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Structural Organization and Promoter Activity of the Human Ryudocan Gene[1]

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To better understand the regulation of ryudocan (syndecan-4) expression, we have determined the structural organization of the human ryudocan gene. The human ryudocan gene extends approximately 24 kilobases and is divided into five exons, which appear to be conserved in syndecan family members. Exon I encodes the signal peptide; exons II-IV, the extracellular domain; and exon V, the transmembrane and cytoplasmic domains, which are highly homologous among syndecan family members. Primer extension analysis showed that human ryudocan gene had a single transcription initiation site, located 3 bases upstream from the described cDNA [Kojima *et al.* (1993) *BBRC* 190, 814-822]. The 5′-flanking sequences of human ryudocan gene contain a TATA-like sequence as well as a variety of other potential binding sites for transcription factors, including Sp1, Ap-2, NF-kB, E-alpha H box, H4TF-2, and LBP-1, and were capable of functioning as a promoter. The determination of the human ryudocan gene structure will allow elucidation of constitutive, cell-specific, tissue-specific, and developmentally regulated expression.

Key words: core protein, gene organization, heparan sulfate proteoglycan, human ryudocan, promoter function.

Ryudocan has been isolated from endothelial cells as an anticoagulant heparan sulfate proteoglycan (HSPG) (*1*) and is now known to be a member of the syndecan family of cell surface HSPG (syndecan-4) (*2*). Several cDNAs of syndecan-4 have been characterized for rat [ryudocan (*1*)], human [ryudocan (*3*), amphiglycan (*4*)], and chicken (*5*). Analysis of the deduced amino acid sequences of these core proteins revealed the presence of four structural domains, namely, signal peptide, extracellular, transmembrane, and cytoplasmic domains, which were similar to those of the other syndecan family members. The signal peptide and extracellular domains have relatively divergent structure in primary sequences, whereas the combined transmembrane and cytoplasmic regions show extremely homologous sequences with conservation at the positions of all four tyrosine groups. Although ryudocan was originally cloned from rat microvascular endothelial cells as an anticoagulant molecule, the majority of purified rat ryudocan possessed no binding activity to antithrombin III, suggesting that it may have another biological function (*6*).

Recently, we have demonstrated that ryudocan is expressed in fibrous connective tissues, peripheral nerve tissues, and placental trophoblasts, and that purified human ryudocan from endothelial-like cells possesses high affinities to basic fibroblast growth factor (bFGF), midkine, and tissue factor pathway inhibitor, suggesting that ryudocan may possess multiple biologic functions, such as bFGF modulation, neurite growth promotion, and anticoagulation, *via* heparan sulfate-binding effectors in the cellular microenvironment (*7*). Shworak *et al.* have reported that rat ryudocan possesses three glycosaminoglycan (GAG) attachment sites that are always occupied, and that each site bears similar proportions of either heparan sulfate (HS) or chondroitin sulfate (CS) (*8*). The attachment of different numbers of HS and CS chains to ryudocan may alter its interactions with specific proteins and hence modify the biologic function of this molecule (*9*). In this study, we determined the genomic organization and nucleotide sequence of the human ryudocan gene to investigate its function and regulation.

EXPERIMENTAL PROCEDURES

Isolation of Human Ryudocan Genomic Clone—A human genomic phage library, which was made by partial *Sau*3AI digestion of human placenta DNA ligated into the *Bam*HI site of bacteriophage λ DASH II (Stratagene, La Jolla, CA), was obtained as a generous gift from Dr. M. Seto (Aichi Cancer Center, Nagoya). Approximately 6×10^5 plaques

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Abbreviations: bFGF, basic fibroblast growth factor; CS, chondroitin sulfate; GAG, glycosaminoglycan; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; PCR, polymerase chain reaction.

were screened with [^{32}P]dCTP-labeled phR1 probe, a partial human ryudocan cDNA, as described previously (*3*). Phage DNAs from positive clones were isolated, then characterized by restriction mapping and Southern blotting. Subfragments were cloned into pBluescript II KS$^+$ (Stratagene) for further characterization. For each independent genomic subclone, the entire length of both strands was enzymatically sequenced by the dideoxynucleotide chain-termination method with Sequenase (United States Biochemical, Cleveland, OH) using both dGTP and dITP according to the manufacturer's protocol. The majority of the sequence was acquired with nested deletion subclones prepared by using exonuclease III/S1 nuclease (Erase-a-Base System: Promega, Madison, WI), while synthetic oligonucleotides based on the preliminary sequence were employed to obtain the remainder of the structure.

PCR Cloning for the 3′ Regions of Human Ryudocan Gene—The clones from the human genomic library contain no 3′-end of the described cDNA (*3*). To isolate the rest of the 3′ region of human ryudocan gene, we carried out the genomic polymerase chain reactions (PCRs) with two primer sets: dTCAGTATCTCCAGCTCTGATTAC (position 21255-21277)/dGGCTCTTCTCTCATTTTCAAGAAA (position 21987-21964), and dAGCTGAGAGTTTATGCTGAAATG (position 21752-21774)/dAGAAAGTACCAGGTTTTATTATCT (position 23124-23101). After amplification, 733-bp and 1,373-bp PCR products were direct-sequenced with Circum Vent™ Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. We also employed the inverse PCR method to isolate the 3′ region of human ryudocan gene beyond the polyadenylation site, as described by Ochman *et al.* (*10*). Briefly, we performed Southern blot analysis with a 401-bp (position 22724-23124) probe to select an appropriate restriction endonuclease for inverse PCR. Ten micrograms of normal human genomic DNA was digested with *Xba*I, and gel-purified fragments of approximately 850 bp, which was the size of the positive band in the Southern analysis, were circularized by self-ligation to use as a template for the subsequent PCR. The nested-PCR method was employed using two primer sets: dCCCTCCCTTGCTGCACCAG (position 22926-22944)/dACGACCTGCCCAGGCTAAG (position 22886-22868), and dTCCCCTTGGGCTGTGTTGGA (position 23018-23037)/dACGACCTGCCCAGGCTAAG (position 22886-22868). A final 719-bp product (pHRG3-XIP) was direct-sequenced as described above.

Primer Extension Analysis—Total RNA and poly(A)$^+$ RNA were purified from the EAhy926 cells (*11*), which is a hybrid of human umbilical vein endothelial cells and a cell line of human lung carcinoma, as described previously (*3*). Primer extension experiments were done using AMV Reverse Transcriptase Primer Extension System (Promega). A gel-purified oligonucleotide primer, PE63-19 (dGCGAACAGACGGGCGGGGG), complementary to nucleotide position +66 to +48 (see Fig. 3), was radiolabeled at the 5′-end, and hybridized to 30 μg of EAhy total RNA or 5 μg of EAhy poly(A)$^+$ RNA in AMV Primer Extension 1× Buffer by incubation at 68°C for 2 h. After further incubation for 10 min at room temperature, AMV (1 unit) and sodium pyrophosphate (final 2.8 mM) were added, and the mixture was incubated at 42°C for 1 h. Primer extension products and sequencing products for the 5′-end subclone of λHRG6 by Sequenase with ^{32}P-endolabeled PE63-19 primer were run on 8% sequencing gel. Dephosphorylated ϕX174/*Hin*fI fragments were radiolabeled at the 5′-end and used as molecular size markers.

Analysis of Promoter Activity—The *Sau*3AI/*Nco*I fragment of the 5′-flanking region of the human ryudocan gene was inserted between the *Bgl*II and *Nco*I sites of the pGL3-Basic vector (Promega) containing luciferase as a reporter gene. This plasmid (pGL3R), covering the gene area from −260 to +47, was used to make the 5′-deleted constructs by restriction endonucleases: *Sac*I for pGL3RSa (−211 to +47); *Eco*RI for pGL3RE (−90 to +47); *Sma*I for pGL3RSm (−5 to +47). A β-galactosidase reporter plasmid driven by the mouse retrovirus promoter, MLV-LacZ (*12*) (a generous gift from Dr. M. Towatari), was cotransfected with the above luciferase constructs for monitoring transfection efficiency. For transfection, 2×10^5 COS-1 cells were plated on 35-mm dishes (FALCON 3046), grown to subconfluence (overnight) in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin sulfate (10% FCS-DMEM-PS), washed once in Opti-MEM (GIBCO/BRL), and incubated at 37°C for 5 h in 2 ml of Opti-MEM/dish containing liposome form of the appropriate luciferase reporter constructs (1 μg), pMLVLacZ (1 μg), and lipofectin reagent (GIBCO/BRL). One milliliter/well of 20% FCS-DMEM-PS was added, and cells were incubated for another 16 h. After removal of medium with liposome, cells were washed, and grown for 24 h to confluence as described above. To measure luciferase activity of the transfected cells, Luciferase Assay System (Promega) was used according to the manufacturer's instructions. Briefly, cells were washed twice with PBS, lysed on the plate with 1×lysis buffer (200 μl/well) for 15 min at room temperature, scraped, and spun down (12,000×*g*) for 15 min at 4°C. Twenty microliters of supernatant from lysed cells, diluted with 1×lysis buffer containing BSA (1 mg/ml), was added to luciferase substrate (100 μl), and the light output was measured immediately for 60 s at room temperature with a luminometer (Lumat LB 9501/16, Berthold; Germany). The baseline background activity in each tube was measured prior to addition of sample, and the activity of the pGL3-Basic vector alone served as a negative control. To measure β-galactosidase activity, 40 μl of cell lysate was diluted with 10 μl of 2dH_2O and assayed using β-Galactosidase Assay System (Promega) and an automated microplate reader (EASY READER/EAR 340AT, SLT-LABINSTRUMENTs, Austria) at 405 nm wavelength.

RESULTS AND DISCUSSION

The human ryudocan gene of approximately 24 kb in size, containing a 260-bp 5′-flanking region, all exons and introns, and a 438-bp 3′-flanking region, was entirely sequenced (Fig. 1A). We have screened a human placenta genomic library with the human ryudocan cDNA probe (phR1) (*3*) and isolated six positive clones from approximately 6×10^5 recombinants. Five of them, however, showed an identical pattern of restriction mapping. Restriction fragments from two independent clones, λHRG1 and λHRG6, were subcloned into pBluescript II KS$^+$ for further restriction mapping and sequencing. Sequencing analysis

revealed that these overlapping clones covered the 5′-flanking region and extended to the start of the fifth exon of the human ryudocan gene. To isolate the rest of the 3′ region, we carried out genomic PCRs. It was found that two PCR products, fragments of 733 bp (pHRG3P1, position 21255-21987) and 1,373 bp (pHRG3P2, position 21752-23124), covered the rest of the 3′ region, which had identical sequences to the described cDNA (*3*). We also employed the inverse PCR method to isolate the 3′ region beyond the polyadenylation site of the human ryudocan gene, as outlined in "EXPERIMENTAL PROCE-DURES." We obtained a 719-bp clone (pHRG3XIP), which contained the 438-bp 3′-flanking region of the human ryudocan gene. Downstream sequences of the polyadenylation site (position 23125) were found to have a lower G/T content than those of the alternative polyadenylation site (position 22320) reported previously (*3*). These data suggest that a typical AATAAA sequence could be more essential than a high G/T content element for an efficient polyadenylation signal function.

The exon-intron organization of the gene is depicted in Fig. 1B, and exon sizes are listed in Table I. Sequences for all intron-exon splice junctions matched with the "GT-AG" rule and consensus sequences for intron splice donors and acceptors (*13*). The splice junctions between exons II/III and IV/V occurred after the first nucleotide of the amino acid codons and in the rest of the intron-exon boundaries between codons, which are similar to mouse syndecan and chicken ryudocan gene structures (*5, 14, 15*). Exon I is 103 bp in length and encodes the entire 5′-untranslated region of the cDNA, the translation initiation site, the signal peptide, and the first two codons of the mature protein. Exon II (139 bp) contains three putative GAG attachment sites, which are conserved in rat, human and chicken ryudocans. Exons III and IV encode the most divergent region among the syndecan family members. Exon III (47 bp) encodes the middle part of extracellular domain. Exon IV (199 bp) contains an additional GAG attachment site, which is not observed in rat and chicken ryudocan. Exon V (2,125 bp) encodes the highly homologous transmembrane

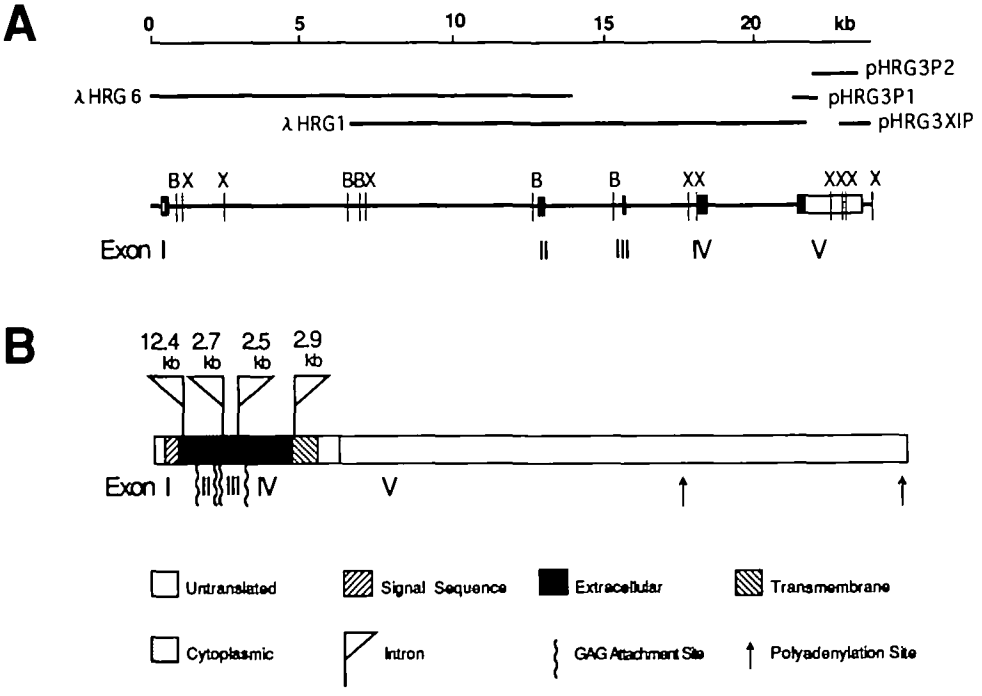

Fig. 1. **Restriction map and exon/intron organization of the human ryudocan gene.** A: Locations of λHRG1, λHRG6, pHRG3P1, pHRG3P2, and pHRG3XIP clones are shown below the scale. Roman-numbered boxes (I-V) indicate exons, of which closed boxes represent coding regions and open boxes 5′- and 3′-untranslated regions. The restriction sites of enzymes are indicated by B (*Bam*HI) and X (*Xba*I). B: The functional domains of exons and the positions and sizes of introns are represented on the 2.6 kb composite human ryudocan cDNA. Roman numbers identify exons I-V, and arrows mark the two polyadenylation sites.

TABLE I. **Exon/intron boundaries of the human ryudocan gene.** Exon sequences are indicated by uppercase letters and intron sequences by lowercase letters. Exon lengths are in bp and intron lengths in kb. The amino acid residues at the splice sites are shown below the exon sequences. Numbering of the amino acid residues is according to Kojima *et al.* (*3*).

Exon (bp)	Splice donor			Intron (kb)	Splice acceptor		
I (103)	GAG	TCG	gtgggt	···(12.4)···	tttctttatcttag	ATC	CGA
	E	S				I	R
	19	20				21	22
II (139)	GAT	CTG	G gtacgg	···(2.7)···	ttctgaccttacag	AT	GAC
	D	L	D				D
	65	66	67				68
III (47)	CCC	TTG	gtaagt	···(2.5)···	ttccgcccatccag	GTG	CCT
	P	L				V	P
	81	82				83	84
IV (199)	CTG	GCA	G gtaagt	···(2.9)···	ctcctcctctccag	CT	CTG
	L	A	A				L
	147	148	149				150
V (2,125)							

and cytoplasmic domains which define the syndecan family members, and the 3′-untranslated region contained potential alternative polyadenylation signals (*3*). This gene organization of human ryudocan, which consists of five exons with a relatively large first intron, is similar to those of other syndecan family members, suggesting that all syndecan genes are likely derived from a common ancestor as described previously (*14*).

The transcription initiation site of the human ryudocan gene was determined by primer extension analysis. Oligonucleotide (PE63-19) was hybridized to EAhy total RNA or poly(A)$^+$ RNA and used to prime reverse transcriptase (Fig. 2). The transcription initiation site is located 43 bp upstream from the first ATG translation start site and 3 bp upstream from the 5′-end of the described cDNA (*3*), and it has been defined as +1 in the numbering of the nucleotide sequence throughout of this paper. Presumably, since the RACE method was used for 5′-cloning of human ryudocan cDNA, the 5′-end of the described cDNA very close to the transcription initiation site was obtained. After the present study was completed, we found a report of molecular cloning of the human ryudocan promoter (*16*). Compared with that, our data have several base differences which might be caused by gene polymorphisms or relatively rare errors in the function of reverse transcriptase, as pointed out previously (*1*).

The 5′-flanking region (260 bp) of the human ryudocan gene is extremely GC-rich (72.3%) and contains a number of potential transcription factor binding sites, including a TATA-like sequence (TATAAGA) located 25 bp upstream from the transcription initiation site (Fig. 3). Five repeats of potential binding sites for the constitutive transcription factor Sp1, including a typical GC-box, are present proximate to the transcription initiation, which is consistent with the constitutive expression of ryudocan as reported previously (*3-5*). Other potential transcription factor binding sites are AP-2, which mediates induction involving cyclic AMP (*17*); NF-kB, which regulates gene expression *via* cytokines (*18*); E-alpha H box, which is involved in cell type-specific gene expression (*19*); and H4TF-2, which regulates gene expression during the cell cycle (*20*). Since it has been reported that ryudocan is expressed selectively in cell-, tissue-, and development-specific patterns (*1, 7, 21*), additional experimentation is needed to elucidate the elements responsible for the regulation of specific expression.

To investigate the functional capability of this region as a promoter, several constructs containing a part of this region in front of a luciferase reporter gene were transfected into COS-1 cells as outlined in "EXPERIMENTAL PROCEDURES." The pGL3R construct covering position −260 to +47 of the human ryudocan gene showed a significant luciferase activity as shown in Fig. 4. On the one hand, the pGL3RSa (−211 to +47), in which the 5′-end of the pGL3R insert was deleted by endonuclease *Sac*I, had a higher level of luciferase activity than the original plasmid. It is interesting that the deleted region (position −260 to −212) contains an LBP-1 binding site, which was reported as a repressor on HIV-1 transcription (*22*). On the other

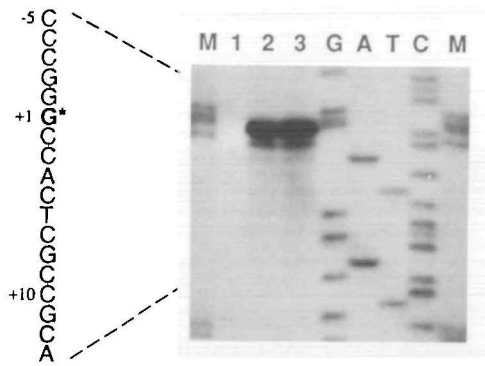

Fig. 2. **Site of transcription initiation as determined by primer extension analysis.** ^{32}P-endolabeled PE63-19 primer complementary to nucleotide position +66 to +48 (see Fig 3) was annealed to total RNA (30 μg, lane 2) or poly(A)$^+$ RNA (5 μg, lane 3) from EAhy926 cell and used to prime reverse transcriptase [lane 1, RNA (−) control] The sequence on the right was produced with the same oligonucleotide as in primer extension reaction The site of initiation is shown with bold letter "**G***," and the numbers indicate the nucleotide positions Dephosphorylated ϕX174/*Hin*fI fragments were radiolabeled at the 5′-end and used as molecular size markers (lane M)

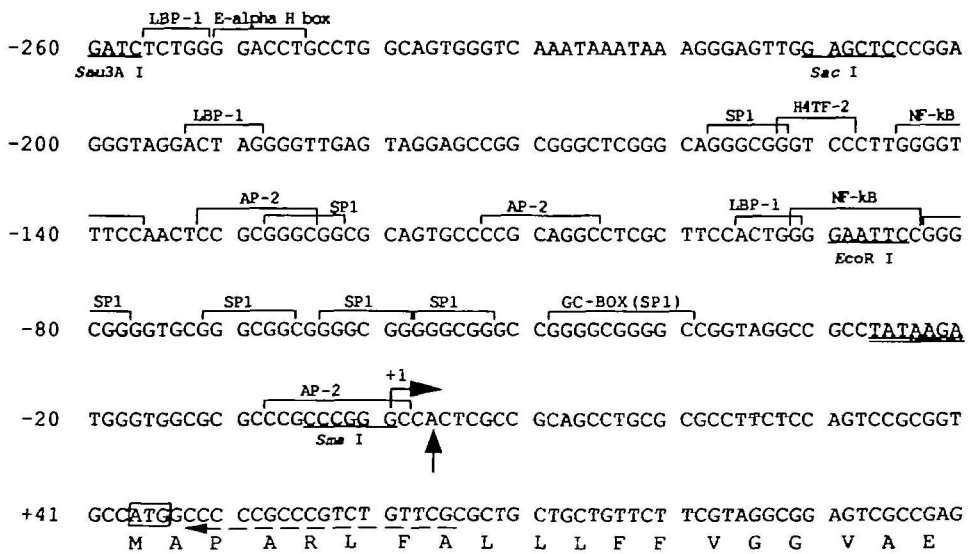

Fig 3 **Nucleotide sequences of the 5′-flanking region of the human ryudocan gene.** The first ATG is boxed, and amino acids encoded by the first exon are indicated below the appropriate codons. Oligonucleotide used in primer extension analysis is represented by the horizontal dashed arrow. The transcription start site is marked with a bent arrow and designated as +1 The 5′-end of cDNA hR5′AP (*3*) is indicated with a vertical arrow. Restriction enzyme sites are underlined. A TATA-like sequence is double-underlined. Consensus binding sequences for transcription factors (Sp1, Ap-2, NF-kB, E-alpha H box, H4TF-2, and LBP-1) are indicated with brackets.

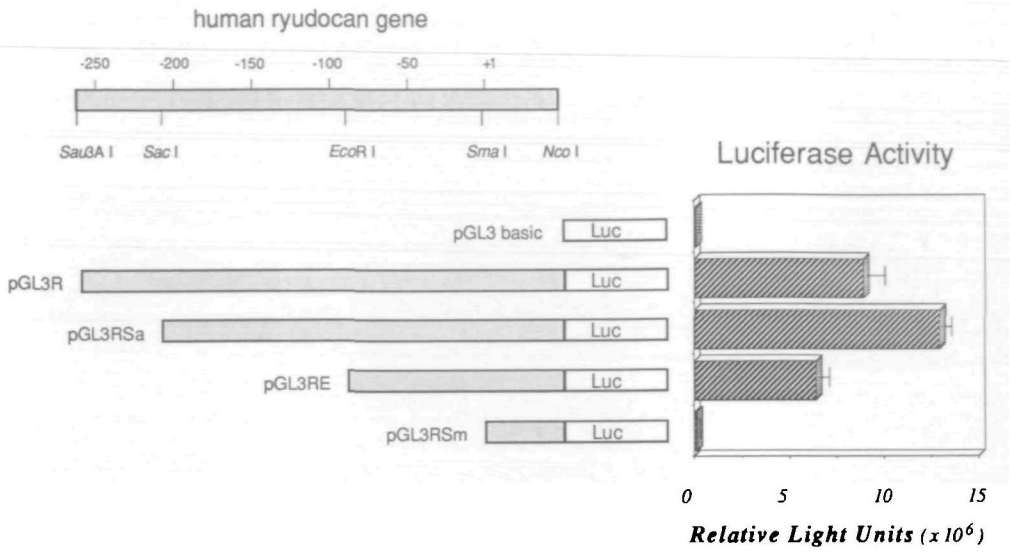

Fig. 4. Upstream sequences of the human ryudocan gene promote activity of a luciferase reporter gene. Left The top represents the relevant restriction sites of the 5′-flanking region of the human ryudocan gene (*Sau*3AI/*Nco*I fragment, −260 to +47) for preparation of the deletion constructs Names assigned to the human ryudocan-luciferase fusion genes are indicated at the left of the corresponding constructs. Right: Relative luciferase activities, corrected for differences in transfection efficiencies by the co-transfected β-galactosidase activities, were determined for extracts from transfected COS-1 cells as described in "EXPERIMENTAL PROCEDURES" Data from triplicate samples (mean ± SD) are presented, and similar data were obtained in two additional experiments

hand, the pGL3RE (−90 to +47) plasmid, which has a TATA-like sequence, five repeats of the constitutive Sp1 site including a GC-box, and an AP-2 binding site, but loses several transcription factor binding sites (2 Sp1, 2 Ap-2, 2 NF-kB, a H4TF-2, and 2 LBP-1 sites) from the pGL3RSa, showed a remarkably reduced luciferase activity. The pGL3RSm (−5 to +47), which loses all potential transcription factor binding sites in the 5′-flanking region, had no promoter activity. These data imply that the 5′-flanking region of the human ryudocan gene has an apparent promoter activity in COS-1 cell system. It is also expected that promoter element(s) would be present between position −90 and −6, enhancer element(s) between −211 and −91, and silencer element(s) between −260 and −212.

In conclusion, we have determined that the human ryudocan (syndecan-4) gene is approximately 24 kb in size and is organized into five exons that appear conserved in syndecan family members. The 5′-flanking sequences of the human ryudocan gene contain a TATA-like sequence as well as a variety of other potential binding sites for transcription factors, including Sp1, Ap-2, NF-kB, E-alpha H box, H4TF-2, and LBP-1, and are capable of functioning as a promoter. The determination of the human ryudocan gene structure will allow elucidation of its constitutive, cell-specific, tissue-specific, and developmentally regulated expression.

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