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The Promoter Structure of TGF-β Type II Receptor Revealed by ''Oligo-capping'' Method and Deletion Analysis

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The promoter of TGF-β type II receptor lacks TATA box or CAAT box. In order to define the exact transcriptional start site(s), we used ''oligo-capping'' method developed by ourselves. The major transcripts were started from GAA located between −35 and −33 relative to 5′ end of cDNA. Other minor transcripts started from −4, +11 and +17. Deletion analysis of this promoter region revealed that it contained two adjacently located promoters (P1 and P2) capable of acting by itself. The P1 is located between −22 and +55 which covers the minor transcriptional start sites described above. The P2 is located just upstream of the P1 between −137 and −22. The results showed all of the transcripts were started either from P1 or P2.

The transforming growth factor-β (TGF-β) has a strong growth inhibitory activity to various types of cells and plays important roles in development and differentiation. The TGF-β signal is transduced by type I and type II TGF-β receptors (TGF-β RI and TGF-β RII) (1). Both receptors are membrane proteins which have the serine/threonine kinase (2-5). Because they are the major components for transducing the TGF-β signal, it is important to understand the regulation of the expression of these receptors.

Humphries et al (6) cloned the promoter region of TGF-β RII. This promoter have no TATA box nor CAT box. The region contains two Sp1 sites at −25 and at −142 relative to cDNA 5′ end (2) and two Ap1 sites at −185 and at −671. The sequence around +1 is somewhat homologous to the initiator element (5′-GCC CTC ATT CTG GAG AC-3′) which was found in mTdT gene (2, 7).

In order to understand transcriptional regulation of TGF-β RII expression, it is an indispensable step to determine the mRNA start sites of this promoter. We previously developed a method called ''oligo-capping'' which enable us to determine the precise 5′ end of mRNAs (8). In this report, we determined the mRNA start site of TGF-β RII gene and analyzed the promoter region for TGF-β RII gene which consists of 1100 bp upstream and 330 bp downstream of the cDNA 5′ end (2). Our results suggest that this promoter consists of two adjacently located regions which can act as promoter by itself.

MATERIALS AND METHODS

Oligo-capping. Oligo-capping was performed as described (8) with some modifications. In brief, 5 to 10 μg of polyA+ RNA from HepG2 cells was treated with 1.2 units of bacteria alkaline phosphatase (TaKaRa) in 100 μl of 100 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol with 100 units of RNasin (Promega) at 37°C for 40 min. After extraction with phenol:chloroform = 1:1 twice and ethanol precipitation, the polyA+ RNA was treated with 20 units

[1] It is with sadness that we announce the death of Professor Nobuo Yamaguchi on 27 January 1996.
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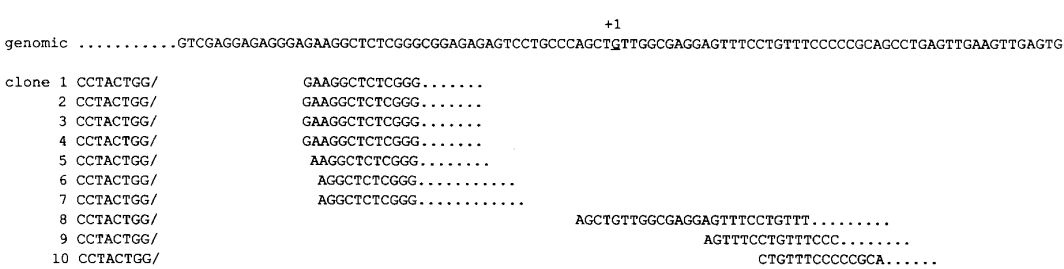

FIG. 1. The 5′ sequence of the oligo-capped mRNA of TGF-β type II receptor. The sequences derived from 5′-oligo are aligned with each other. The sequences corresponding TGF-β type II receptor gene were aligned along with the genomic sequence shown above. Gaps shown between the sequence derived from 5′-oligo and the sequence corresponding to TGF-β type II receptor does not exist in real sequence. +1 shows the 5′ end of cDNA (2).

of tobacco acid pyrophosohatase (purified by authors, 5) in 100 μl of 50 mM sodium acetate (pH 5.5), 1 mM EDTA, 5 mM 2-mercaptoethanol with 100 units of RNasin at 37°C for 45 min. After phenol:chloroform extraction and ethanol precipitation, 2 to 4 μg of the BAP-TAP treated polyA+ RNA were ligated with 0.4 μg of 5′-oligo (KM-02; 5′-AGC AUC GAG UCG GCC UUG UUG GCC UAC UGG-3′) using 250 units of RNA ligase (TaKaRa) in 100 μl of 50 mM Tris-HCl (pH7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.5 mM ATP, 25% PEG8000 with 100 units of RNasin at 20°C for 3 hour to 16 hour.

RT-PCR. A antisense primer +120 (5′-ACA ACG TTC GAG GGA AGC TGC ACA GG-3′) which correspond +118 to +136 of TGF-β RII cDNA (2) plus a HindIII site or random primers were used to make cDNA of oligo-capped mRNA. The 5′ end of TGF-β RII mRNA was amplified using a part of KM-02 (5′-AGC ATC GAG TCG GCC TTG TTG-3′) and primer +55 (5′-GCA AGC TTG ACT CAC TCA ACT TCA ACT CAG CGC T-3′) which correspond +22 to +55 of TGF-β RII promoter. Amplified band are cloned and sequenced. The clones which had the sequence GCC TAC TGG (which corresponds 3′ 9 bases of KM-02) at its 5′ end were scored as having the 5′ end of the mRNA.

Transfection and luciferase assay. The CV1 cells (9) were transfected with 2.5 μg of various promoter mutants and 1 μg of pACT-βGAL (10) plasmid DNA using Lipofectamine (GIBCO-BRL). After 44 hours of transfection, the CV1 cells were washed with 1 × PBS for two times. The cells extracts were prepared with 200μl of 1× Lysis buffer, mixed 15μl of cell extract with 100μl Luciferase Assay Reagent (Promega). The luciferase activity was assayed with a luminometer (Berthoid LB9500C) by measuring the light produced for a period of 10 seconds.

βgal assay. The cell lysate (50 μl) was mixed with 50 μl of 15 mM chlorophenol red-galactopyranoside (CPRG, Boehringer Co.), 100 μl of 1×Z buffer, and 100μl of H_2O and was incubated at 37 °C for 30 minutes.

RESULTS AND DISCUSSION

Identification of transcriptional start sites is essential for establishing the promoter region of a gene, especially when it is a ‘‘TATA- less’’ promoters, in which the start sites are not easily predictable. We previously developed ‘‘oligo-capping’’ method (8) which allows us to replace the cap structure with an oligonucleotide. Here we used it to determine the transcriptional start sites of TGF-β RII gene. In brief, polyA+ RNA from HepG2 cells were first treated with bacterial alkaline phosphatase (BAP). Then, the RNA was treated with tobacco acid pyrophosphatase (TAP). This sequential treatment with BAP and TAP leaves 5′ phosphate at the capped end of mRNA, while none-capped end becomes 5′ OH. Further treatment with RNA ligase and oligonucleotides (5′-oligo) allows the ligation only at capped end. The 5′ portion of TGF-β RII mRNA was then amplified with RT-PCR using a part of 5′-oligo and a part of TGF-β RII mRNA as primers (for detail see materials and methods). The amplified band was cloned and their sequence were determined.

Of the ten clones which we sequenced, seven started from GAA located from −35 to −33 relative to cDNA 5′ end. Other three clones started from −4, +11 and +17 (Fig. 1). Humphrie et al (6) used a RNA protection assay to determine transcriptional start sites. They observed one major transcriptional start site at or near cDNA 5′ end (+1) with several minor starts from upstream (6). Bae et al (11) found heterologous start sites which span from −33 to +57

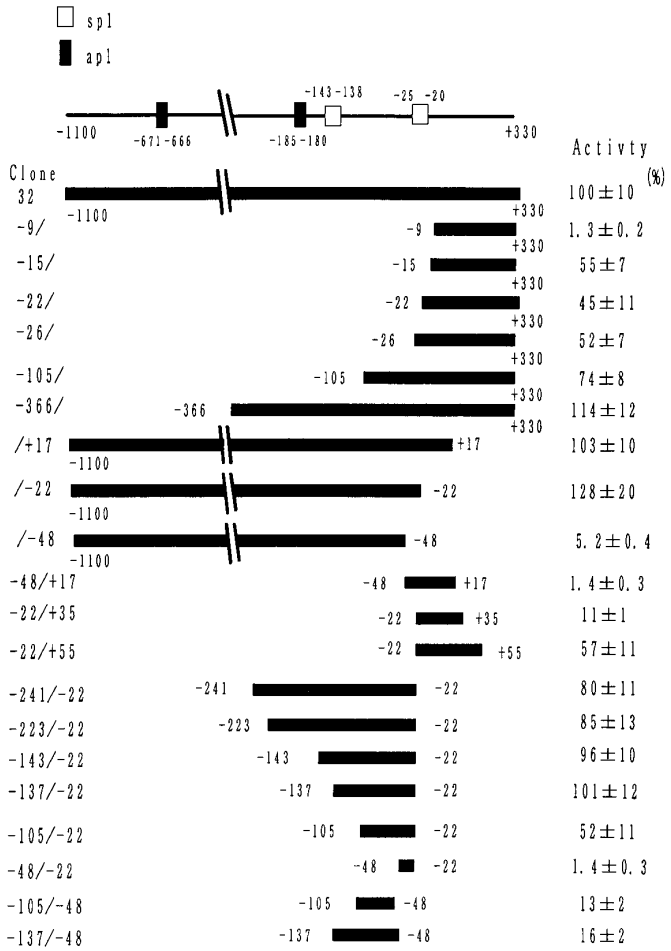

FIG. 2. Deletion analysis of TGF-β type II receptor promoter. The activity of pGL32 which was about 36 times higher than that of pGL-Basic (Promega) was defined as 100%. The data of luciferase assay was normalized against the data of β-gal assay.

using S1 analysis. They also found, by 5′ RACE method, transcriptional start sites at −35, −4, +30, +36 and +38. Our results were somewhat similar to that of Bae et al, although down stream transcripts starts more upstream than those found by Bae et al.

In order to analyze the structure of the TGF-β RII promoter, we cloned a 1430 bp human genomic DNA which consists of 1100 bp upstream and 330 bp downstream of the 5′ end of cDNA (2). The sequence of 1100 bp upstream region was identical with those reported by Humphrie et al (6) up to −888. The fragment was inserted into pGL-basic (Promega) and the resultant clone was named pGL32. The cell lysate from pGL32 transfected CV1 cells showed the 36 times higher luciferase activity than that of pGL-basic transfected cells (data not shown). This result is consistent with that of Humphrie et al (6).

We have made various deletion mutants of pGL32 and assayed for their promoter activity by transient transfection assay. The result is shown in Fig. 2. When the 5′ deletion mutants were assayed, 5′ boundary of the promoter located between −15 and −9. In contrast, the 3′ deletion mutants revealed that 3′ end of the promoter located between −48 and −22. This results suggest the presence of the two active promoters within this region. We designated the

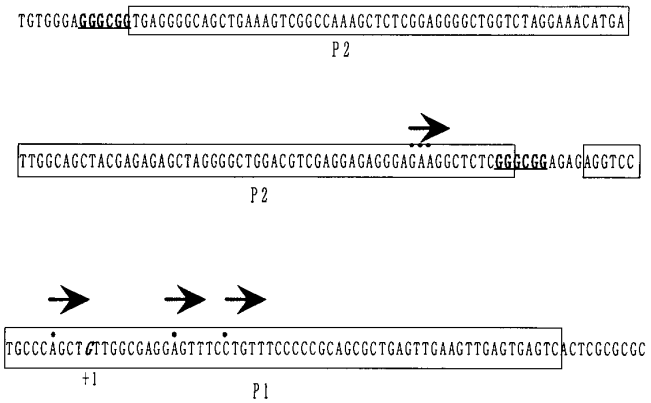

FIG. 3. Putative structure of promoter region of TGF-β type II receptor gene. P1 and P2 were boxed. mRNA start sites revealed by ''oligo-capping'' method were shown by a dot and a arrow. Sp1 sites were underlined. Note that Sp1 sites are not included in P1 or P2. 5′ end of cDNA (+1) was shown as italic.

promoter which is close to the start codon as promoter 1 or P1, and the other promoter which is distant to the start codon as promoter 2 or P2.

We then tried to locate the 3′ end of P1 and 5′ end of P2. As shown in Fig. 2, the 3′ end of P1 seemed to be located between +35 and +55. The 5′ end of P2 seemed to be located between −137 and −105. Thus, P1 seemed to be locate within a 71 bp region which spans from −15 to +55. P2 seemed to be contained within a 116 bp region which spans from −137 to −22.

Our results suggest that P1 and P2 regions may support transcription initiations by itself in transient assay. The transcriptional start sites we found were also located with in these two regions. The major start sites which located from −35 to −33 contained within P2. Other sites were contained within P1 (Fig. 3). This finding is consistent with the notion that P1 and P2 are acting as the transcriptional initiator within the cell.

Both P1 and P2 did not contain Ap1 or Sp1 binding sequence. Thus, it appears that Ap1 and Sp1 sites in these region do not have significant effect to the transcription activity at least in our assay system. This is somewhat unexpected, because the Sp1 site acts as a major activity determinants in many of the TATA less promoters. P1 contains the sequence which is similar to the initiator element found in mTdT gene (7). Although this sequence alone was not enough to support the full activity of P1 (see the results of clone −48/+17), it is consistent with the notion that P1 is acting as transcriptional initiator.

In summary, the promoter of TGF-β RII gene consists of two adjacently located promoters. Elucidating the promoter structure would be the first step for understanding the expression of this important gene.

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