sequenced. The DNA sequence corresponding to nucleotides 2130–692 is shown in Fig. 1. Nucleotides 335 to −179 correspond to the 5′-untranslated region of a previously reported rat cDNA clone, which was originally numbered with reference to the first nucleotide of the initial ATG codon (5). The sequence was analyzed by the Wisconsin University Genetics Computer Group program and MatInspector data bank searches (25, 29) to assess restriction enzyme cleavage sites and nuclear factor-binding elements. Analogous to the TRI and TRII gene promoters (25, 30), this sequence lacks a TATA box.

Two nuclear factor Sp1-binding sequences (31), at nucleotides −31 to −424 and nucleotides −527 to −518 (a GC box), are located within 0.7 kb upstream of the initial ATG codon. Another GC box is located further upstream (−1880 to −1871). The 0.5-kb region between nucleotides −179 to −692 is highly GC enriched to 69%, consistent with CpG islands often associated with the initiation of gene transcription (32, 33). Three sequences consistent with binding sites for nuclear factor CCAAT/enhancer-binding protein (C/EBP, 34) occur at nucleotides −2073 to −2065, −1523 to −1515, and −574 to −565. In addition, the data bank searches suggested a variety of other elements that might be responsible for conditional, hormone-dependent, or tissue-specific TRIII gene expression. These sequences can be located and evaluated through GenBank™ accession number AF117811.
cis-acting SP1 Elements in the Basal TRIII Promoter—The basal TRIII promoter region defined by nested DNA fragment analysis was then examined by DNaI footprinting with nuclear extract from osteoblast-enriched cell cultures. As shown in Fig. 4, two major protected regions termed FP1 and FP2 correspond to the GC box at nucleotides −527 to −518 and to the Sp1-binding site at nucleotides −431 to −424. Consistent with this, oligonucleotide probes spanning each of the FP1 and FP2 sites (Table II) formed nuclear protein-DNA complexes that were identical to those obtained with a consensus Sp1 oligonucleotide probe (Fig. 5). Oligonucleotides with mutations within either the GC box or the Sp1-binding sequences failed to compete for nuclear factor binding to radiolabeled consensus Sp1 probe or by themselves to form nuclear protein/DNA complexes. Furthermore, antibody preparations specific for Sp1 or Sp3 depleted or supershifted complex formation. The lower molecular mass Sp3-DNA complex is thought to represent a processed, less abundant form of Sp3 (35). However, mutations introduced at either Sp1-binding site within TRIII promoter/reporter transfection constructs (Fig. 6) only partially decreased reporter gene expression. Importantly, mutation of the GC box in pTR3/0.3P did not reduce promoter activity to the lower level of the truncated promoter construct pTR3/0.2B, in which the 94 bp upstream of pTR3/0.3P, which contain the GC box, were removed.

cis-acting AP-2 Elements in the Basal TRIII Promoter—To analyze the basal promoter region further, gel shift analysis with three overlapping oligonucleotide probes (GS1, GS2, and GS3) that spanned the area between the GC box and the Sp1-binding site described above showed distinct nuclear factor-binding patterns. Nuclear factor binding was strongest with probes GS2 (nucleotides −485 to −455) and GS3 (nucleotides −464 to −434) (Fig. 7, left panel). The several nuclear factor/DNA complexes formed by these probes may correspond to less distinct footprints observed between FP1 and FP2 (Fig. 4). The Genetics Computer Group and MatInspector sequence analyses of the region between FP1 and FP2 suggested possible binding sites for nuclear factors Sp1, AP-2, and several E-box and zinc finger DNA-binding proteins (Table II and Refs. 34–37). No discernible Sp1-like complexes formed with GS1 (nucleotides −511 to −480), GS2, or GS3, by comparison with a consensus Sp1 probe (Fig. 7, left panel), by competition with radiolabeled consensus Sp1 oligonucleotide, or by reactivity with anti-Sp1 or anti-Sp3 antibody (data not shown). In contrast, GS2 and GS3 each competed with a probe containing consensus AP-2-binding sequence (Fig. 7, middle panel). By sequence analysis, GS3 contains consensus nuclear factor AP-2 and AP-4-binding sites (36). Complex formation was resistant to antibody to AP-4 (data not shown), whereas complexes consistent with AP-2 were readily evident with GS-3. Studies with specific anti-AP-2 antisera (37) showed that AP-2α and AP-2γ, but not AP-2β, were present in extract from osteoblast-en-
moterless transfection vector pGL2-Basic, as shown in Table I. Uosteoblasts.

C9–12 replicate samples per condition. 37 and 38 and Fig. 8, cocorticoid (13), consistent with changes in TRIII mRNA (Refs. 37, 38, and Fig. 8, 2194 and 2194, 2013, and 2014) were cloned into promoterless transfection vector pGL2-Basic, as shown in Table I. U, StuI; C, SacI; N, NcoI; M, MscI; K, KpnI; S, SalI; E, EagI; B, BssHII; A, ApaI. Constructs were co-transfected with pSV-bgalactosidase into primary osteoblast-enriched cell cultures with LipofectAMINE. Luciferase reporter gene activity was measured after 48 h and corrected for protein content and relative β-galactosidase expression. Bars represent means ± S.E. of data from 3–4 independent overlapping studies and 9–12 replicate samples per condition.

![Diagram of promoter/reporter constructs](Image)

**Fig. 3. Expression of rat TRIII promoter activity in fetal rat osteoblasts.** The XhoI and NcoI flanking sites in the genomic clone λ22 are indicated on the upper bar on the left. DNA fragments from the 5’ portion of the rat TRIII gene, shown below λ22, were cloned into promoterless transfection vector pGL2-Basic, as shown in Table I. U, StuI; C, SacI; N, NcoI; M, MscI; K, KpnI; S, SalI; E, EagI; B, BssHII; A, ApaI. Constructs were co-transfected with pSV-β-galactosidase into primary osteoblast-enriched cell cultures with LipofectAMINE. Luciferase reporter gene activity was measured after 48 h and corrected for protein content and relative β-galactosidase expression. Bars represent means ± S.E. of data from 3–4 independent overlapping studies and 9–12 replicate samples per condition.

![Diagram of DNase I footprinting assay](Image)

**Fig. 4. DNase I footprinting assay of the rat TRIII gene promoter.** Nuclear extract obtained from primary osteoblast-enriched cultures was hybridized with a 3’ 32P-end labeled DNA probe encompassing nucleotides −664 to −350 of the rat TRIII gene promoter. Undigested DNA fragments were analyzed by electrophoresis on a sequencing gel and visualized by autoradiography. Lanes 1–4 show results with 40, 80, 160, and 0 μg of nuclear extract, as indicated. The sequence of the two major protected regions, FP1 and FP2, are shown on the right, and the Sp1-binding sequences that they encompass are shown by the vertical bars.

**Table I**

| Name          | Size (bp) | Flanking sites | Flanking nucleotides | Characteristic |
|---------------|-----------|----------------|----------------------|---------------|
| pTR3/3.7      | 3.7       | KpnI/SacI      | −4045/−253           | Full length   |
| pTR3/3.19     | 1.9       | SacI/SacI      | −2194/−253           | 5’ truncation |
| pTR3/1.98     | 0.9       | SacI/NcoI/SacI | −2194/−2062; −979/−253 | Internal deletion |
| pTR3/1.8      | 1.8       | MscI/SacI      | −2013/−253           | 5’ truncation |
| pTR3/0.4K     | 0.4       | Knl/SacI       | −687/−253            | 5’ truncation |
| pTR3/0.4S     | 0.4       | SmaI/SacI      | −641/−253            | 5’ truncation |
| pTR3/0.3P     | 0.3       | PCR primer/SacI| −534/−253            | 5’ truncation |
| pTR3/0.2B     | 0.2       | BstHIII/SacI   | −440/−253            | 5’ truncation |
| pTR3/0.2E     | 0.2       | EcoRI/SacI     | −411/−253            | 5’ truncation |
| pTR3/0.1A     | 0.1       | ApaI/SacI      | −35μ/−253            | 5’ truncation |
| pTR3/1.9R     | 1.9       | SacI/SacI      | −253/−2194           | Reversed      |
| pTR3/0.3Pμ    | 0.3       | PCR primer/SacI| −534/−253            | GC box mutation |
| pTR20.2Bμ     | 0.2       | PCR primer/SacI| −440/−253            | Sp1 site mutation |

* SacI, SacI, NcoI, and NcoI refer to the 5’ and 3’ SacI and NcoI restriction enzyme cleavage sites diagrammed in Fig. 1.

Regulation of TRIII Promoter Activity in Osteoblasts—On fetal rat osteoblasts, TGF-β binding to TRIII is rapidly suppressed by treatment with BMP-2 (12) and increased by glucocorticoid (13), consistent with changes in TRIII mRNA (Refs. 37 and 38 and Fig. 8, left panel). To assess whether these differences occur at least in part through variations in TRIII gene promoter function, we examined effects by BMP-2 and cortisol on four transfection reporter constructs (pTR3/3.7, pTR3/1.9, pTR3/1.8, and pTR3/0.4K) that span active upstream and downstream regions of the TRIII promoter. Treatment with cortisol enhanced reporter gene expression by all four constructs, predicting a glucocorticoid-dependent regulatory element located at minimum between nucleotides −687 and −253. Also consistent with its effect on TRIII mRNA and protein in these cells, BMP-2 suppressed TRIII promoter activity. However, this effect was only evident with pTR3/1.9 and TR3/3.7, predicting that it augments the effect of the putative si-lencer region noted between nucleotide −2194 and −2014 (Fig. 8, right panel). In contrast, neither glucocorticoid nor BMP-2 significantly altered TRIII promoter activity in fetal rat fibroblasts transfected with TR3/3.7 or with TR3/1.8, which are enhanced by glucocorticoid and/or suppressed by BMP-2 in transfected osteoblasts. Although these findings reflect the similarly limited effects by glucocorticoid and BMP-2 on radio-labeled TGF-β binding to TRIII on fibroblasts (Fig. 9), chronic exposure to either of these factors may perhaps cause more obvious differences in TRIII expression by these cells.

Overexpression of TRIII Suppresses the Stimulatory Effect of TGF-β on Osteoblast Protein Synthesis—Our earlier studies suggest that hormone- and growth factor-dependent changes in TRIII occur in parallel with variations in the sensitivity of bone cells to TGF-β. In particular, a relatively higher level of TRIII correlates well with a reduced response to treatment with
TABLE II
Oligonucleotide probes used in electrophoretic mobility shift assay

| Oligonucleotide probes | Sequence | Possible sites |
|-----------------------|----------|---------------|
| G                     | AAGGAGAAGAAGAGAGCCAGAGGAGGA | GC box (Sp1) |
| G<sub>µ</sub>          | AAGGAGGAGTGGCAGAGGAGGA | GC box mutation |
| S                     | TCCCCGCCGCACCGCCCGGGCGTTGCGC | Sp1 |
| S<sub>µ</sub>          | TGGGAGGCGGTGCGCGCCGCGGGCGT | Sp1 mutation |
| GS1                   | TGGGAGGCGGTGCGCGCCGCGGGCGT | Sp1/AP-2 |
| GS2                   | TGGGAGGCGGTGCGCGCCGCGGGCGT | Sp1/AP-2 |
| GS3                   | TGGGAGGCGGTGCGCGCCGCGGGCGT | Sp1/AP-2 |

<sup>a</sup> Regions containing possible nuclear factor binding sites were determined by MatInspector, version 2.2 (32), and are underlined.

FIG. 5. Gel mobility shift assays of the GC box and Sp1-binding sequences in the rat TRIII gene promoter. 32P-Labeled oligonucleotide probes described in Table II were incubated with nuclear extracts from primary osteoblast-enriched cultures without or with a 100-fold molar excess of unlabeled oligonucleotides (left panel) or anti-Sp1 or anti-Sp3 antibody preparations (right panel) as indicated. Protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gel and visualized by autoradiography. No reaction occurred with normal rabbit serum (not shown).

FIG. 6. Effects of mutations in the GC box and Sp1-binding sequences on rat TRIII gene promoter activity. Plasmids pTR3/0.3P, pTR3/0.3P<sub>µ</sub>, pTR2/0.2B, and pTR2/0.2B<sub>µ</sub> containing the rat TRIII gene promoter fragment inserts with native and mutated GC box and Sp1-binding sequences described in Table I are shown on the left. Constructs were transfected for 48 h into primary osteoblast-enriched cultures, and reporter gene expression was assessed as described in the legend to Fig. 3. Luciferase activity was corrected for protein content and relative β-galactosidase expression. Data represent means ± S.E. from 3 independent overlapping studies and 9 replicate samples per condition. The numbers in parentheses are results expressed as a percentage of control.

TGF-β1 (10, 12, 13, 40). To address this in a way that would limit the contribution of variations in TRI, TRII, downstream signaling components, or nuclear effectors of osteoblast activity (13, 39), osteoblasts were transiently transfected to overexpress rat TRIII. By comparison to vector transfected osteoblasts, radioligand binding to TRIII increased 2.8-fold in cells transfected with the TRIII expression construct. Consistent with situations where higher levels of TRIII are expressed, such as on less differentiated bone cells or on osteoblasts treated with glucocorticoid, PTH, or its related protein, PTH-related protein (10, 12, 13, 40, 41), the stimulatory effect of TGF-β on collagen and noncollagen protein synthesis was significantly lower, reduced by 43 and 32% relative to control (Fig. 10).

DISCUSSION
On many cells, changes in the TR profile can significantly alter TGF-β activity (7). However, mechanisms that regulate TR expression or stability are still poorly understood. Our earlier studies in osteoblasts showed that TR levels are controlled by transcriptional and post-transcriptional events. In isolated bone cells, TR mRNAs and proteins exhibit relatively short half-lives, offering the opportunity for rapid changes in TGF-β sensitivity (40). Moreover, osteotropic factors like BMP-2, glucocorticoid, PTH, and PTH-related protein specifically alter the TR profile and modify the effects of TGF-β on osteoblasts (10, 12, 13, 38, 39, 41). To understand these events at the molecular level, we first isolated and cloned the rat TRII gene promoter and characterized several cis- and trans-acting elements that control constitutive and hormone-dependent TRIII expression (24, 25, 39, 42). TRIII is often the most abundant TR and can help to define TGF-β isoform activity or its biological effects (6–17). In the current study, we therefore cloned the rat...
FIG. 8. Regulation of rat TRIII gene promoter activity by glucocorticoid and BMP-2. Left panel, osteoblast-enriched cultures were treated for 24 h with 10 nM cortisol or 1 nM BMP-2 and extracted, and total RNA was assessed by Northern blot analysis with a rat-specific cDNA probe (4). Numbers to the left refer to an ethidium-stained sizing ladder from a parallel gel lane. Ethidium stained rRNA profiles are shown below, with 28 and 18 S rRNA bands indicated. Right panel, the relative sizes of plasmids pTR3/3.7, pTR3/1.9, pTR3/1.8, and pTR3/0.4K with the rat TRIII gene promoter fragment inserts described in Table I are shown to the left. Constructs were transfected for 24 h into osteoblast-enriched cultures. Cells were then treated for 24 h with vehicle, 10 nM cortisol, or 1 nM BMP-2 in serum-free medium, and reporter gene expression was assessed as described in the legend to Fig. 3. Promoter regions with potential cortisol or BMP-2-responsive sequences are shown as dark segments overlying the rectangles representing promoter fragments. Luciferase activity was corrected for protein content and relative β-galactosidase expression. Data bars represent means ± S.E. from 4–6 independent overlapping studies and 12–30 replicate samples per condition. The numbers in parentheses are results expressed as a percentage of control, set as 1 in untreated cells transfected with each promoter/reporter construct. Their individual control activities differed from each other precisely as shown in Fig. 3.

FIG. 9. Effects by glucocorticoid and BMP-2 on TRIII expression by fetal rat fibroblasts. Left panel, fetal rat fibroblasts were transfected for 24 h with plasmid constructs pGGL2-Basic, pGGL2-Control, pTR3/1.8, or pTR3/3.7, treated for 24 h with vehicle, 10 nM cortisol, or 1 nM BMP-2 in serum-free medium, and reporter gene expression was assayed as described in the legend to Fig. 8. Data bars represent means ± S.E. from 2 independent studies and 6 replicate samples per condition. pGGL2-Control enhanced reporter gene activity by 479 ± 48-fold, pTR3/1.8 enhanced reporter gene expression by 120 ± 7-fold, and pTR3/3.7 enhanced reporter gene expression by 68 ± 6-fold, relative to pGGL2-Basic. By analysis of variance, no significant effects were induced by cortisol or BMP-2 on TRIII reporter gene expression. Right panel, fetal rat fibroblasts were treated for 24 h with vehicle, 10 nM cortisol, or 1 nM BMP-2 in serum-free medium. Cultures were labeled with 125I-labeled TGF-β1 and extracted, and TR profiles were assessed by polyacrylamide gel electrophoresis and autoradiography, as described (12, 40).

TRIII gene promoter and have begun to define elements that can account for basal and conditional TRIII expression by bone cells.

Sequence analysis showed that DNA within 5.0 kb upstream of the coding region of the rat TRIII gene lacks TATA and CCAAT boxes. However, it contains two nuclear factor Sp1-binding sites in the highly GC-enriched 3′ basal promoter region, comprising a so-called CpG island. Multiple transcription initiation sites occur within the basal TRIII promoter, a situation often associated with genes lacking TATA and CCAAT box elements (32, 33). In general, Sp1 can activate gene expression, whereas Sp3 can be stimulatory or suppressive (33). Proximal Sp1-binding sites are thought to function in a cooperative way, forming complexes that initiate transcription from multiple sites. Furthermore, several transcription factors associate with Sp1, and in this way enhance or reduce the activation of specific gene promoters (43). Mutation of either of the two Sp1-binding sequences in this region of the TRIII gene promoter reduced its activity by approximately one-third. However, a truncation removing 94 nucleotides that included the more upstream Sp1-binding site caused an 85% decrease in promoter function. The sequence between these sites is GC enriched to 75% and contains several possible nuclear factor-binding sites, including Sp1, AP-2, and AP-4. By gel shift analysis and anti-nuclear factor antibody reactivity, little or no binding by Sp1 or AP-4 occurred in this region, whereas complexes consistent with AP-2 were evident. Nonetheless, overexpression of AP-2 by transfection did not further stimulate this region of the TRIII promoter. This suggests that the amount of endogenous AP-2 may be sufficient for TRIII expression or that complex interactions between Sp1 and AP-2 may govern overall TRIII expression (44). Other oligonucleotide probes from this region also formed several gel shift complexes, but their identity is not yet known. Thus, our current findings suggest effects by Sp1 and AP-2, perhaps among other nuclear factors within this region, although none of these sites by themselves appears to have a dominant influence.

Organization of the basal region of the TRIII gene promoter is similar to that for other growth factor receptor genes (25) and does not itself seem to account for differences in TRIII expression among various tissues, during development, or in response to regulatory factors or events. In osteoblasts, we found that TRIII promoter activity was induced by glucocorticoid and suppressed by BMP-2. Although promoter-dependent reporter gene expression only represents an indication of relative changes in authentic TRIII gene expression, the magnitude of the effects that we observed were consistent with our earlier evidence for changes in TGF-β binding to TRIII and on TRIII mRNA (12, 13, 38, 39). Importantly, analogous effects are either not seen or less evident in undifferentiated periosteal cells or fibroblasts (Refs. 12, 40, and 48 and our current studies), suggesting phenotype-related differences. Sensitivity to glu-
corticoid occurs near the 3′ end of the TRIII promoter. Initial studies to locate possible cis-acting elements that allow this effect suggest at least two response regions between nucleotides −687 and −495 and between nucleotides −440 and −386.3 The more upstream region contains a consensus C/EBP-binding site, consistent with the stimulatory effect of glucocorticoid on C/EBP expression in adipocytes (45, 46) and bone cells.4 However, the more downstream response region contains no identifiable glucocorticoid response element or C/EBP-binding site, suggesting interactions with other trans-acting factors. The inhibitory effect of BMP-2 is only evident with TRIII promoter fragments above nucleotide −2013, consistent with its ability to enforce the effect of an endogenous silencer region initially apparent by nested fragment deletion analysis. Sequence analysis shows a variety of possible binding sites in this region. Notably, it contains two binding domains for Myc/Max nuclear factors whose activity may be suppressed when Mad subunit expression increases during tissue and organ development (47). Further studies to define this element may therefore help to explain the significant decrease in TRIII expression that occurs with native or BMP-2 induced differentiation of osteoblasts (10, 12).

In summary, we have cloned the rat TRIII gene promoter and have begun to indentify regulatory regions that control basal, hormone, and growth factor induced changes in TRIII expression previously observed on rat osteoblasts. Indeed, forced overexpression of TRIII reduced the effectiveness of TGF-β1 treatment, consistent with the relatively lower activity of TGF-β in less differentiated bone cell cultures where proportionately more TRIII is endogenously expressed (12, 40, 48). Future studies to define in more detail the conditional elements that alter TRIII expression may help to decipher the complex events that control the changes in TGF-β sensitivity and its biological effects in bone and in other tissues. A better understanding of the nuclear factors that regulate the loss of TRIII expression with differentiation may further increase our understanding of the gene repression that must occur to limit tissue growth during development and to avoid hyperplastic disease.