Characterization of the Promoter Region of the Human Transforming Growth Factor-\(\beta\) Type II Receptor Gene*

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Diminished cellular responsiveness to transforming growth factor-\(\beta\) (TGF-\(\beta\)) is frequently correlated with decreased transcription of the type II receptor for TGF-\(\beta\) (TGF-\(\beta\) RII). We have cloned and characterized the human TGF-\(\beta\) RII promoter and, using \(S1\) nuclease mapping and \(5'\) rapid amplification of cDNA ends polymerase chain reaction, have identified five alternative transcription start sites within the region \(-33\) to \(+57\).

DNA transfection experiments and electrophoretic mobility shift assays have revealed the existence of five distinct regulatory regions including two positive regulatory elements and two negative regulatory elements in addition to the core promoter region. The first positive regulatory element (\(-219\) to \(-172\)) interacts with two distinct nuclear protein complexes, at least one of which appears to be a previously unidentified transcription factor. The second positive regulatory element (\(+1\) to \(+35\)) also interacts with two separate protein complexes, both of which appear to be novel transcription factors. Deletion of either positive regulatory element markedly decreased expression of the target gene, suggesting that both positive regulatory elements are necessary for basal expression levels of TGF-\(\beta\) RII.

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is a homodimeric, 25-kDa peptide that plays a critical role in many cellular processes, including regulation of the cell cycle, cell differentiation, extracellular matrix synthesis, and modulation of the synthesis of other growth factors and their receptors (Massagué, 1990; Roberts and Sporn, 1990). Aberrant TGF-\(\beta\) function has been implicated in the pathogenesis of many diseases including arthritis (Lafyatis et al., 1989), hepatitis (Castilla et al., 1991), atherosclerosis (Chen et al., 1987; Grainger et al., 1993), and glomerulonephritis (Border et al., 1990). It has also been suggested that in some cases, diminished responsiveness to TGF-\(\beta\) may underlie the process of malignant transformation (Wakefield and Sporn, 1990). This decreased responsiveness to TGF-\(\beta\) could be caused by defects not only in TGF-\(\beta\) expression or activation but also by defects in the regulation of TGF-\(\beta\) receptors.

Much work has recently been directed toward characterizing the different types of TGF-\(\beta\) receptors and their intracellular signaling pathways as well as identifying their role in cell regulation and pathologies (Miyazono et al., 1994; Kingsley, 1994; Massagué, 1992). Three distinct cell surface receptors, types I, II, and III, have been cloned and characterized (Wang et al., 1991; Lopez-Casillas et al., 1991; Lin et al., 1992; Moren et al., 1992; Franzen et al., 1993; He et al., 1993; Attisano et al., 1993). Type I and type II receptors are transmembrane serine/threonine kinases that together are sufficient for signal transduction. The type III receptor is a transmembrane proteoglycan without intrinsic signaling ability but that may facilitate the binding of TGF-\(\beta\) to the type II receptor (Wrana et al., 1992). The most commonly held model for receptor activation proposes that the type I and type II receptors form a heteromeric complex that is essential for signaling responses (Wrana et al., 1994). It is therefore likely that a mutation in either receptor could result in a loss of responsiveness to TGF-\(\beta\) (Wrana et al., 1992; Bassing et al., 1994; Cárcamo et al., 1994).

Several tumor cell lines, including retinoblastoma, pheochromocytoma, neuroblastoma, and breast carcinoma, which are resistant to the growth inhibitory effects of TGF-\(\beta\), also fail to express the type II receptor (Park et al., 1994; Kimchi et al., 1988; Sun et al., 1994). In a previous study, our laboratory described a series of gastric cancer cell lines in which resistance to TGF-\(\beta\) correlated with gross structural mutations in the type II receptor gene. There were two notable exceptions in which Southern analysis yielded a gene without gross deletions or rearrangements, but no type II receptor protein or mRNA was produced. This suggested that abnormalities in transcriptional regulation of the type II receptor may also be involved in the escape from TGF-\(\beta\) growth control frequently observed in the process of carcinogenesis.

In order to study the transcriptional regulation of human TGF-\(\beta\) RII, we cloned and sequenced 1.9 kilobase pairs of the 5'-flanking region and used \(S1\) nuclease mapping and \(5'\) RACE PCR studies to identify five alternative transcription start sites within a region from \(-33\) to \(+57\). The human hepatoma HepG2 cell line was selected for this study because of its high level of TGF-\(\beta\) RII expression. Using a series of promoter-CAT deletion constructs transfected into HepG2 cells, we identified two distinct positive regulatory elements at \(-219\) to \(-172\) and \(+1\) to \(+35\). Electrophoretic mobility shift assays (EMSAs) and mutational analysis were then utilized to define two target sequences in the first positive regulatory element and one target sequence in the second positive regulatory element. One protein interacting with the first positive regulatory element may be an AP1 or CREB-like transcription factor. The other

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‡‡ The abbreviations used are: TGF-\(\beta\), transforming growth factor-\(\beta\); TGF-\(\beta\) RII, TGF-\(\beta\) type II receptor; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assays.
two target sequences do not share homology with any previously reported consensus sequences and may be recognized by novel transcription factor complexes.

MATERIALS AND METHODS

Cloning the Promoter Region of the TGF-β Type II Receptor—A human genomic library was obtained (Clontech) and screened by standard methods using the 5′-end fragment of the human TGF-β RII cDNA. Four overlapping clones of the promoter region were isolated, and subfragments were cloned into the pTZ18 vector (Pharmacia) and sequenced in both directions by the Sanger dideoxynucleotide method (U.S. Patent, sequence kit).

51 Nuclease Determination of Transcription Start Sites—A 32P-labeled DNA probe was generated spanning the putative transcription start sites(s). A plasmid containing the 2.7-kilobase pair Xbal-HindII genomic DNA fragment (10 μg) was digested by the Eagl restriction enzyme, which cut 138 base pairs 3′ of the published cDNA end (Lin et al., 1992) and left a 5′ overhang. The end was then labeled as described (Geiser et al., 1991), and the 5′ end of the probe was released by digestion with XbaI. The probe (100,000 cpm) was then hybridized to 80 μg of total RNA from human adenocarcinoma A549 and DU145 human prostatic adenocarcinoma cell lines in hybridization buffer (80% formamide, 400 mM NaCl, 0.1% SDS, 20 mM Tris, pH 7.4, and 1 mM EDTA) over 14 h. The nucleotide digestion was then done with 150 units of enzyme (Boehringer Mannheim) for 1 h at 37 °C. Samples were extracted with phenol/chloroform and ethanol precipitated and then loaded (in 50% formamide dye) onto a 6% denaturing acrylamide gel. RACE PCR to Determine 5′ RNA Ends—Total RNA from A549 (4 μg) was reverse-transcribed (Perkin-Elmer RT-PCR kit) at 42 °C for 1 h using random primers. The resulting cDNA was then tailed with dGTP using terminal transferase (Life Technologies Inc.) to create a 5′ end with an oligo(dG) stretch. This product was then amplified by PCR using the oligonucleotide 5′-GGCCGAGGAACTGTACAG (137 to +119 relative to the published cDNA end) and an oligo(dC) (Geiser et al., 1991). The amplified product was run on an agarose gel, blotted to nitrocellulose, and hybridized to a labeled upstream oligonucleotide (+137 to +119 relative to the amplified receptor product). The position of the probe was then amplified a second time using the oligonucleotide 5′-GAGTCGCGGTCCTGTTCCCCAG (118 to +98) and oligo(dC). The product was then blotted onto the AT cloning vector (Invitrogen), and individual clones were sequenced to determine the 5′ ends.

Nuclear Extracts—Nuclear extracts of HepG2 cells were prepared as described (Kim et al., 1999) with minor variations. Monolayers of HepG2 cells (3 × 105 to 5 × 105) were harvested by scraping, washed in cold phosphate-buffered saline, and incubated in 2 packed cell volumes of buffer A (10 mM Hepes, pH 8.0, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 200 mM sucrose, 0.5 mM phenylmethanesulfonyl fluoride, 1 μg of both leupeptin and aprotinin/ml, and 0.5% Nonidet P-40) for 4 min at 5°C. The crude nuclear extract was then precleared by addition of 20% glycerol to 5% before 1 h at 4°C. Nuclear extracts were harvested on a rocking platform at 4°C for 10 min and centrifuged at 15,000 × g for 10 min, and the supernatant was used as a template. Amplified DNA fragments were cloned into the promoterless CAT expression plasmid (pGEM-SVOCAT) (Kim et al., 1989) using HindIII and KpnI or XbaI restriction sites built into the oligonucleotides used for amplification. The sequences of the PCR-generated portions of all constructs were verified by DNA sequencing. The constructs were named pTβRII-nt, n is the distance in nucleotides from the transcription initiation site. The plasmid containing the CAT gene alone was used as the control. All CAT construct plasmids were purified by two sequential CsCl banding steps.

EMSA—Double-stranded oligonucleotides representing the first and second enhancer regions as well as a series of mutant oligonucleotides for each region were generated using an oligonucleotide synthesizer. Two oligonucleotides, TβRII(−219−172) and TβRII(+1/−50), were labeled using a fill in reaction with [α-32P]dCTP (50 μCi at 3,000 Ci/mmol) and the Klenow fragment of Escherichia coli DNA polymerase I. These fragments were then gel purified using a 6% polyacrylamide gel and autoradiography to locate the specific fragment. Binding reactions contained 10 μg of nuclear extract protein, 10 μg of fetal calf serum, 10 μg of leupeptin, 1 μg of aprotinin/ml, and 0.5% Nonidet P-40 for 5 min at 1°C. Competition reactions were performed by adding an unlabeled double-stranded oligonucleotide to the reaction mixture. Reactions were electrophoresed on a 6% Novex precast nondenaturing polyacrylamide gel at 100 V for 1 h in a 100 mM Tris/borate-EDTA buffer. Gels were vacuum dried and analyzed by autoradiography.

RESULTS

Isolation of 5′-specific Human TGF-β RII Genomic Clones—A 5′ segment of the TGF-β RII cDNA was used to screen a human lambda phage genomic DNA library for clones containing the promoter region. Four independent clones were isolated that overlapped in the 5′-untranslated and promoter regions. Restriction fragments of the lambda phage inserts were subcloned and sequenced to derive the promoter sequence shown in Fig. 1. The sequence obtained includes 1,883 nucleotides upstream and 35 nucleotides downstream of the 5′ most residue of the human TGF-β RII precursor cDNA (Lin et al., 1992).

Analysis of the 5′-flanking Region of the Human TGF-β Type II Receptor Gene—Examination of the human TGF-β RII gene sequence 5′ to the first major transcription initiation site designated as −1 reveals several notable features. No consensus CAAT or TATA boxes exist near the published cDNA end. The sequence GGGCGG is found at two positions −25 and −143 (Fig. 1) and corresponds to the sequence identified as a possible signal for the transcription factor Sp1, commonly found in promoters of viral and cellular housekeeping genes. The conserved sequence of the transcription factor AP1 is also identified at two positions, −124 (TGACTCA) and −195 (TGTAGCA).

Transcription Initiation Sites of the 5′-flanking Region of the Human TGF-β Type II Receptor Gene—To identify the true start site(s) of transcription, S1 nuclease mapping was used on RNA isolated from two cell lines known to express the type II receptor, A549 human lung adenocarcinoma and DU145 human prostatic adenocarcinoma. A DNA probe was generated that would hybridize to the first 138 nucleotides of the mRNA (from the published cDNA sequence) and any RNA upstream (5′) of the published sequence. As shown in Fig. 2A, several putative start sites were repeatedly seen that represent RNA ends both longer and shorter than the published cDNA 5′ end. These heterologous start sites appear to span 90 nucleotides, from +33 to +57 relative to the cDNA 5′ end. The same probe hybridized to trNA did not result in any protected bands, thus demonstrating the completion of the S1 nuclease digestion.

Although S1 nuclease mapping of RNA ends is frequently a good indicator of transcriptional start sites, the multiple bands revealed by this assay prompted examination of the 5′ ends of the TGF-β RII mRNA through S1 RACE PCR. Fig. 2B shows that heterogeneous clones representing heterologous start sites
were observed. Of the six clones sequenced, two were longer than the published cDNA (by 4 and 35 nucleotides) and four were shorter (by 30, 36, and 38 nucleotides). These results indicate a range of transcripts spanning from 235 to 138, confirming the heterogeneous nature of transcriptional start sites observed in the S1 assays. Whereas the cloned 5'-9 ends approximated the S1 nuclease bandsizes, some differences are evident that probably reflect deficiencies inherent in the assays (i.e. RNA secondary structure inhibiting reverse transcriptase in the 5'-9 RACE PCR or S1-sensitive sequence sites).

Cellular Expression Directed by the 5'-flanking Region of the Human TGF-β Type II Receptor Gene Reveals Two Distinct Positive Regulatory Elements and Two Negative Regulatory Elements—To study in vitro transcriptional regulation of TGF-βRII, we selected the human hepatoma HepG2 cell line, which has been shown to have the highest basal level of expression of TGF-βRII of any line studied. In order to identify the sequences essential for transcription of the TGF-βRII gene, progressively shorter fragments of the 5'-flanking region fused with the coding region of the bacterial CAT gene in the plasmid pGEM-SV0CAT were transfected into HepG2 cells. As seen in Fig. 3, construct pTβRIIP(-1240/+50) generated a similar level of CAT activity as the longer constructs, pTβRIIP(-1430/+50), pTβRIIP(-1670/+50), and pTβRIIP(-1883/+50). Transcription doubled upon deletion of the sequence between -1240 and -504 (pTβRIIP(-504/+50)), suggesting the presence of a weak negative regulatory element in this region. Eliminating the sequence -274 to -137 (pTβRIIP(-137/+50)) resulted in a dramatic drop in transcriptional activity pointing to a very strong positive regulatory element localized to this region. Deletion of the sequence -137 to -47 (pTβRIIP(-47/+50)) led to a 10-fold increase in activity pointing to the presence of a second strong negative regulatory element within this region. Finally, the shortest construct, pTβRIIP(-12/+50), demonstrated a significant level of activity compared with the control SV0CAT construct, indicating the presence of a second functional positive regulatory element. Of all constructs evaluated, pTβRIIP-274 and pTβRIIP-504 displayed the highest level of activity. The most dramatic change in activity was seen with deletion of the region -274 to -137 containing the putative first positive regulatory element. Examination of this region revealed at least two potential recognition sequences for transcription factors AP1 (-195; TTAGTCA) and Sp1 (-143; GGCGCGG, Fig. 1).

To further define the first positive regulatory element (-274 to -137), an additional series of CAT deletion constructs was created from nucleotide -274 to -47, each ending at +2 (Fig. 4). Deletion of the sequences from -274 to -219 led to significant changes in the level of activity. However, removal of
sequences from −219 to −200 decreased activity 20-fold, and further deletion to −172 abolished nearly all activity. This localized the positive regulatory element to within this 48-base pair sequence where there is an AP1-like binding site (−219; TTAGTCA; Fig. 1). Levels of transcription remained minimal with sequential deletion of nucleotides −217 through −100. However, when the region −100 to −67 was deleted, activity returned to previous levels, indicating the presence of a strong negative regulatory element in this region. Finally, the promoter fragment −47/+2 displayed a relatively high level of activity, which was significantly diminished by a substitution mutation of the Sp1 site, implicating a role for Sp1 in transcriptional activation from this region.

Identification of Nuclear Proteins Interacting with the First Positive Regulatory Element (−219 to −172)—To identify any nuclear proteins associating with the first positive regulatory element (−219 to −172), EMSA was performed as described above using a double-stranded 32P-labeled oligonucleotide containing the sequence for the first positive regulatory element. The reaction mixture was then electrophoresed on a polyacrylamide gel and viewed by autoradiography. The results are shown in Fig. 5A. In the absence of an unlabeled competitor oligonucleotide (lane 1), two strong upper bands (complex a and complex b) and multiple weak lower bands are apparent. It is clear that these bands represent specific binding of protein to the target oligonucleotide sequence, because binding to the labeled probe diminishes with increasing concentrations of unlabeled competitor (lanes 2–6). Complex a was competed out more readily than complex b, suggesting that complex b binds with greater affinity or to a longer target sequence.

To determine whether the observed AP1-like consensus sequences present in the first positive regulatory element are operative or whether other previously identified transcription factors might be responsible for the strong enhancer activity, a second mobility shift assay was performed. This time, the radiolabeled first positive regulatory sequence was incubated with HepG2 nuclear protein in the presence of a 100-fold molar excess of the consensus sequences for AP1, AP2, and CRE. As shown in Fig. 5B (lanes 3 and 5), both the AP1 and the CRE recognition sequences were successful in competing with the first positive regulatory element (−219/−172) for binding with complex a but not complex b or the proteins represented by the lower bands. AP2 failed to compete with the first positive regulatory element for any protein. The target sequences for AP1 and CRE are very similar. Complex a may therefore represent an AP1 or CRE-like factor. The data further suggest that a novel transcription factor complex or an uncommon consensus sequence is responsible for the specific protein-DNA binding represented by complex b.

Identification of Nuclear Protein Recognition Sequences within the First Positive Regulatory Element—To determine which sequences within the first positive regulatory element of the TGFβ RII promoter are required for specific binding to complexes a and b, we synthesized a series of mutant oligonucleotides derived from the first positive regulatory element (Fig. 6A). Each mutant oligonucleotide contained a 4-base pair substitution in which pyrimidine pairs were converted to purine pairs and vice versa. A mobility shift assay was then
performed using a radiolabeled first positive regulatory element probe incubated with HepG2 nuclear protein in competition with the series of mutant oligonucleotides. As shown in Fig. 6B, substitution of nucleotides −207 to −192 (lanes 5–8, M4–M7, ACTGTGTGACCTAGT) led to decreased competition for binding to complex b with the most marked reduction resulting from mutation of the central nucleotides −203 to −199 (lane 6, M5). The target sequence for complex b must therefore reside within this 16-nucleotide segment. Muta- tion of nucleotides −195 to −188 (lanes 8 and 9, M7 and M8, TAGT-CATT) led to decreased competition for binding to complex a. This region shares homology with AP1 and CREB consensus sequences. The first positive regulatory element therefore contains at least two distinct sequences demonstrating specific binding to different nuclear proteins (Fig. 6C).

Identification of Nuclear Proteins Interacting with the Second Positive Regulatory Element—To identify specific binding of proteins to the second positive regulatory element (+1 to +50), we employed the same strategy. An oligonucleotide representing the second positive regulatory element was synthesized and radiolabeled with 32P. HepG2 nuclear protein was combined with the labeled second positive regulatory element probe and incubated with increasing concentrations of unlabeled oligonucleotide. This assay was repeated several times, and representative results are shown in Fig. 7A. Two strong upper bands consistently appeared (complexes c and d) along with at least one weaker lower band (complex e) and represented specific binding because these bands progressively disappeared with increasing concentrations of unlabeled competitor.

To determine whether any of these bands represented known transcription factors, the second positive regulatory element probe was mixed with nuclear protein and incubated with the oligonucleotide target sequences for AP1, AP2, CREB, and Sp1 (Fig. 7B). There was no evidence of binding to any of these target sequences by complexes c, d, or e (lanes 3–6).

Identification of Nuclear Protein Recognition Sequences within the Second Positive Regulatory Element—Another set of oligonucleotides was synthesized in which the wild type second positive regulatory element was serially mutated with sequential 5-base pair substitution mutations (Fig. 8A). EMSA was performed using the second positive regulatory element probe, nuclear protein, and the mutant oligonucleotides. The results are shown in Fig. 8B. Competition for binding to complex c was abolished by mutation of nucleotides +16 to +20 (AAGTG, M4), whereas competition for binding to complexes c, d, and e was abolished by mutations through a longer sequence from +11 to +29 (Fig. 8B, M3–M6). Therefore, the second positive regulatory element appears to contain at least one nuclear protein recognition sequence from +11 to +29 and possibly a second nested within the first (Fig. 8C). This sequence does not match any published binding site for previously described transcription factors, suggesting that the second positive regulatory element, as well as the first positive regulatory element, may be regulated by an unidentified transcription factor(s).

Comparing Transcriptional Activity Directed by the First and Second Positive Regulatory Elements—To evaluate the relative contributions of the first and second positive regulatory elements to the overall promoter activity levels, another series of
CAT constructs was created containing various combinations of mutations and deletions in the two target sequences of the first positive regulatory element, designated X and Y, and the single target sequence of the second positive regulatory element, designated Z. Fig. 9A presents a schematic of the construct series. Construct −219/35 contained the wild type human TGF-β RII promoter sequence from nucleotide −219 to +35. The presence of a bar represents the intact wild type target sequence, and absence of the bar indicates that the sequence has been mutated. Thus, construct −219/+35M3 carried the promoter sequence with a 5-base pair substitution mutation in Z (+11 to +16, AGTTT–CTGGG). Similarly, −219M7/35 carried a substitution mutation in Y (−195 to −192, TAGT–GCTG), whereas −219M5/35 carried a mutation in X (−203 to −200, TGTG–GTGT). −219M5/+35M3 combined mutations in both X and Z. −219M7/+35M3 combined mutations in X and Y. Constructs −219M5/+2, −219M7/+2, and −219/+2 contained a truncated promoter sequence from −219 to +2 in which the second positive regulatory element was deleted. These CAT constructs were transfected into HepG2 cells, and the transcriptional activity was assayed. As anticipated, the highest level of transcription occurred with both intact first and second positive regulatory elements (−219/+35).

Isolated mutations of sequences Y and X in the first positive regulatory element (−219M7/+35 and −219M5/+35, respectively) or of sequence Z in the second positive regulatory element (−219/+35M3) caused only a small decrease in activity. Among the three individual mutations, the largest decrease in activity to 82% of baseline, occurred with the isolated mutation of sequence Y, which contains the putative AP1/CRE site. Mutations in the first positive regulatory element were then paired with mutation of the second positive regulatory element and, as expected, led to much more dramatic decreases in transcriptional activity. When both X and Z were mutated (−219M5/+35M3), activity fell to 56% of baseline levels. Combined Y and Z mutations (−219M7/+35M3) decreased activity to 14% of baseline. Again, mutation of sequence Y led to a more significant decrease in transcription than mutation of X. Deletion of the second positive regulatory element decreased transcription to a greater degree than simply mutating the target sequence Z, confirming that sequence Z is essential to activity of the second positive regulatory element but suggesting that the mutation was not sufficient to inactivate the entire target sequence. Comparing all constructs, the lowest level of activity occurred with mutation of both the target sequences for the first and second positive regulatory elements. Thus, the two target sequences in the first positive regulatory element and the single target sequence in the second positive regulatory element are critical to confering enhancer activity, and both positive regulatory elements interact to contribute significantly to basal promoter activity.

**DISCUSSION**

In 1985, Sornn and Roberts first suggested that defects in the TGF-β receptor system might, in some situations, account for resistance to its effects on growth in some situations. There is now substantial evidence to support this early speculation. For example, human esophageal epithelial cells stably transfected with cyclin D1 are resistant to the growth inhibitory effects of TGF-β1; these cells express normal levels of the type I receptor.
but markedly reduced levels of the type II receptor (Okamoto et al., 1994). Murine myeloid cells infected with the src oncogene express significantly higher levels of the type II receptor and show increased sensitivity to the growth inhibitory effects of TGF-β1 (Birchenall-Roberts et al., 1991). Transfecting human breast carcinoma and hepatoma cells lacking type II receptor with wild type TGF-βRII restores sensitivity to TGF-β and decreases tumorigenicity in transplanted breast cancer cells (Sun et al., 1994; Inagaki et al., 1993). Recently, we have reported that a majority of human gastric carcinoma cell lines acquired resistance to growth inhibition by TGF-β and possessed structural mutations in TGF-βRII (Park et al., 1994). Instances in which cells failed to express RII mRNA despite the absence of apparent structural deletions or rearrangements of the gene introduced the possibility of a promoter defect and first suggested that transcriptional regulation may play an important role in controlling TGF-βRII expression. Most recently, Markowitz et al. (1995) have identified a subset of colon cancer cell lines in which defective DNA repair mechanisms consistently lead to characteristic mutations in the TGF-βRII gene causing resistance to growth inhibition by TGF-β.

**Fig. 7.** Detection of nuclear proteins that interact with the second positive regulatory element of the TGF-β type II receptor promoter. A, EMSA. Labeled double-stranded oligonucleotide +1/+50 was incubated with HepG2 nuclear extract, and the resulting DNA-protein complexes were resolved by native polyacrylamide gel electrophoresis and autoradiography. Four bands are visualized. The two upper bands were consistently present with multiple repetitions of the assay. Lower bands of higher mobility were variably present at variable intensities. Specific binding is demonstrated by progressive disappearance of the bands with increasing concentrations of unlabeled competitor oligonucleotide. B, the same labeled oligonucleotide and nuclear extract in competition with consensus sequences for AP1, AP2, CRE, and Sp1. Lane 2 shows competition with unlabeled +1/+50 oligonucleotide.

**Fig. 8.** Identification of second positive regulatory target sequences. A, sense strand sequence for series of mutant oligonucleotides. WT gives the wild type sequence. M1-M8 contain the 5-nucleotide base substitutions as shown. B, EMSA performed with labeled +1/+50 double-stranded oligonucleotide incubated with HepG2 nuclear extract in competition with mutant oligonucleotides. C, wild type sequence of second positive regulatory element showing the target sequences for complex a2, b2, c2, and d2.

**Fig. 9.** Relative contribution of first and second positive regulatory elements to overall promoter activity. A, schematic representation of series of TGF-β type II receptor promoter-CAT constructs. X and Y mark the positions of the two target sequences within the first positive regulatory element, and Z marks the position of the second positive regulatory target sequence. The presence of the shaded bar signifies the wild type sequence, and its absence indicates that the sequence has been mutated. The arrow marks the transcriptional start site +1. B, CAT assay results after transfection of constructs into HepG2 cells and 72 h of incubation. The bottom row shows unacetylated forms, the middle row shows monoacetylated forms, and the top row shows diacetylated forms.

**Fig. 10.** Promoter Region of the Human TGF-β RII Gene.
vation of TGF-β RII mRNA may be a common occurrence in epithelial malignancies. By permitting escape from regulation by TGF-β, such mutations confer a strong growth advantage to affected cell populations. Decreased transcription of RII mRNA can have the same effect as mutation of the structural gene.

In this report we present an expanded sequence for the promoter region of TGFβ RII and describe the existence of at least five distinct regulatory regions including two positive regulatory elements (−219 to −172 and +1 to +35) and two negative regulatory elements (−1240 to −504 and −100 to −67; Fig. 10) in addition to the core promoter region (−47 to −1; Fig. 10). One negative element located between 0.5 and 1.2 kilobase pairs upstream from the transcriptional start site(s) was not extensively examined in this study. Deletion of this region increased transcription approximately 2-fold. The first positive regulatory element (−219 to −172) is required for basal transcriptional activity because its deletion allows the powerful second negative regulatory element (−100 to −67) to repress transcription completely regardless of the presence of the core promoter and second positive regulatory element (see −137/+50 in Fig. 3 and −172/+2 in Fig. 5). Transcription directed by the core promoter region is dependent on an Sp1 consensus sequence at +25. Mutation of this sequence reduces transcription by 70% (−475Sp1 mt/+2 in Fig. 4).

Two distinct protein complexes demonstrate specific binding to the first positive regulatory element. Complex a, which may be identical to AP1 or CREB, seems to bind to sequence Y (−196 to −189), and complex b binds to sequence X (−207 to −197). The second positive regulatory element is also recognized specifically by two different protein complexes. In this case complexes c, d, and e all bind to the same target sequence Z (+11 to +25), although complex c appears to bind to a more limited portion (+16 to +21).

Methylation analysis reveals that the two positive regulatory elements cooperate with the promoter region to sustain basal levels of promoter activity. Maximum levels of transcription were achieved with intact first and second positive regulatory elements. Mutation of individual target sequences in either first or second positive regulatory element impaired transcription only slightly; however, mutation of both first and second positive regulatory sequences together led to marked declines in transcriptional activity (Fig. 9, A and B).

This study presents sequencing data for the human TGF-β RII promoter region that agrees well with a previously published report (Humphries et al., 1994) and also extends the known sequence an additional 930 base pairs upstream. However, unlike the earlier report, this study shows the heterogeneous nature of the transcriptional start sites and presents functional data regarding the regulation of transcription from the human TGF-β RII promoter region. The human TGF-β RII promoter is similar to other promoters lacking TATA and CAAT boxes in that transcription is initiated from multiple start sites separated by as much as 90 nucleotides surrounding the previously published cDNA 5′ end (Lin et al., 1992). The identification of start sites at +30 and further downstream complements the recognition of a positive regulatory element at +11 to +25. Sequence analysis reveals multiple sites homologous to known transcription factor consensus sequences. Two putative Sp1 sites are located at −143 and −25. The −25 site is responsible for at least 70% of basal activity from the −47/−1 core promoter region. Two putative AP1/CRE binding sites have also been recognized at −669 and −196. The −669 site is located in a region that contains a weak negative regulatory element, but further analysis is required to determine if this site is functional. The −196 site is located in the first positive regulatory element and corresponds to the binding site for complex a (Fig. 6C). EMSA performed with the labeled first positive regulatory element and HepG2 nuclear extract in competition with unlabeled AP1 and CRE consensus sequences confirmed that complex a specifically bound to AP1/CRE-like sequences. Purified AP1 and CRE/ATF protein also demonstrated specific binding to the first positive regulatory element.

This study has shown that the promoter region of the human TGF-β RII gene contains multiple components including two positive regulatory elements and two negative regulatory elements in addition to the core promoter. Such a high level of structural complexity suggests a correspondingly high level of functional intricacy. Multiple nuclear proteins have been shown to bind specifically to the two positive regulatory elements, and it is likely that these proteins include previously unidentified transcription factors. Studies are currently underway to define the activity of the TGF-β RII promoter in different cell lines as well as to purify and characterize the involved binding proteins.

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