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# Structure of the Gene Encoding the Human Differentiation-Stimulating Factor/Leukemia Inhibitory Factor Receptor[1]

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**The human gene encoding the differentiation-stimulating factor (D-factor)/leukemia inhibitory factor (LIF) receptor was cloned and its structure was analyzed. The gene spans more than 70 kilobases and contains 20 exons. The D-factor/LIF receptor can be subdivided into several regions: cytokine receptor homologous domain 1, an Ig-like domain, cytokine receptor homologous domain 2, three fibronectin type III domains, a transmembrane domain and a cytoplasmic region. Each domain of the receptor is encoded by a set of exons. There is a TATA sequence upstream of the transcription initiation site. One unit of the *Alu* sequence is present in the 5′ flanking region. An NF-IL6 site is located 31 bases downstream of the transcription initiation site.**

**Key words: D-factor/LIF receptor, gene structure, TATA box.**

We and others have purified and cloned the differentiation-stimulating factor (D-factor)/leukemia inhibitory factor (LIF) as a cytokine that induces macrophage differentiation and inhibits the proliferation of myeloid leukemia M1-T22 cells (*1-3*). The D-factor has a wide variety of biological activities besides its actions on normal and leukemic hematopoietic cells (*4, 5*). It is necessary for development of the placenta. In D-factor-gene disrupted mice, implantation of the blastocyst is defective (*6*) and the numbers of hematopoietic stem cells are reduced in the spleen and bone marrow (*7*). Targeted disruption of the D-factor receptor gene causes placental, skeletal, neural, and metabolic defects, and thereby results in perinatal death (*8, 9*). The D-factor receptor is a member of the hematopoietin receptor family (*4, 10, 11*). It is structurally most similar to gp 130, granulocyte colony-stimulating factor (G-CSF) receptor, IL-12 receptor, and oncostatin M (OSM) receptor (*12-17*). Cardiotrophin-1 and human OSM can act through the D-factor receptor (*18, 19*). The ciliary neurotrophic factor receptor complex also includes the D-factor receptor (*20*).

The soluble D-factor receptor is present in normal mouse serum and its amount increases about 30-fold in the late stages of pregnancy (*21, 22*). We isolated mouse cDNAs encoding the membrane receptor and the soluble receptor for the D-factor, from a cDNA library prepared from the liver of a pregnant mouse (*11, 23*). The mRNA encoding the soluble receptor has the insertion of a nucleotide sequence, which is absent in the cDNA encoding the membrane receptor, and contains a stop codon and polyadenylation signals. Transcripts utilizing these signals are 3 kb in size, and are very abundantly present in the liver, but not detectable in other tissues. The pregnancy-related change in the expression of the 3 kb mRNA in the liver parallels the change in the level of the soluble D-factor receptor in the mouse serum. In the placenta, in contrast to in the liver, the 5 kb mRNA encoding the membrane receptor continues to increase during pregnancy. It is not known yet whether or not the soluble D-factor receptor is present in human serum. We analyzed cDNAs encoding the human D-factor receptor (unpublished results) and found that there were many kinds of cDNAs other than that encoding the membrane receptor which has been reported (*10*). To elucidate the mechanisms of expression of the D-factor receptor gene and production of various kinds of mRNAs, we isolated the human chromosomal gene for the D-factor receptor. In this study, we determined the exon/intron organization of the gene and characterized its promoter region.

## MATERIALS AND METHODS

*Cloning of the Human Chromosomal Gene for the D-Factor Receptor*—The cDNA for the D-factor receptor was obtained by the PCR method with primers 5F and 4R, and the human liver cDNAs (Clontech) as templates. The synthetic oligonucleotides used in this work are summarized in Table I, and were purchased from Takara Shuzo Corp. The nucleotide sequences of the primers were based on the cDNA sequence reported by Gearing *et al.* (*10*).

The isolated cDNA was used as a probe to screen a human genomic library (Clontech) constructed with the EMBL3 Sp6/T7 vector. A genomic clone containing the 5′ flanking region of the gene was isolated by plaque hybridization using the cDNA fragment which was obtained by 5′ RACE.

*Polymerase Chain Reaction (PCR)*—The lengths of introns were determined by PCR with pairs of oligonucleotide

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Abbreviations: bp, base pair; CRH, cytokine receptor homology; D-factor, differentiation-stimulating factor; FNIII, fibronectin type III; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin M; RACE, rapid amplification of cDNA end; RT-PCR, reverse transcription-polymerase chain reaction.

primers that were in two adjacent exons. Long-range PCR was carried out with a GeneAmp PCR System 9600 (Perkin-Elmer) for 30 cycles (denaturation for 20 s at 94°C and extension for 15 min at 68°C), using a Long and Accurate PCR Kit Ver. 2 (Takara) and phage DNA or chromosomal DNA from human placenta. The products were analyzed by electrophoresis on a 1% agarose gel. They were subcloned into pCRII vectors (Invitrogen). Exon/intron junctions were sequenced with an ALF DNA sequencer (Pharmacia) using double-stranded plasmid DNAs. The length of intron 5 was determined by long-range PCR using 750 ng of human placental DNA as a template. The junctions on both sides of introns 1 and 5 were determined by amplification with an exon-specific primer and a primer for the arms of the vector phage.

*Rapid Amplification of the cDNA End (RACE)*—5′ RACE was carried out using a 5′ AmpliFINDER RACE KIT (Clontech). The first-strand cDNA was synthesized from 1 μg of poly(A)$^+$ RNA from human placenta using primer 21R. The single strand cDNA was ligated to the AmpliFINDER anchor for 48 h using T4 RNA ligase (NEW ENGLAND Biolabs). The reaction mixture (20%) was subjected to the first round PCR using primer 21R and the anchor primer. Two percent of the product was subjected to the second round PCR using primer 52R and the anchor primer.

## RESULTS AND DISCUSSION

*The cDNA Sequence of the Human D-Factor Receptor*—We screened a human placenta cDNA library and a human liver cDNA library with a probe, which was prepared by the RT-PCR method using primers 1F and 3R (Table I). A total of 15 cDNA clones encoding the human D-factor receptor were isolated, but none was a full length one. A cDNA clone which covered the entire coding sequence was obtained by the PCR method with primers 5F and 4R, and liver cDNAs as templates. The sequence of the cDNA was analyzed and compared with the cDNA sequence published by Gearing *et al.* (*10*). Three nucleotide differences, which caused three amino acid differences, were found between the two clones. In the published sequence, there is a C at position 329, T at position 2079, and T at position 3287, which are a T, C, and A at the corresponding positions in our clone. The former clone encoded a histidine, isoleucine, and tryptophan, while the latter clone encoded a tyrosine, threonine, and arginine. These differences could be due to polymorphism in the genomic sequence. The sequence of our clone was confirmed by another experiment in which two cDNA fragments were prepared by the RT-PCR method using mRNAs from human kidney 293 cells, and primers (5F, 2R, 1F, and 4R). Furthermore, it was confirmed that our human cDNA was functional by expression of the cDNA in mouse myeloid leukemia WEHI-3B cells. The cells expressing the human D-factor receptor, as well as those transfected with the cDNA for the mouse D-factor receptor, were induced to differentiate by treatment with the human D-factor (*24*).

To identify the transcriptional initiation site and obtain the cDNA for the 5′ untranslated region, we carried out 5′ RACE, as described under "MATERIALS AND METHODS." A single band was obtained after the second round PCR using a nested primer. We subcloned and sequenced 12 clones. Three cDNAs began at 164 bp upstream of the translation initiation site (position 179 in the published cDNA, *10*). Seven cDNAs began at 2 bp further downstream. cDNA which began at one bp upstream and one bp downstream of these cDNAs were also present. We found that the 25 bp sequence of the reported cDNA end (*10*) was derived from the adaptor used for construction of the cDNA library (*14*).

*Cloning of the Human D-Factor Receptor Gene*—Screening of $10^6$ clones of the human genomic library with the 3.4 kb cDNA encoding the human D-factor receptor yielded nine positive clones, that were isolated and characterized. The restriction map of these clones was determined using *Eco*RI. To isolate the chromosomal gene coding for the 5′ portion of the D-factor receptor, the genomic library was screened with the 0.6 kb *Eco*RI fragment of λ2, which contained the intron 1 sequence of the gene. Two clones were positive and thus characterized. These clones contained the 5 or 7 kb upstream of the λ2 sequence. However,

TABLE I. **PCR primers used to amplify introns and cDNAs.**

| Primer | Sequence | Intron No. |
|---|---|---|
| 51 F | 5′-TAATCCCAGCTCAGAAAGGGAGC-3′ | 1 |
| 55 F | 5′-TCCAGCTTTAAAGTGACTTTGGGC-3′ | |
| 56 R | 5′-TCCCCAATCGCTTTTACTTTCCAGTG-3′ | |
| 21 R | 5′-AGAACAGTTCCACACTTGCA-3′ | |
| 5 F | 5′-AGGACTGACTGCATTGCACA-3′ | 2 |
| 21 R | 5′-AGAACAGTTCCACACTTGCA-3′ | |
| 37 F | 5′-TTGCAAGTGTGGAACTGTTCTTGG-3′ | 3 |
| 45 R | 5′-ATCACCATGTGAAAGAGCTGGAAT-3′ | |
| 22 F | 5′-GAAAACAGGTCCCGTTCTTG-3′ | 4 |
| 23 R | 5′-CCTGTCGTTCCACTTTAGGT-3′ | |
| 38 F | 5′-ATACCTAAAGTGGAACGACAGGGG-3′ | 5 |
| 39 R | 5′-CACAGGGCTCCAGTCACTCCACTC-3′ | |
| Sp6 | 5′-GGCCATTTAGGTGACACTATAGAA-3′ | |
| T 7 | 5′-GCCTAATACGACTCACTATAGGGA-3′ | |
| 42 F | 5′-AAGAGTGGAGTGACTGGAGC-3′ | 6 |
| 43 R | 5′-GTTCCACTACTTGCAGAAACAG-3′ | |
| 24 F | 5′-CTGTTTCTGCAAGTAGTGGA-3′ | 7 |
| 25 R | 5′-AGTTGTTGAGGAGTATCTGG-3′ | |
| 49 F | 5′-CCAGATACTCCTCAACAACT-3′ | 8 |
| 50 R | 5′-GTTGATTGTGATCGACCCAG-3′ | |
| 26 F | 5′-CTGGGTCGATCACAATCAAC-3′ | 9 |
| 27 R | 5′-GAATGAAGTAGGAGTATGGG-3′ | |
| 41 F | 5′-CTTGGCATTTACCAGGCAAC-3′ | 10 |
| 33 R | 5′-GCTCCATTTGCTCCATTTCC-3′ | |
| 7 F | 5′-GGAAATGGAGCAAATGGAGC-3′ | 11 |
| 8 R | 5′-TCCAAGTATCAGGCCCCTTT-3′ | |
| 28 F | 5′-AAAGGGGCCTGATACTTGGA-3′ | 12 |
| 29 R | 5′-CGCTATTTTGGAAGGTGGTG-3′ | |
| 9 F | 5′-CACCACCTTCCAAAATAGCG-3′ | 13 |
| 12 R | 5′-AGGAGAATCCCCTTTCCCAT-3′ | |
| 13 F | 5′-CCCTCAAACAGCACTGAAAC-3′ | 14 |
| 14 R | 5′-CTTATACCTGGTCGAAACTC-3′ | |
| 15 F | 5′-CGCTCCATGATTGGATATATA-3′ | 15 |
| 16 R | 5′-ATTTGGTGCAACAATGGGAG-3′ | |
| 17 F | 5′-CTCCCATTGTTGCACCAAAT-3′ | 16 |
| 18 R | 5′-AACTTTTATGTCAGAACGACC-3′ | |
| 6 F | 5′-TGGTCTTGCGAGCCTATACA-3′ | 17 |
| 3 R | 5′-AGGATACTTGTCACCACTCC-3′ | |
| 34 F | 5′-GGAGTGGTGACAAGTATCCT-3′ | 18 |
| 35 R | 5′-TGGAATATCAGGGTAGAAGG-3′ | |
| 20 R | 5′-CCTCCTACAGGGTCATTTTC-3′ | 19 |
| 36 F | 5′-CCTTCTACCCTGATATTCCA-3′ | |
| 21 R | 5′-AGAACAGTTCCACACTTGCA-3′ | 5′-RACE |
| 52 R | 5′-TCATCTGTGCAATGCAGTCAGTCC-3′ | |
| 1 F | 5′-GACCCCAACATGACTTGCGACTACGTCATTAAGTG-3′ | cDNA |
| 2 R | 5′-GGTTCCGACCGAGACGAGTTACACCACTTAATGA-3′ | |
| 3 R | 5′-AGGATACTTGTCACCACTCC-3′ | |
| 4 R | 5′-GAAGTGACACGGTGACACTG-3′ | |
| 5 F | 5′-AGGACTGACTGCATTGCACA-3′ | |

they did not encode exon 1. A clone coding exon 1 was isolated by screening of the genomic library with the cDNA fragment which was obtained by 5′ RACE.

*Structure of the Human D-Factor Receptor Gene*—The gene is split by 19 introns (Fig. 1). The exon-intron junctions were sequenced, and all of the splice donor and splice acceptor sites conform to the GT/AG consensus (Table II). The lengths of introns were determined by PCR. The length of intron 1 was estimated to be 23 kb, because the 9 kb band was amplified using primers 55F (7 kb downstream of exon 1) and 56R (7 kb upstream of exon 2), and the genomic DNA as a template. Assuming that the length of intron 1 is 23 kb, the gene spans approximately 70 kb. The gene consists of 20 exons. The 5′ untranslated region is encoded by exon 1, and exon 2 corresponds to the signal sequence containing the initiator ATG codon. Exons 3 to 6 and 8 to 11 encode the CRH1 and CRH2 domains (*4*), respectively. The Ig-like domain is encoded by a single exon, 7. Each of the three FNIII domains is encoded by two exons (exons 12 to 17). Exon 18 encodes the hydrophobic transmembrane domain. The cytoplasmic domain is encoded by exons 19 and 20. The translation termination codon is encoded by exon 20, which is followed by a large 3′ untranslated sequence. At present, the 3′ end of the mRNA for the D-factor receptor is unknown. We isolated a cDNA clone including an extra 1.8 kb downstream of the published cDNA sequence. Although it does not contain a poly A tail or polyadenylation signals, it seems to include the near end, since the estimated length of the cDNA, 5.4 kb, is similar to the mRNA sizes reported by Gearing *et al.* (*10*); they detected two major RNA species of ~6 and ~4.5 kb, and a minor 5 kb species in human placenta on Northern blot analysis.

The cDNAs encoding the mouse soluble D-factor receptor have an extra 280 or 502 bp between the second and the third FNIII repeats of the transmembrane receptor cDNA (*11, 23*). This suggests that the mouse soluble receptor is generated by an alternative splicing of the transcript. We analyzed the corresponding intron (15 in Fig. 1) of the human gene. However, there was no homology between the sequence of human intron 15 and the extra nucleotide sequence in the mouse soluble receptor cDNA. Recently, it was shown that the extra-exon specific to the mouse soluble receptor contained a part of a mouse B2 repetitive element (*25*). This provides a possible explanation for the genesis of the mRNA specific to mouse.

The D-factor receptor is structurally most similar to gp130, G-CSF receptor, IL-12 receptor, and OSM receptor (*12-17*). Among these receptors, the G-CSF receptor alone has been examined as to its genomic organization (*13*). The human G-CSF receptor and human D-factor receptor genes consist of 17 exons and 20 exons, respectively. The D-factor receptor has one more CRH domain (4 exons) than the G-CSF receptor. The 5′ noncoding region of the D-factor receptor consists of one exon, whereas that of the G-CSF receptor includes 2 exons.

The intron phases for the D-factor receptor gene are shown in Fig. 1B. Phase 0 introns are inserted between the codons. Phase 1 and 2 introns interrupt the codons after the first and second nucleotides, respectively. This intron organization of the D-factor receptor gene is identical to that of the G-CSF receptor gene, and is well conserved in the genes of the cytokine receptor superfamily (*13, 26*).

Typical CRH domains contain four sequentially paired cysteine residues and a WSXWS motif. Each pairs of cysteine residues of the four cysteine residues is usually encoded by a different exon (*26*). In the CRH 1 domain of the D-factor receptor, three cysteine residues are separat-

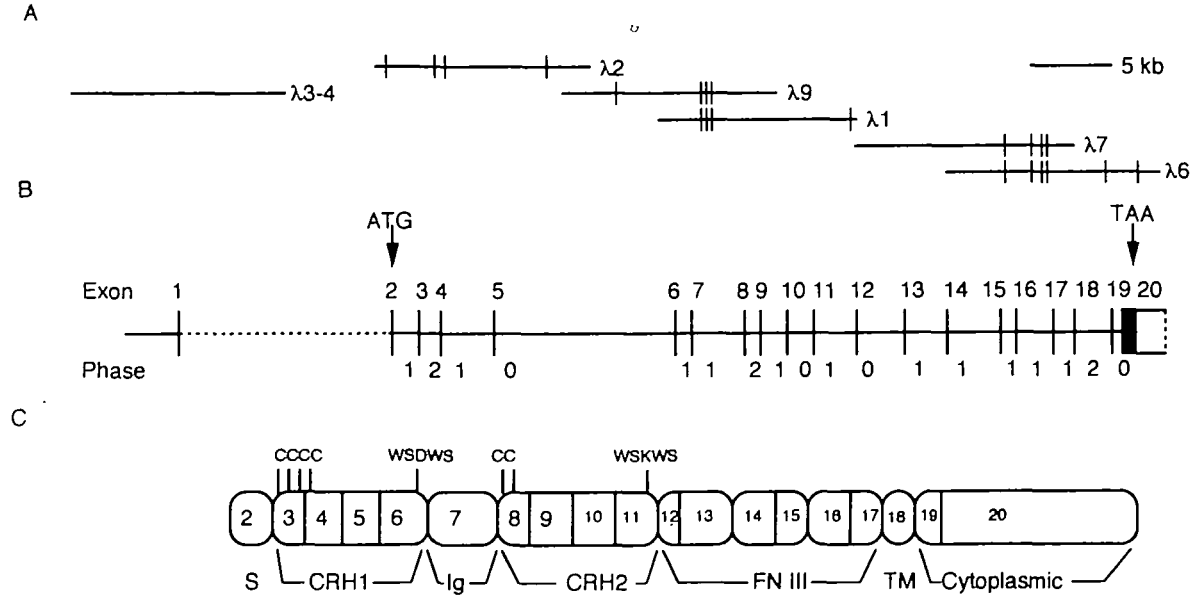


Fig. 1. **Organization of the human D-factor receptor gene.** A: Schematic representation of six λ clones carrying the human D-factor receptor gene. The restriction enzyme sites for *Eco*RI are shown by vertical bars. B: The exon-intron organization of the human D-factor receptor gene. Numbers under the line represent the phases of the introns. C: Correlation of each exon to the putative domains of the D-factor receptor. Conserved cysteine residues and the WSXWS motif are shown above the boxes. S, Ig, CRH, FNIII, and TM indicate signal sequence, Ig-like domain, cytokine receptor homologous domain, fibronectin type III domain, and transmembrane domain, respectively.

ed from the other one by an intron (Fig. 1C). The CRH 2 domain has only the first two of the four conserved cysteine residues. These results suggest that the first two cysteine residues are more important than the second two. The mouse IL-3 binding protein (AIC2A) also has two repeats of a CRH domain. Mutational analysis of the binding protein indicated that both CRH domains were necessary for the binding of IL-3 (27). However, structure-function analysis of the D-factor receptor remains to be performed.

*Sequence of the 5′ Flanking Region*—The 5′ flanking region of the human D-factor receptor gene is shown in Fig. 2. Human D-factor receptor mRNA in the placenta mainly starts at 162 bp upstream of the translation initiation site (the position 1 indicated in Fig. 2). The minor products of 5′

TABLE II. **Exon-intron junctions of the human D-factor receptor gene.**

| Exon No. | Size (bp) | Intron donor | Size (kb) | Acceptor |
|---|---|---|---|---|
| 1 | 143 | TCCAG gtacagcact | 23 ~ | cctaacctag GACTG |
| 2 | 161 | A AAG G gtaagtgcat | 1.7 | tctcctccag GG GCT |
| 3 | 115 | AAC AG gtaattttaa | 1.5 | cttattacag G TCC C |
| 4 | 140 | C GTT T gtaagtattt | 3.6 | ctttttaaag CC TTA |
| 5 | 164 | AA TTA gtaagtttga | 11.2 | tcatttttag GTG AC |
| 6 | 175 | T TCT T gtaagcttat | 1.2 | ttctttctag GG ATA |
| 7 | 255 | T GGA T gtaagtatat | 3.8 | tttctgttag AT CCA |
| 8 | 130 | GAA AG gtaatgataa | 0.4 | taattaacag T TTT T |
| 9 | 170 | A AAA G gtaagctgta | 1.8 | atatttgcag TT TAT |
| 10 | 146 | AG CAG gtaagattct | 1.3 | tttccaatag CGG AA |
| 11 | 163 | A GCC A gtaagttaac | 2.9 | tccttttcag GT CCT |
| 12 | 71 | GG AAG gtaaatatct | 2.9 | tttttcatag CCT TT |
| 13 | 214 | A AAT G gtgagtgctt | 2.8 | cttattgaag AT GAT |
| 14 | 180 | A TCT G gtaagatgaa | 3.1 | catcctatag AT GAA |
| 15 | 102 | A TTG G gtaagttaaa | 0.9 | ccttttacag CT CCC |
| 16 | 168 | A TCA G gtgagtaaat | 2.4 | ttaaaaatag GT CGT |
| 17 | 162 | A AAT T gtaagtttca | 1.0 | ctcgtttcag CT GTG |
| 18 | 94 | GAA TG gtaatagttc | 2.1 | ttaattaaag G ATT A |
| 19 | 79 | GT GAG gtaatctttt | 0.4 | ttatttctag GGA AG |
| 20 | 2.5k~ | | | |

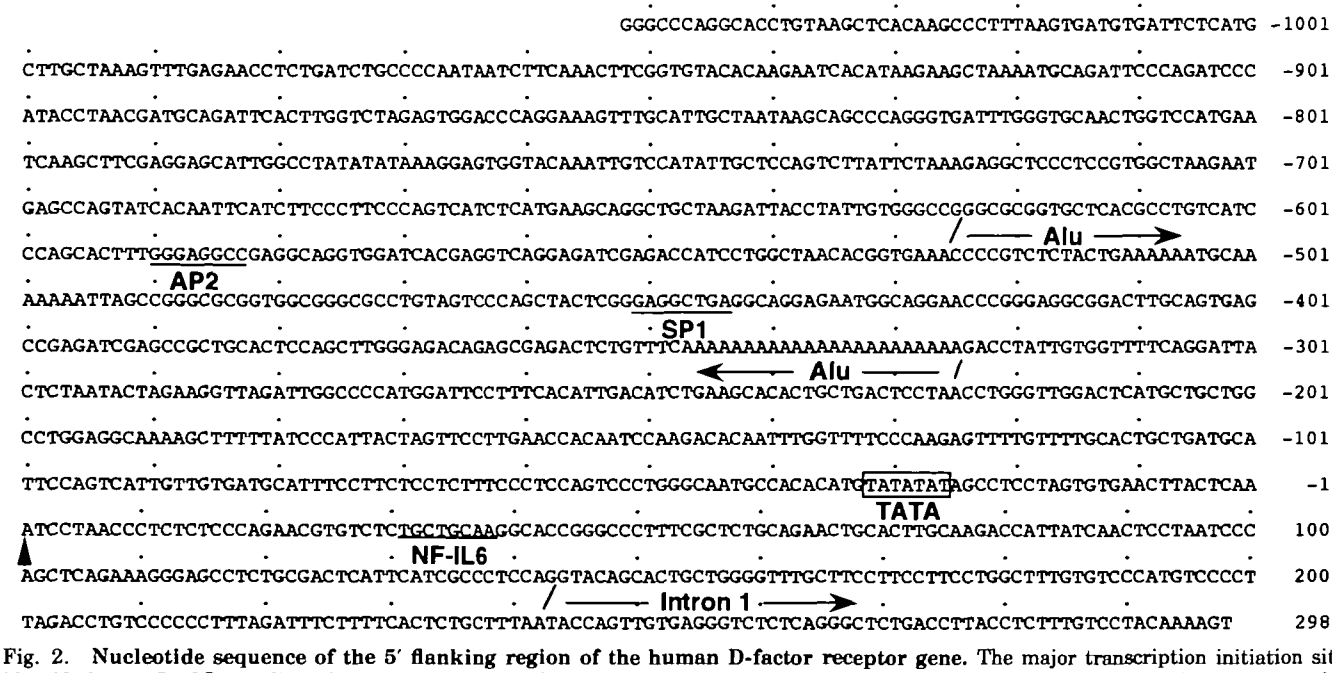


Fig. 2. **Nucleotide sequence of the 5′ flanking region of the human D-factor receptor gene.** The major transcription initiation site identified on 5′ RACE is indicated by an arrowhead and corresponds to +1. The TATA motif is boxed. Potential recognition sequences for transcription factors (AP2, Sp1, and NF-IL6) are underlined.

RACE indicate minor transcription initiation sites around the major initiation site.

There is a consensus TATA motif at an appropriate position (about 30 bp upstream of the initiation site). An *Alu* sequence is present at −325 to −624. Although its function is not well understood, it may affect the expression of some genes. It contains possible regulatory elements for transcription (*28*), such as AP2 (−583 to −590) and Sp1 (−443 to −451). An NF-IL6 binding site is located at +31. NF-IL6 is induced in hepatocyte and myeloid leukemic M1 cells by treatment on IL-6 or D-factor (*29*). In mouse placenta, mRNAs for the D-factor and D-factor receptor increase with growth of the placenta (*23*). Therefore, it would be interesting to examine the physiological significance of these possible regulatory elements.

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