Journal of Cellular Biochemistry 63:478–490 (1996)

Cloning, Characterization, and Expression of the Transforming Growth Factor-β Type I Receptor Promoter in Fetal Rat Bone Cells

Changhua Ji, Sandra Casinghino, Thomas L. McCarthy, and Michael Centrella

Section of Plastic Surgery, Department of Surgery, Yale University School of Medicine, New Haven, Connecticut 06520-8041

Abstract Transforming growth factor (TGF-β) binds several discrete membrane proteins. Of these, a type I receptor appears indispensable for signal transduction. Previous examination of TGF-β receptor expression has been limited to changes in cell surface protein, and more recently, mRNA abundance. In order to learn more about TGF-β function and receptor expression during osteogenesis, we have now cloned a 4 kilobase (kb) DNA fragment 5′ proximal to the coding region of the rat TGF-β type I receptor gene. Sequence analysis revealed multiple elements compatible with transcription initiation, including a properly positioned and oriented CCAAT box, six Sp1 binding sites (three defining GC boxes), and two strong AP2 binding sites within a 0.7 kb span directly upstream of the coding region. The 3′ terminal 0.3 kb span comprises a GC-enriched (77%) so-called CpG island that, like other similarly organized promoters, lacks a TATA box. Primer extension and RNase protection studies with cRNAs from this area show multiple initiation sites within 220 bp 5′ proximal to the initial methionine codon. Transient transfections using nested, deleted, and inverted promoter sequences demonstrated maximal reporter expression by a 1 kb fragment encompassing all of these elements. Truncation of the 1 kb fragment from the 5′ and 3′ ends indicated the need for several elements for peak promoter activity. These results, and transfections in fetal rat bone and dermal cells, suggest that this promoter contains elements that specify basal and conditional expression of the TGF-β type I receptor in bone.

Key words: transcription initiation, CpG island, transcription factor AP2, transcription factor Sp1, osteoblasts, differentiation

Signal-transducing transforming growth factor type β (TGF-β) receptors occur on most cells [Massague et al., 1994]. Variations in TGF-β receptor expression have been observed with growth, differentiation, and neoplasia [Centrella et al., 1995a; Massague et al., 1994; Miyazono et al., 1994; Sankar et al., 1996], and different biological effects appear to correspond with the gain or loss of individual receptor subtypes [Centrella et al., 1995b; Chen et al., 1993; Miyazono et al., 1994; Sankar et al., 1995, 1996]. In many situations TGF-β binding to a 73 kDa type II receptor recruits and activates a 53 kDa type I receptor [Wrana et al., 1994]. In other cases only a subset of TGF-β induced events seems to persist when preferential binding to type I receptors occurs [Centrella et al., 1995b; Chen et al., 1993; Sankar et al., 1995, 1996]. Consequently, type I receptors are thought to have an indispensable role in TGF-β dependent signalling. The coding regions of several type I TGF-β receptor proteins have now been cloned. After their transfection and expression in naive or chemically mutated cells, some type I receptor clones have been shown to confer sensitivity to TGF-β treatment [Bassing et al., 1994; Ebner et al., 1993; Franzen et al., 1993]. Two of these, termed R4 in rat and ALK-5 in humans, are thought to encode primary, high affinity TGF-β type I receptors [Bassing et al., 1994; Franzen et al., 1993]. Additionally, the levels of type I receptor protein and mRNA expression have been examined in various tissue systems including bone cells. For example, binding studies with radiolabeled TGF-β show that type I receptor levels decrease after glucocorticoid treatment and increase in response to BMP-2 in osteoblast-enriched cultures from fetal rat bone. In each

Received May 15, 1996; accepted June 21, 1996.

Address reprint requests to Michael Centrella, Section of Plastic Surgery, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208041, New Haven, CT, 06520-8041. E-mail: CentrellMA@MASPO3.MAS.YALE.EDU.

case, the extent to which TGF-β can stimulate collagen synthesis directly corresponds to the level of type I receptor relative to other cell surface TGF-β binding components [Centrella et al., 1991, 1995b]. However, little is still known about how TGF-β receptor expression is controlled. To continue our studies of type I receptor with respect to osteoblast differentiation, we have now cloned the promoter for the rat TGF-β type I receptor termed R4. Our results are consistent with the prevalent, constitutive expression of TGF-β type I receptor at the transcript level in differentiated bone cells and support other studies predicting that cell surface TGF-β receptor levels can be determined in part by transcriptional events [Centrella et al., 1991, 1995b, 1996].

METHODS

Genomic Library Screening

About 6×10^5 plaques obtained from the EMBL-3 rat liver genomic DNA library (Clontech, Palo Alto, CA) were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and screened by hybridization (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) with a DNA probe containing the first 402 base pairs (bp) of rat TGF-β type I receptor cDNA (R4-Puc) (GenBank accession number L26110) [Bassing et al., 1994] previously labeled by random priming with [α-^{32}P]dCTP and [α-^{32}P]dTTP (Dupont/NEN, Boston, MA) [Feinberg and Vogelstein, 1984]. Positive clones were re-screened with the same probe, recombinant bacteriophage were plaque purified, and phage DNA was isolated by the plate lysate method [Sambrook et al., 1989].

DNA Sequencing

Phage inserts were subcloned into the Xho I site of pBluescript-KSII vector (Stratagene, Inc, La Jolla, CA), mapped with an assortment of restriction endonucleases, and subclones were generated by cleavage at appropriate restriction sites (Fig. 1). Double-stranded plasmid DNA was denatured with 0.2 N sodium hydroxide and sequenced by the dideoxy-chain termination method [Sanger et al., 1977], using a T7 Sequenase sequencing kit (U.S. Biochemical Corp., Cleveland, OH). Results were computer analyzed with the Wisconsin Sequence Analysis Package (GCG program).

Cell Cultures

Using procedures approved by the Yale Animal Care and Use Committee, parietal bones from 22-day-old Sprague-Dawley-derived rat fetuses (Crl:CD®(SD)BR); Charles River Breeding Laboratories, Wilmington, MA) were dissected free of sutures and digested for five 20-min intervals with collagenase. The first digestion releases less differentiated cells (population 1), while the last three digestions (populations 3–5) are enriched with cells exhibiting differentiated osteoblast characteristics. Primary cultures from each group were plated at $6–9 \times 10^3$ cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM Hepes buffer, pH 7.2, 100 μg/ml ascorbic acid, penicillin and streptomycin, and 10% fetal bovine serum (FBS). Clonal rat osteosarcoma-derived osteoblast-like ROS 17/2.8 cultures (obtained from Drs. Masaki Noda and Gideon Rodan; Merck Sharp and Dohme Research Laboratories, West Point, PA) and first passage rat skin fibroblasts obtained from the rat fetuses used to isolate bone cells were cultured and treated by the same procedures [Centrella et al., 1995b; McCarthy et al., 1988].

RNA Preparation

RNA lysis buffer (5 M guanidine monothiocyanate, 25 mM trisodium citrate, 0.5% Sarkosyl, 100 mM 2-mercaptoethanol) was added to 9.6 cm^2 cultures containing about 5×10^5 cells, and the lysates were extracted with phenol-chloroform-isoamyl alcohol (75:25:1) containing 200 mM sodium acetate. Total RNA was precipitated with isopropanol and collected by centrifugation [Chomczynski and Sacchi, 1987]. RNA pellets were redissolved in lysis buffer, reprecipitated with isopropanol, ethanol washed, dried, and solubilized in diethylpyrocarbonate-treated water to a concentration of 1 μg/μl.

Primer Extension Analysis

An oligonucleotide complementary to 19 bases upstream and the 3 bases encoding the initial methionine codon (5′-CATGGTCCCGCTGCCACTGTTT-3′) of the rat TGF-β type I receptor gene termed R4 [Bassing et al., 1994] was synthesized commercially and 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Twenty

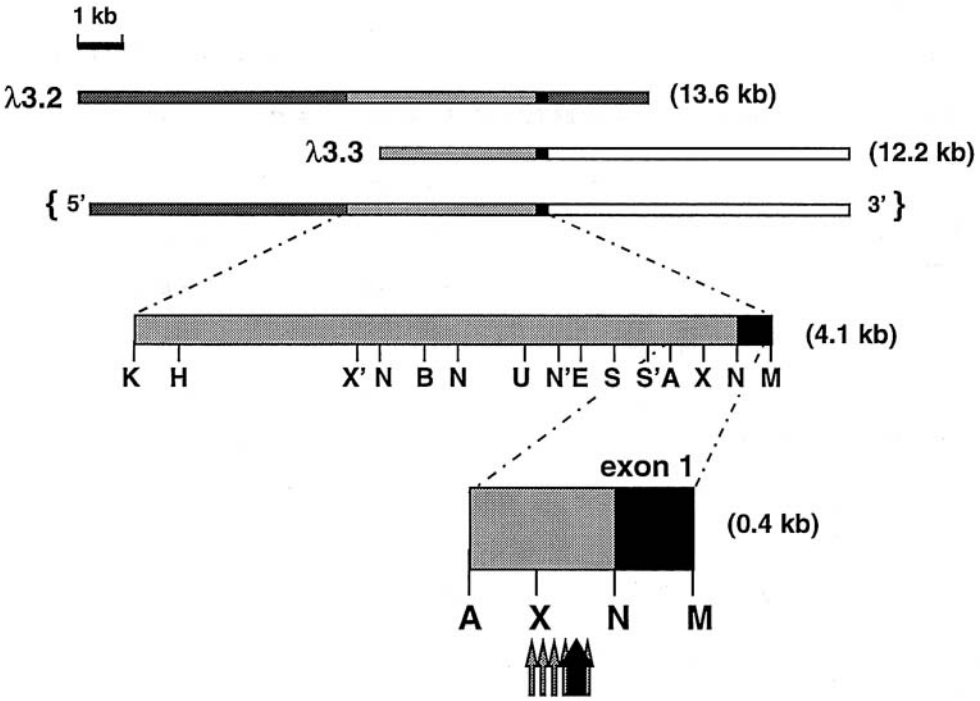


Fig. 1. Structure of rat TGF-β type I receptor genomic clones. Two overlapping rat genomic clones, termed λ3.2 and λ3.3, shown as thin bars, were aligned by restriction analysis. The area encompassing the largest cloned promoter-reporter construct, pKS4.1 (see Fig. 3) is contained in the region overlapped by both, and is enlarged and shown as the bar of intermediate thickness (Kpn I to Sma I) beneath them. The region that contains many features associated with promoter activity is shown as the thickest bar (Apa I to Sma I), and the area encompassing exon 1 (Nco I to Sma I) is shown as the black box on each bar. Arrows identify the region where multiple initiation sites of varying strength are thought to occur (see Fig. 4). The letters refer to the following restriction sites: K, Kpn I; H, Hind III; X', Xba I; X, Xho I; N, Nco I; N', Not I; B, Bgl II; U, Stu I; S, Sac I; S', Sac II; A, Apa I; E, EcoR I; M, Sma I.

micrograms of total cell RNA and 3×10^5 cpm of primer were hybridized in annealing buffer (80% formamide, 1 mM EDTA, 100 mM sodium citrate, 300 mM sodium acetate, pH 6.4) for 16 h at 50°C, and precipitated with ethanol. The product was solubilized and further incubated for 1 h at 42°C in a buffer containing 10 mM magnesium chloride, 40 mM KCl, 1 mM dithiothreitol, 1.25 mM each dATP, dCTP, dGTP, and dTTP, 50 mM Tris-Cl, pH8.0, and 20 U of avian myeloblastosis virus (AMV) reverse transcriptase. The samples were then digested with DNase-free RNase at 37°C for 15 min, and the products were run on denaturing 6% polyacrylamide gels alongside sequencing ladders.

RNase Protection Assay

A 1.0 kb EcoR I to Sma I fragment of cloned genomic DNA containing 965 bp of upstream sequence and 74 bp of downstream sequence (numbered as shown in Fig. 2) was cloned into pGEM-4Z (Promega Corp, Madison, WI) to produce a fragment that we named pGEM-ES1.0. pGEM-ES1.0 was linearized with Apa I and a 0.4 kb cRNA probe was generated by in vitro transcription with T7 RNA polymerase using the Maxiscript kit (Ambion, Inc, Austin, TX). The cRNA probe was labeled with [α-^{32}P]UTP.

Fig. 2. Nucleotide sequence of the 5' region of a rat TGF-β type I receptor gene. Genomic fragments shown in Figure 1 were subcloned at convenient restriction sites and overlapping fragments were sequenced and aligned. The sequence was analyzed by a computer-based data search, and several regions consistent with previously identified areas of transcriptional regulation are indicated with a larger text, underlining, and identification above the sequence. Double underlines correspond to overlapping regions between adjacent identified sequences. The arrows beneath the TATA box shown at position −734 to −739 indicate its reversed orientation. The bent arrows, indicated at nucleotides 1 and 38, show the positions of strong transcriptional initiation, demonstrated by both RNase protection and primer extension analyses. The region of protein coding sequence and corresponding amino acids are identical to those identified in R4 cDNA [Bassing et al., 1994; He et al., 1993]. The upstream genomic sequence of the promoter that we cloned has been deposited in the GenBank data base under accession number U48401.

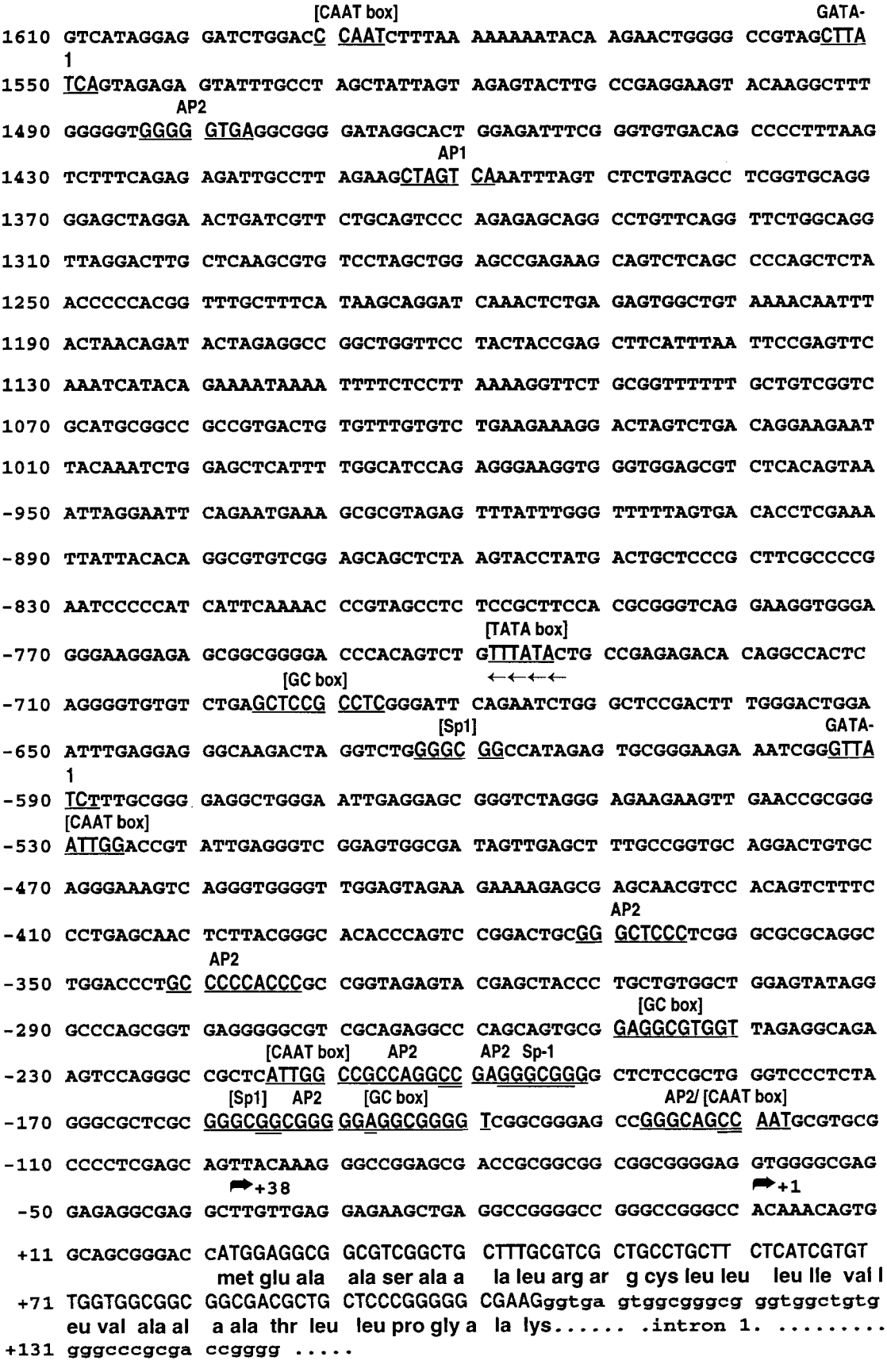

[CAAT box]
GATA-
-1610 GTCATAGGAG GATCTGGACC CAATCTTTAA AAAAAATACA AGAACTGGGG CCGTAGCTTA
1
-1550 TCAGTAGAGA GTATTTGCCT AGCTATTAGT AGAGTACTTG CCGAGGAAGT ACAAGGCTTT
AP2
-1490 GGGGGTGGGG GTGAGGCGGG GATAGGCACT GGAGATTTCG GGTGTGACAG CCCCTTTAAG
AP1
-1430 TCTTTCAGAG AGATTGCCTT AGAAGCTAGT CAAATTTAGT CTCTGTAGCC TCGGTGCAGG
-1370 GGAGCTAGGA ACTGATCGTT CTGCAGTCCC AGAGAGCAGG CCTGTTCAGG TTCTGGCAGG
-1310 TTAGGACTTG CTCAAGCGTG TCCTAGCTGG AGCCGAGAAG CAGTCTCAGC CCCAGCTCTA
-1250 ACCCCCACGG TTTGCTTTCA TAAGCAGGAT CAAACTCTGA GAGTGGCTGT AAAACAATTT
-1190 ACTAACAGAT ACTAGAGGCC GGCTGGTTCC TACTACCGAG CTTCATTTAA TTCCGAGTTC
-1130 AAATCATACA GAAAATAAAA TTTTCTCCTT AAAAGGTTCT GCGGTTTTTT GCTGTCGGTC
-1070 GCATGCGGCC GCCGTGACTG TGTTTGTGTC TGAAGAAAGG ACTAGTCTGA CAGGAAGAAT
-1010 TACAAATCTG GAGCTCATTT TGGCATCCAG AGGGAAGGTG GGTGGAGCGT CTCACAGTAA
-950 ATTAGGAATT CAGAATGAAA GCGCGTAGAG TTTATTTGGG TTTTTAGTGA CACCTCGAAA
-890 TTATTACACA GGCGTGTCGG AGCAGCTCTA AGTACCTATG ACTGCTCCCG CTTCGCCCCG
-830 AATCCCCCAT CATTCAAAAC CCGTAGCCTC TCCGCTTCCA CGCGGGTCAG GAAGGTGGGA
[TATA box]
-770 GGGAAGGAGA GCGGCGGGGA CCCACAGTCT GTTTATACTG CCGAGAGACA CAGGCCACTC
[GC box]
←←←←
-710 AGGGGTGTGT CTGAGCTCCG CCTCGGGATT CAGAATCTGG GCTCCGACTT TGGGACTGGA
[Sp1]
GATA-
-650 ATTTGAGGAG GGCAAGACTA GGTCTGGGGC GGCCATAGAG TGCGGGAAGA AATCGGGTTA
1
-590 TCTTTGCGGG GAGGCTGGGA ATTGAGGAGC GGGTCTAGGG AGAAGAAGTT GAACCGCGGG
[CAAT box]
-530 ATTGGACCGT ATTGAGGGTC GGAGTGGCGA TAGTTGAGCT TTGCCGGTGC AGGACTGTGC
-470 AGGGAAAGTC AGGGTGGGGT TGGAGTAGAA GAAAAGAGCG AGCAACGTCC ACAGTCTTTC
AP2
-410 CCTGAGCAAC TCTTACGGGC ACACCCAGTC CGGACTGCGG GCTCCCTCGG GCGCGCAGGC
AP2
-350 TGGACCCTGC CCCCACCCGC CGGTAGAGTA CGAGCTACCC TGCTGTGGCT GGAGTATAGG
[GC box]
-290 GCCCAGCGGT GAGGGGGCGT CGCAGAGGCC CAGCAGTGCG GAGGCGTGGT TAGAGGCAGA
[CAAT box] AP2 AP2 Sp-1
-230 AGTCCAGGGC CGCTCATTGG CCGCCAGGCC GAGGGCGGGG CTCTCCGCTG GGTCCCTCTA
[Sp1] AP2 [GC box] AP2/ [CAAT box]
-170 GGGCGCTCGC GGGCGGCGGG GGAGGCGGGG TCGGCGGGAG CCGGGCAGCC AATGCGTGCG
-110 CCCCTCGAGC AGTTACAAAG GGCCGGAGCG ACCGCGGCGG CGGCGGGGAG GTGGGGCGAG
+38
+1
-50 GAGAGGCGAG GCTTGTTGAG GAGAAGCTGA GGCCGGGGCC GGGCCGGGCC ACAAACAGTG
+11 GCAGCGGGAC CATGGAGGCG GCGTCGGCTG CTTTGCGTCG CTGCCTGCTT CTCATCGTGT
met glu ala ala ser ala a la leu arg ar g cys leu leu leu lle val l
+71 TGGTGGCGGC GGCGACGCTG CTCCCGGGGG CGAAGggtga gtggcgggcg ggtggctgtg
eu val ala al a ala thr leu leu pro gly a la lys..... .intron 1. .....
+131 gggcccgcga ccgggg .....

Figure 2.

Ten micrograms of total cell RNA from primary osteoblast-enriched population 3–5 cultures and 1×10^5 cpm of probe cRNA were combined in 30 μl of hybridization buffer (80% formamide, 1 mM EDTA, 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 mM EDTA, 300 mM NaCl, 10 mM Tris-Cl [pH 7.5], 1 U/ml RNase A and 40 U/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.

Transfection Studies

Restriction fragments containing various cloned DNA segments were inserted into the multiple cloning site of pGL3-Basic, a promoter-deficient luciferase reporter plasmid (Promega), to test for promoter activity. To produce the construct named pES1.0, pGEM-ES1.0 was digested with EcoR I and Hind III to release the 1.0 kb EcoR I to Sma I fragment and 36 bp of polylinker. The Xho I and Hind III sites of the polylinker region in pGL3-Basic were exposed by restriction digestion. The EcoR I tail of the insert and Xho I tail of the reporter plasmid were filled in using *Escherichia coli* DNA polymerase I Klenow fragment, and the insert was ligated into the blunted Xho I site and the intact Hind III site of pGL3-Basic. A larger construct, named pKS4.1, was produced by inserting a 3.9 kb Kpn I to Xho I fragment (obtained from the phage mapping described above) next to an internal Xho I site (at −107) of pES1.0 and the Kpn I site on the reporter plasmid polylinker. pKS4.1 was restriction digested, deleting the Kpn I to Bgl II sector, to produce the construct named pBS2.3. Similarly, pKS4.1 was internally deleted with Bgl II and EcoR I to produce pBΔE2.8.pES1.0 was truncated at the 3′ end by digestion with Nco I to produce pEN1.0; with Xho I and Hind III to produce pEXH0.8; and with Sac II and Hind III to produce pESH0.4.pEN1.0 was truncated at the 5′ end with Sac I to produce pSN0.7; with Sma I and Apa I to produce pAN0.4; with Nhe I and Xho I to produce pXN0.1; and with Sma I and Sac II to produce pSN0.1.pAN0.4 was truncated at the 3′ end with Xho I and Nco I to produce pAX0.2. The inverted construct named pSH3.6rev was generated by inserting a 3.6 kb Hind III-Sma I sector from pKS4.1 into pGL3-Basic that was first digested with Sma I and Hind III, utilizing the opposing orientation of those restriction sites on the insert and the reporter plasmid. A smaller inverted promoter named pSE1.0rev was produced from pSH3.6rev by EcoR I and Hind III restriction deletion. The relative positions of pertinent restriction sites and the cloned constructs are shown in Figure 3 and Table I. All deleted, truncated, and re-oriented constructs were made blunt-ended by Klenow fragment of DNA polymerase I where necessary, and re-circularized by treatment with T4 DNA ligase. Purified plasmid constructs were co-transfected with pSV-β-galactosidase (Promega) to assess for transfection efficiency in several experiments. Variations in β-galactosidase expression were minimal (SE of ±5.9% among groups within an experiment) and correction for differences in its expression never altered the patterns of luciferase activity. For transfections, 50–75% confluent cultures were rinsed in serum-free medium and exposed to plasmid (1.5 μg of cloned promoter-reporter plasmid and 0.5 μg of pSV-β-galactosidase per 4.5 cm^2 culture) with 1% Lipofectin™ (GIBCO-BRL, Gaithersburg, MD) for 3 h. The cells were then re-fed with growth medium containing 5% fetal bovine serum and cultured 48–72 h to achieve confluence. The medium was aspirated and the cultures were rinsed with PBS and extracted with cell lysis buffer (Promega). Nuclei were cleared by centrifugation at 12,000*g* for 5 min, and supernatants were stored at −75°C until assay. Commercial kits were used to measure luciferase (Promega) and β-galactosidase (Tropix, Inc., Bedford, MA). Protein content was determined by the method of Bradford [1976].

RESULTS

Isolation of Rat TGF-β Type I Receptor Genomic Clones

Three positive clones were identified from a total of 6×10^5 recombinant phage screened with a probe encoding the 5′ 402-bp sequence of the rat TGF-β type I receptor cDNA termed R4 [Bassing et al., 1994]. By restriction enzyme analysis, two clones (designated λ3.2 and λ17.2) contained similar sized genomic DNA inserts of 13.6 kb and comprised 10.5 kb of upstream sequence and 3.1 kb of sequence downstream from the initial methionine codon. The third clone (λ3.3) contained a 12.2 kb insert which overlapped with λ3.2 by about 6.8 kb at the 5′

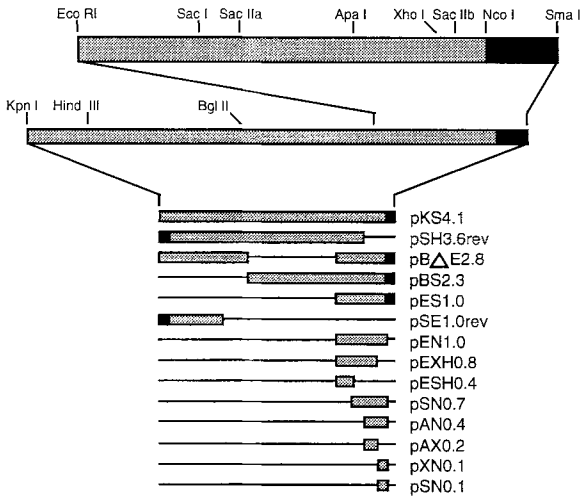


Fig. 3. Panel of promoter constructs derived from rat genomic TGF-β type I receptor clones. The upper two bars show the locations of restriction sites used for subcloning truncated, reversed, and deleted constructs. The grey areas indicate upstream sequences encompassing sequences tested for promoter activity. The black boxes indicate the area corresponding to translated sequences. The black lines on each construct indicate areas of the full length pKS4.1 where sequences were deleted. The names assigned to the transfection constructs are shown on the right.

end (Fig. 1). λ3.3 and λ3.2 were digested with Xho I, and the genomic inserts were cloned into pBluescript for sequencing and further characterization.

Sequence Analysis

Figure 2 shows the nucleotide sequence corresponding to the amino-terminal coding region and 1.7 kb of upstream DNA for the rat TGF-β type I receptor promoter that we cloned. Sequence analysis revealed that this region lacks a properly oriented consensus TATA box within the first 0.7 kb region upstream of the initial methionine codon. Six binding sites for transcription factor Sp1 occur within this area, upstream of a region encoding a putative cap site (numbered as +1; designated by reference to the transcription initiation site studies described below). A CpG island often associated with the beginning of a gene [Gardiner-Garden and Frommer, 1987; Tazi and Bird, 1990] is present between nucleotides at −297 to −1, with a GC content of 77%. Similarly, the region encoding the amino terminal portion of the coding region of the type I receptor is also GC-enriched to the level of 71%. Four of the Sp1 binding sites are located in the GC-rich area (at −140 to −149; −155 to −160; −193 to −198; and −241 to −250), and three of the six Sp1 binding sites (at −140 to −149; −241 to −250; and −687 to −696) are consensus GC boxes. A seventh Sp1 binding site occurs within the first intron at +116 to +121.

Several studies revealed that the promoters for many growth factor receptor genes lack both TATA and CCAAT boxes [Araki et al., 1987; Cooke et al., 1991; Humphries et al., 1994; Ishii et al., 1985; Pang et al., 1995; Saito et al., 1992; Sehgal et al., 1988]. However, this promoter sequence contains two CCAAT boxes in the GC-rich region (one at −122 to −118 in the forward orientation, and a second at −211 to −215 in the backward orientation) and two CCAAT boxes further upstream (at −526 to −530 in the backward orientation, and −1591 to −1587 in the forward orientation), which are possible binding sites for transcription factors C/EBP, CTF/NF1, and CBF [Jones et al., 1987; Sinha et al., 1996]. One AP1 and one AP2 site occur far upstream and four AP2 sites occur within the GC-rich promoter region, while two others occur less than 100 nucleotides upstream. Two overlapping AP2 sites (at −211 to −202; −203 to −195) are thought to be very high affinity, consensus elements [Williams and Tjian, 1991]. The upstream sequence also contains two potential binding sites for a major human DNA-binding protein termed GATA-1 that appears critical for erythroid development [Pevny et al., 1991], while many potential regions for various other tissue specific or regulated transcription factors are scattered throughout the sequence (data not shown).

Determination of the Transcription Initiation Site

To locate sequences where this promoter initiates gene transcription, we first performed primer extension analysis. A 22-mer DNA primer corresponding to nucleotides +25 to +3 (numbered as indicated in Fig. 2) was synthesized, 5′ end-labeled, combined with total RNA from primary osteoblast-enriched population (3–5 cultures from fetal rat bone as template, and extended with AMV reverse transcriptase. This process generated several extended DNA products (Fig. 4A), indicating multiple transcription initiation sites from this promoter. One major product occurred only 2 nucleotides beyond the primer, and another extended 38 nucleotides further. Several less prominent but distinct products were also observed within the next 165 nucleotide span.

TABLE I. Characteristics of Constructs Containing Rat TGF-β Type I Receptor 5′ DNA Sequences Used to Evaluate Promoter Activity*

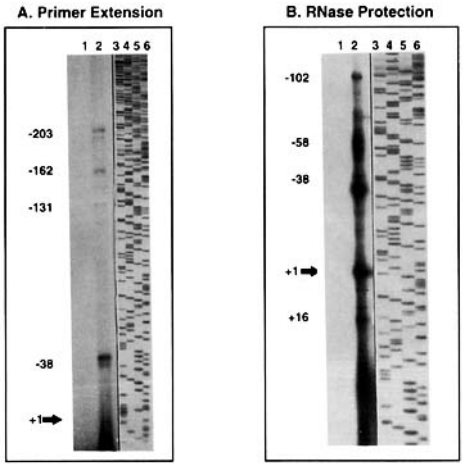
Construct name	≈ Size (kb)	Restriction fragment	Characteristics
pKS4.1	4.1	Kpn I-Sma	Full length
pSH3.6rev	3.6	Hind III-Sma I	0.5 kb 5′ truncation and reversed orientation
pBΔE2.8	2.8	Kpn I-Bgl II, Eco RI-Sma I	1.3 kb internal deletion
pBS2.3	2.3	Bgl II-Sma I	1.8 kb 5′ truncation
pES1.0	1.0	Eco RI-Sma I	3.1 kb 5′ truncation
pSE1.0rev	1.0	Eco RI-Sma I	3.1 kb 5′ truncation and reversed orientation
pEN1.0	1.0	Eco RI-Nco I	3.1 kb 5′ truncation, exon 1 fragment deletion
pEXH0.8	0.8	Eco RI-Xho I	3.1 kb 5′ truncation, 0.2 kb 3′ truncation
pESH0.4	0.4	Eco RI-Sac IIa[a]	3.1 kb 5′ deletion, 0.6 kb 3′ truncation
pSN0.7	0.7	Sac I-Nco I	3.4 kb 5′ truncation, exon 1 fragment deletion
pAN0.4	0.4	Apa I-Nco I	3.7 kb 5′ truncation, exon 1 fragment deletion
pAX0.2	0.2	Apa I-Xho I	3.7 kb 5′ truncation, 0.2 kb 3′ truncation
pXN0.1	0.1	Xho I-Nco I	4.0 kb 5′ truncation, exon 1 fragment deletion
pSN0.1	0.1	Sac IIb-Nco I[a]	4.0 kb 5′ truncation, exon 1 fragment deletion

*Constructs were prepared by restriction digestions and subcloned into pGL3-Basic (Promega), as described in Methods. Size refers to the length of the fragment inserted within the multiple cloning site of pGL3-basic.
[a]Sac IIa and Sac IIb refer to the two separate Sac II restriction sites present in pKS4.1, as indicated in the uppermost bar of Figure 3.

To characterize the rat bone cell transcription initiation sites further, we then used an RNase protection assay. A cRNA probe that encompassed the area between the Apa I site at −287 (which included the longest products observed by primer extension) to nucleotide +98 (which was 77 nucleotides downstream from the putative translation start site) was generated by in vitro transcription labeling with T7 RNA polymerase. When the cRNA probe was combined with total RNA from the fetal rat osteoblast-enriched cultures and the hybrids were digested with RNase, a number of protected fragments occurred (Fig. 4B), in agreement with results from the primer extension assay. Although some of the upstream sites consistent with transcriptional initiation that were found with primer extension were not observed by RNase protection assay, some overlapping sites were evident. By comparison with a ladder of known sequence and length, a common prominent fragment was seen at position −38, and a second occurred 21 nucleotides upstream of the initial methionine codon. Since this was the most downstream initiation site demonstrated by both methods, we used it to set the nucleotide numbering sequence, and it is designated as +1 in Figure 2. In addition, several putative initiation sites fell within sequences encompassing the published 5′ end of human type I receptor cDNA (corresponding to −56 of the sequence in Figure 2 and −76 of the transcribed but not translated region of the human cDNA sequence, which is numbered by reference to the initial methionine codon) [Franzen et al., 1993]. This area was 60 nucleotides downstream from the most proximal forward CCAAT box (Fig. 4C).

Promoter Activity in Transfected Cells

To demonstrate that this cloned DNA sequence specifies a functional promoter, various complete, deleted, truncated, and reversed-orientation fragments (described in Table I) were subcloned into a promoter-free reporter transfection vector, PGL3-Basic, that can be used to drive gene expression with high efficiency in fetal rat bone and skin cell cultures [McCarthy et al., 1995, 1996]. As shown in Figure 5, insignificant reporter activity was present in primary osteoblast-enriched fetal rat cell population 3–5 cultures transfected with the promoter-free pGL3-Basic construct alone. In contrast, very high expression occurred in cultures transfected with a commercial positive control plasmid (pGL3-Control; Promega), which is driven by a combination of simian virus 40-derived promoter and enhancer sequences. Transfection with plasmid constructs containing several of the many DNA fragments that we subcloned into PGL3-Basic elicited very high reporter expression. No significant differences were observed among the constructs containing the largest cloned fragment (pKS4.1), and those where the 5′ end was successively truncated down to



C. Possible Transcription Initiation Sites

Apa I
↓
-310 TGCTGTGGCT GGAGTATAGG GCCCAGCGGT GAGGGGGCGT CGCAGAGGCC CAGCAGTGCG

-250 **GAGGCGTGGT** TAGAGGCAGA AGTCCAGGGC CGCTCATTGG CCGCCAGGCC GA*GGGCGG*GG
↑ ↑

-190 CTCTCCGCTG GGTCCCTCTA GGGCGCTCGC *GGGCGG*CGGG G**GAGGCGGGG** TCGGCGGGAG
↑

-130 CCGGGCAGCC AATGCGTGCG CCCCTCGAGC AGTTACAAAG GGCCGGAGCG ACCGCGGCGG
↑ **hum** ⇑ ↑↑ ↑↑ ↑

-70 CGGCGGGGAG GTGG**G**GCGAG GAGAGGCGAG GCTTGTTGAG GAGAAGCTGA GGCCGGGGCC
⇑ ⇑ ⇑ ⇑ ↑▲ ↑↑ ⇑ ⇑ ⇑ ⇑

-10 GGGCCGGGCC ACAAACAGTG GCAGCGGGAC C ATG GAG GCG GCG TCG GCT
↑ ▲ ⇑ ⇑ ⇑ ⇑ met glu ala ala ser ala

Fig. 4. Transcription initiation site analysis for rat TGF-β type I receptor. **A:** RNA isolated from yeast (**lane 1**) or from primary fetal rat osteoblast-enriched cultures (**lane 2**) was annealed with a 5′-end labeled primer corresponding to the putative initial methionine codon and 19 upstream nucleotides of rat TGF-β type I receptor, and extended by incubation with AMV reverse transcriptase. DNA remaining after RNase digestion was separated by gel electrophoresis and visualized by autoradiography. **B:** Yeast RNA (**lane 1**) or bone cell-derived RNA (**lane 2**) was hybridized with a radiolabeled 0.4 kb probe extending 0.3 kb upstream from the putative initial methionine codon of rat TGF-β type I receptor, and digested with RNase. Protected fragments were separated by gel electrophoresis and visualized by autoradiography. Identical results were seen in 2 separate studies. The arrows in A and B indicate the location of the nucleotide designated as +1 in Figure 3. **Lanes 3–6** show a sequencing ladder used to determine the length of the radioactive fragments, and the lengths of several major sites are indicated on the left. **C:** Sequence from Figure 3 corresponding to the region encompassing common major bands in the vicinity of the initial methionine codon. The Apa I restriction site and the published 5′ end of a human sequence cloned from an erythroleukemia cDNA library (hum) are indicated from above, the positions of GC boxes are shown in bold, putative Sp1 binding sites are shown in italics, and the forward oriented CCAAT box is *underlined*. Positions of transcription initiation, determined by primer extension analysis (↑), by RNase protection assay (⇑), or by both methods (▲) are indicated below their corresponding sites. The first six amino acids including the putative initial methionine are also shown.

1.0 kb (pEN1.0). Maximal reporter activity was retained when the 0.1 kb sequence beyond the initial methionine codon (the Nco I to Sma I restriction fragment; shown in Fig. 3 and Table I) was deleted (comparing pES1.0 with pEN1.0). Furthermore, using constructs that were further truncated from the 5′ end, we found that preserving as little as 0.4 kb of this region (pAN0.4) still permitted 50–60% of maximal promoter activity in osteoblast-enriched cell cultures. Only very low reporter activity was induced by the 0.1 kb fragments (pXN0.1 and p SN0.1) immediately upstream of exon 1, and was not significant by comparison to results with the very active promoter constructs. No significant reporter activity above that driven by pGL3-Basic occurred in cells transfected with constructs containing active fragments cloned in the inverted orientation (pSH3.6rev; as shown in Fig. 5; and the inverse of pES1.0, designated as pSE1.0rev, data not shown). However, maximal promoter activity was still evident when the 1.3 kb internal third (the Bgl II to Eco RI restriction fragment described in Fig. 3 and Table 1) of the largest promoter fragment that we cloned was deleted (comparing pKS4.1 with pBΔE2.8).

As shown in Figure 6, to develop a better focus on the region of the promoter that is necessary for efficient transcription, several additional truncated constructs were prepared by restriction digestion from the maximally active promoter construct, pEN1.0. Deletion of only the 0.1 kb fragment comprising pXN0.1 from the 3′ end of pEN1.0 (producing pEXH0.8) decreased reporter expression in the osteoblast-enriched cell cultures by approximately 70%, and deletion of the next 0.5 kb (producing pESH 0.4) caused only a marginal additional loss in activity. As shown earlier, eliminating 0.6 kb of DNA from the 5′ end of pEN1.0 (producing pAN0.4) maintained 50–60% of maximal promoter activity. However, although pAN0.4 contained sequence information that was able to drive significant promoter activity, the two fragments that comprised this area were by themselves either ineffective (pAX0.2) or, as noted previously, minimally effective (pXN0.1). Consequently, optimal promoter activity in osteoblast-enriched cultures appeared to require several elements or regions within the 944 bp upstream sequence spanned by pEN1.0. These regions appeared to include one or more elements between the Eco RI and Apa I restriction cleavage sites, between

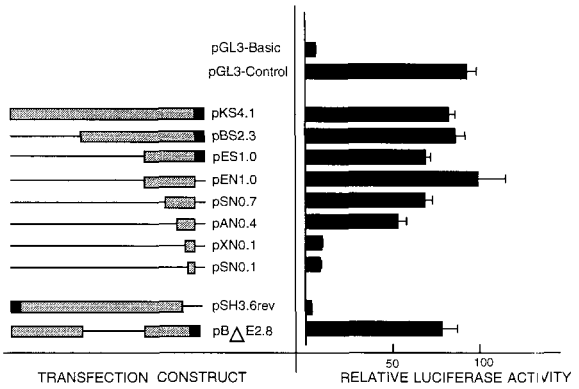


Fig. 5. Identification of basal TGF-β type I receptor promoter sequences in osteoblast-enriched primary cell cultures from fetal rat bone. DNA sequence regions described in Figure 3 and Table I were subcloned into a promoter-free transfection vector encoding firefly luciferase (pGL3-Basic). On the left, the grey (putative promoter) and black (exon 1) bars indicate preserved sequences of pKS4.1, and black lines show where the sequences were deleted by restriction enzyme digestions. Subconfluent cultures were transfected with 1.5 μg of each plasmid shown and 0.5 μg of pSV-β-galactosidase, encoding β-galactosidase, and analyzed for luciferase or β-galactosidase activity 2 days later. Luciferase was measured in an aliquot of cell lysate equivalent to 12,500 cells, and β-galactosidase was measured in a 50,000 cell equivalent. Data are shown as relative luciferase activity, corrected for protein content (3.3 ± 0.1 μg) and relative β-galactosidase expression (100 ± 2%). Bars are means ± SE of an experiment where all constructs were examined simultaneously, and represent the results from 2–7 independent, overlapping studies where each plasmid was examined in 6–25 replicate cultures.

the Apa I and Xho I sites, and between the Xho I and Nco I sites.

For comparative studies, pEN1.0, which induced maximal reporter activity in osteoblast-enriched population 3–5 cultures, was then transfected into 3 other fetal rat cell cultures (fetal rat dermal fibroblasts, less differentiated fetal rat bone cells termed population 1, and an immortalized, highly differentiated fetal rat osteoblast-like cell line termed ROS 17/2.8), where we previously observed differences in the steady levels of transcripts encoding TGF-β type I receptor [Centrella et al., 1995b]. As shown in Figure 7, pGL3-Basic had very little activity and pEN1.0 was expressed with very high efficiency in each cell culture. Nonetheless, variations in pEN1.0-driven reporter expression were apparent among these four culture models. Notably, a 2–3-fold greater level of reporter expression occurred in ROS 17/2.8 and osteoblast-enriched population 3–5 cultures relative to dermal fibroblasts and less differentiated population 1 cell cultures. Analogous to the data in Figures 5 and 6, less

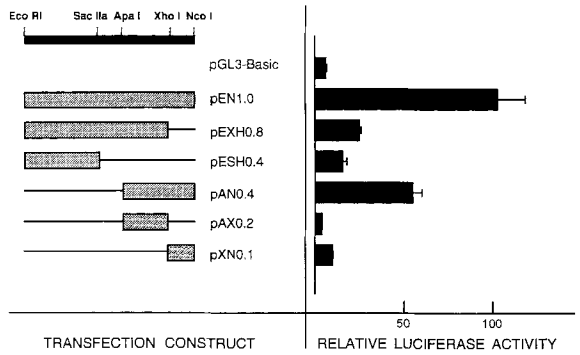


Fig. 6. Restriction dissection of a TGF-β type I receptor promoter that is maximally active in osteoblast-enriched primary cell cultures from fetal rat bone. DNA sequence regions described in Figure 3 and Table I were subcloned into a promoter-free transfection vector encoding firefly luciferase (pGL3-Basic). On the left, the grey bars indicate preserved sequences of pEN1.0, and black lines show where the sequences were deleted by restriction enzyme digestions. Subconfluent cultures were transfected with 1.5 μg of each plasmid shown and 0.5 μg of pSV-β-galactosidase, encoding β-galactosidase, and analyzed for luciferase or β-galactosidase activity 2 days later. Luciferase was measured in an aliquot of cell lysate equivalent to 12,500 cells, and β-galactosidase was measured in a 50,000 cell equivalent. Data are shown as relative luciferase activity, corrected for protein content (3.3 ± 0.1 μg) and relative β-galactosidase expression (100 ± 2%). Bars are means ± SE of an experiment where all constructs were examined simultaneously, and represent the results from 4–7 independent, overlapping studies where each plasmid was examined in 13–25 replicate cultures. A scale is shown over the transfection constructs indicating the approximate positions of the restriction digestion sites used to subclone them.

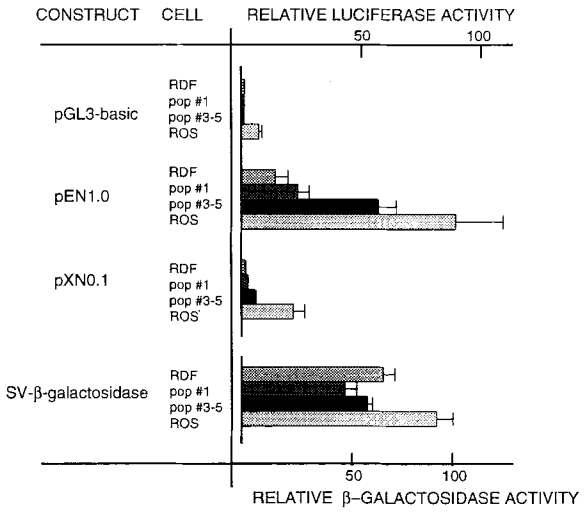


Fig. 7. TGF-β type I receptor promoter-reporter expression in various fetal rat cell cultures. Promoter regions derived from pKS4.1 with maximal (pEN1.0) and minimal (pXN0.1) activity, as shown in Figure 5, were examined in subconfluent dermal fibroblasts (RDF), less differentiated fetal rat bone cells (population 1), osteoblast-enriched cultures (populations 3–5), and a highly differentiated fetal rat osteoblast-like transformed cell line (ROS 17/2.8). The cultures were transfected with 1.5 μg of each plasmid shown and 0.5 μg of pSV-β-galactosidase, encoding β-galactosidase, and analyzed for luciferase or β-galactosidase activity 2 days later. The activity of the promoterless parental plasmid, termed pGL3-Basic, is also indicated. Data are shown as net luciferase activity corrected for protein content, and compared to net β-galactosidase activity, determined as in Figure 5. Bars are means ± SE and represent results from 4–7 independent, overlapping studies where each plasmid was examined in 12–22 replicate cultures.

promoter activity was seen in cultures transfected to express either weakly active or inactive TGF-β type I receptor promoter fragments in each cell type. However, even using the minimally active promoter fragment termed pXN0.1 that appears to contain essential information for optimal reporter expression, a similar pattern in relative promoter activity was seen. Small variations also occurred in the expression of a co-transfected plasmid that encodes β-galactosidase, but they were less divergent and did not parallel the differences in expression of the pEN1.0 or pXN0.1 reporter constructs. This suggests inherent dissimilarities in TGF-β type I receptor promoter activity that may be related to specific complements of transcription factors in each cell model, some of which may be phenotype specific, rather than to large differences in transfection sensitivity.

DISCUSSION

Independent variations in one or more TGF-β receptors appear to limit certain biological effects in some mesenchymal tissue derived cells [Centrella et al., 1991, 1995b; Chen et al., 1993; Sankar et al., 1995, 1996], but very little is known about how receptor expression is regulated. Our previous studies in bone cells demonstrated that transcriptional and post-transcriptional events determine overall TGF-β binding profiles, and suggested the need for constitutive receptor transcription. Specifically, we found that TGF-β receptors have very short half-lives [Centrella et al., 1996], and treatment with agents that regulate osteoblast function produces variations in apparent TGF-β receptor levels that correlate well with changes in biological activity in primary bone cell cultures [Centrella et al., 1991, 1995b, and unpublished results). To dissect these findings further, we chose to examine receptor promoter activity to determine if some variations occur by differences in gene activation. The sequence of the promoter for human TGF-β type II receptor has been reported previously [Humpries et al., 1994], and

we have examined type II receptor promoter driven reporter constructs in preliminary studies [Centrella et al., unpublished results]. However, there was no information about the cloning or the creation of reporter constructs under control of the TGF-β type I receptor promoter. For these reasons, we then cloned genomic DNA adjacent to the previously characterized cDNA for the rat TGF-β type I receptor [Bassing et al., 1994; He et al., 1993], and produced a variety of deletion constructs in order to characterize its expression in cells with various levels of commitment to the osteoblast phenotype. These tools should also become useful to assess regions of the gene that might allow regulation of TGF-β type I receptor expression in various tissues, under different growth conditions, or in response to external stimuli.

Like several growth factor receptor promoters, the rat TGF-β type I receptor promoter sequence lacks a consensus TATA box adjacent to the apparent sites of transcription initiation. In contrast, it does contain several CCAAT box sequences in the region that confers maximal reporter expression. One of these CCAAT boxes is oriented and positioned in a region consistent with promoter function, but point mutation and gel shift studies suggest that it is not involved in basal promoter expression (Ji et al., unpublished results). Moreover, several elements encoding Sp1 binding sites are clustered within a region that is highly GC-enriched (a so-called CpG island) [Gardiner-Garden and Frommer, 1987; Tazi and Bird, 1990] approximately 0.3 kb upstream of the initial methionine codon, and some of these do bind factors within nuclear extracts from population 3–5 cultures [Centrella et al., unpublished results]. Many genes that are controlled by promoters of this sort also tend to lack a CCAAT box, but analogous to our present results, have multiple sites of transcription initiation. By two separate analytical methods, two strong regions of transcriptional initiation were found positioned very close to the initial methionine codon, and several others were scattered throughout the upstream 0.2 kb region. This area overlaps with the 5′ end of the DNA sequence encoding TGF-β type I receptor that was cloned from a human erythroleukemia cDNA library [Franzen et al., 1993]. Consequently, the TGF-β type I receptor promoter that we cloned contains various basal transcription elements, and its organization is consistent with promoters found in the genes encoding receptors for epidermal growth factor [Ishii et al., 1985], insulin [Araki et al., 1987], nerve growth factor [Sehgal et al., 1988], insulin-like growth factors [Cooke et al., 1991], fibroblast growth factor [Saito et al., 1992], platelet-activating factor [Pang et al., 1995], erythropoietin [Youusoufian et al., 1990], interleukin 1 [Ye et al., 1993], and the TGF-β type II receptor [Humpries et al., 1994], among others. A promoter organization of this sort is consistent with constitutive gene expression in many cells. More so, it helps to explain the wide range of TGF-β sensitive cells in the body, and the apparent requirement of a type I TGF-β receptor for all phases of TGF-β signalling [Massague et al., 1994; Miyazono et al., 1994].

Although much of the information that we derived from the sequence of this promoter predicts an unregulated and constitutive level of TGF-β type I receptor gene expression, it does not eliminate the possibility that there are other elements dispersed throughout the active 4.0 kb fragment that we cloned that might impart situational diversity. As much as 50% of maximal promoter activity appears to be controlled by a 0.3 kb region adjacent to the initial methionine codon, while maximal activity is present within a 1.0 kb nucleotide span. Curiously, in fetal rat osteoblast-enriched cultures virtually all of this activity is retained in the largest 4.0 kb fragment that we examined. This is unlike our results with large promoter clones for insulin-like growth factor I (IGF-I) and IGF binding protein 5 (IGFBP-5), where upstream elements apparently repress reporter expression [McCarthy et al., 1995, 1996]. Nevertheless, we do not yet know if this broad spectrum of promoter activity will occur in cells from every tissue source. In this regard, extracts from various unstimulated fetal rat cells probed with a type I receptor cDNA show an increase in relative TGF-β type I receptor mRNA transcripts in parallel with greater levels of commitment to the osteoblast-like phenotype [Centrella et al., 1995b]. This observation is similar to the differences we now find in reporter expression in cultures transfected with a maximally active type I receptor promoter construct. However, when we have probed cells treated with agents that increase cell surface type I receptors, we have not yet detected similar changes in steady state type I receptor transcript levels [Centrella et al., unpublished results]. Overall, these findings suggest that basal type I receptor gene expression might

be tempered by upstream regulators that in some instances may reduce transcription, or that post-transcriptional events complement transcription. With regard to the former possibility, a DNA sequence bank search revealed a large number of elements within and upstream of the 0.3 kb GC-enriched cluster, and these areas will need to be examined for regulatory effects.

In summary, we have cloned a fragment of rat genomic DNA encoding a promoter of transcription that lies directly upstream of the TGF-β type I receptor termed R4. Sequence analysis reveals that it contains complex and multiple regulatory elements that specify constitutive expression from many initiation sites, consistent with a high GC content, several Sp1 binding sites, and the lack of a proximal consensus TATA box. Initial promoter-reporter analysis demonstrates that this promoter drives very strong transcriptional activity. However, various regulatory elements are also present that could modulate type I receptor expression under specific circumstances. Prospective studies in various cell culture models where type I receptor expression can be regulated will be useful to separate the importance of transcriptional and post-transcriptional controls.

ACKNOWLEDGMENTS

We are grateful for rat TGF-β type I receptor cDNA sequence information from Drs. J. Teixeira and P.K. Donahoe (Massachusetts General Hospital, Boston, MA), and for support from NIH grants AR-39201, DK-47421, the Section of Plastic Surgery and the Department of Surgery (Yale University) during these studies.

REFERENCES

Araki E, Shimada F, Uzawa H, Mori M, Ebina Y (1987): Characterization of the promoter region of the human insulin receptor gene: evidence for promoter activity. J Biol Chem 262:16186–16191.

Bassing CH, Yingling JM, Howe DJ, Wang T, He WW, Gustafson ML, Shah P, Donahoe PK, Wang X-F (1994): A transforming growth factor β type I receptor that signals to activate gene expression. Science 263:87–89.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Centrella M, McCarthy TL, Canalis E (1991): Glucocorticoid regulation of transforming growth factor β1 (TGF-β1) activity and binding in osteoblast-enriched cultures from fetal rat bone. Mol Cell Biol 11:4490–4496.

Centrella M, Rosen V, Horowitz MC, Wozney JM, McCarthy TL (1995a): Transforming growth factor-β gene family members, their receptors, and bone cell function. In Bikle DD, Negro-Vilar A (eds): "Hormonal Regulation of Bone Mineral Metabolism." Bethesda, MD: Endocrine Society Press, pp 211–226.

Centrella M, Casinghino S, Kim J, Pham T, Rosen V, Wozney J, McCarthy TL (1995b): Independent changes in type I and type II receptors for transforming growth factor β induced by bone morphogenetic protein-2 parallel expression of the osteoblast phenotype. Mol Cell Biol 15:3273–3281.

Centrella M, Ji C, Casinghino S, McCarthy TL (1996): Rapid flux in transforming growth factor-β receptors on bone cells. J Biol Chem 271:18616–18622.

Chen R-H, Ebner R, Derynck R (1993): Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-β activities. Science 260:1335–1338.

Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Cooke DW, Bankert LA, Roberts CT Jr, LeRoith B, Casella SJ (1991): Analysis of the human type I insulin-like growth factor receptor promoter. Biochem Biophys Res Commun 177:1113–1120.

Ebner R, Chen R-H, Shum L, Lawler S, Zioncheck TF, Lee A, Lopez AR, DeRynck R (1993): Cloning of a type I TGF-β receptor and its effect on TGF-β binding to the type II receptor. Science 260:1344–1348.

Feinberg AP, Vogelstein B (1984): A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267.

Franzen P, tenDijke P, Ichijo H, Yamashita H, Schulz P, Heldin C-H, Miyazono K (1993): Cloning of a TGFβ type I receptor that forms a heteromeric complex with the TGFβ type II receptor. Cell 68:681–692.

Gardiner-Garden M, Frommer M (1987): CpG islands in vertebrate genomes. J Mol Biol 196:261–282.

He WW, Gustafson ML, Hirobe S, Donahoe PK (1993): Developmental expression of four serine/threonine kinase receptors homologous to the activin/transforming growth factor-β type II receptor family. Dev Dyn 196:133–142.

Humphries DE, Bloom BB, Fine A, Goldstein RH (1994): Structure and expression of the promoter for the human type II transforming growth factor-β receptor. Biochem Biophys Res Commun 203:1020–1027.

Ishii S, Xu Y-H, Stratton RH, Roe BA, Merlino GT, Pastan I (1985): Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc Natl Acad Sci USA 82:4920–4924.

Jones KA, Kadonaga JT, Rosenfeld J, Kelly TJ, Tjian R (1987): A cellular DNA binding protein that activates eukaryotic transcription and DNA replication. Cell 48:79–89.

Massague J, Attisano L, Wrana JL (1994): The TGF-β family and its composite receptors. Trends Cell Biol 4:172–178.

McCarthy TL, Centrella M, Canalis E (1988): Further biochemical and molecular characterization of primary rat parietal bone cell cultures. J Bone Min Res 3:401–408.

McCarthy TL, Thomas MJ, Centrella M, Rotwein P (1995): Regulation of insulin-like growth factor I transcription by cyclic AMP in fetal rat bone cells through an element within exon 1: Protein kinase A dependent control without a consensus cAMP response element. Endocrinology 136:3901–3908.

McCarthy TL, Casinghino S, Mittanck DW, Ji C, Centrella M, Rotwein P (1996): Promoter-dependent and indepen-

dent activation of insulin-like growth factor binding protein-5 gene expression by prostaglandin E_2 in primary rat osteoblasts. J Biol Chem 271:6666–6671.

Miyazono K, ten Dijke P, Ichijo H, Heldin C-H (1994): Receptors for transforming growth factor-β. Adv Immunol 55:181–220.

Pang J-HS, Hung R-Y, Wu C-J, Fang Y-Y, Chau L-Y (1995): Functional characterization of the promoter region of the platelet activating factor receptor gene: Identification of an initiation element essential for gene expression in myeloid cells. J Biol Chem 270:14123–14129.

Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, Dagati V, Orkin SH, Constantini F (1991): Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature (London) 349:257–260.

Saito H, Kouhara H, Kasayama S, Kishimoto T, Sato B (1992): Characterization of the promoter region of the murine fibroblast growth factor receptor gene. Biochem Biophys Res Commun 183:688–693.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger F, Nicklen S, Coulsen AR (1977): DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Sankar S, Mahooti-Brooks N, Centrella M, McCarthy TL, Madri JA (1995): Expression of transforming growth factor β type III receptor in bovine endothelial cells increases their responsiveness to transforming growth factor β2. J Biol Chem 270:13567–13572.

Sankar S, Mahooti-Brooks N, McCarthy TL, Centrella M, Madri JA (1996): Modulation of transforming growth factor β receptor levels on microvascular endothelial cells during in vitro angiogenesis. J Clin Invest 97:1436–1446.

Sehgal A, Patil N, Chao M (1988): A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol Cell Biol 8:3160–3167.

Sinha S, Kim I-S, Sohn K-Y, deCrombrugghe B, Maity SN (1996): Three classes of mutations in the A subunit of the CCAAT-binding factor CBF delineates functional domains involved in the three step assembly of the CBF-DNA complex. Mol Cell Biol 16:328–337.

Tazi J, Bird A (1990): Alternative chromatin structure at CpG islands. Cell 60:909–920.

Williams T, Tjian R (1991): Analysis of the DNA binding and activation properties of the human transcription factor AP-2. Genes Dev 5:670–682.

Wrana J, Attisano L, Wieser R, Ventura F, Massague J (1994): Mechanism of activation of the TGF-β receptor. Nature (London) 370:341–347.

Ye K, Dinarello CA, Clark B (1993): Identification of the promoter region of human interleukin-1 type I receptor gene: Multiple initiation sites, high G+C content, and constitutive expression. Proc Natl Acad Sci USA 90:2295–2299.

Youussoufian H, Zon LI, Orkin SH, D'Andrea AD, Lodish HF (1990): Structure and transcription of the mouse erythropoietin receptor gene. Mol Cell Biol 10:3675–3682.