

Molecular Cloning and Expression Analysis of a Putative Nuclear Protein, SR-25¹

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We cloned a full-length mouse cDNA and its human homologue encoding a novel protein designated as "SR-25." In Northern blot analysis, SR-25 mRNA was expressed in all organs tested, and relatively abundant in testis and thymus. Deduced amino acid sequences of mouse SR-25 and human SR-25 showed 77.7% identity. SR-25 has a serine-arginine repeat (SR repeat) and two types of amino acid clusters: a serine cluster and a highly basic cluster. Based on the presence of many nuclear localizing signals and a similarity to RNA splicing proteins, SR-25 is strongly suggested to be a nuclear protein and may contribute to RNA splicing. © 2000 Academic Press

To obtain a novel cDNA clone from the mouse pancreatic β -cell line, MIN6, we constructed a cDNA library from mRNA of MIN6 cells, and randomly sequenced 1,173 clones from their 3' ends (1). The obtained sequences were compared with each other at first to assess the frequency of their appearance in our library, and then with the DNA data base in GenBank using a FASTA program. There are 898 independent clones in the whole 1,173 clones. Out of the 898 clones, 600 clones were unknown. Among them, we encoun-

Abbreviations used: FBS, fetal bovine serum; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; snRNP, small nuclear ribonucleoprotein particle; SR repeat, serine-arginine repeat; TdT, terminal deoxynucleotidyl transferase.

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tered a cDNA clone having intriguing repetitive sequences. From deduced amino acid sequence, the cDNA is thought to be encoding a nuclear protein, and it was designated as SR-25.

MATERIALS AND METHODS

Cultured cell lines. Human insulinoma cells dispersed and cultured for 10 passages were used as the source of human islet β cells. MIN6 and NIT-1 were derived from mouse insulinomas, whereas α -TC1 originated from a mouse α -cell tumor. QGP-1 was derived from a human pancreatic endocrine tumor, and AR42J was from a rat pancreatic exocrine tumor. AtT20 and α -TSH were from a mouse pituitary tumors, and GH3 was from a rat pituitary tumor. PC12 was from a rat pheochromocytoma. NIH/3T3 and TIG-1 were from mouse and human fibroblasts, respectively.

Cloning of mouse SR-25 cDNA. MIN6 cDNA library was constructed as described previously (1). The fragment (740 bp) of mouse SR-25 cDNA was obtained from the random sequencing of this library. To obtain the full-length cDNA, rapid amplification of cDNA ends (RACE) (2) was performed for both 3' and 5' terminal regions.

3' RACE. Total RNA (5 μ g) purified from MIN6 cells with ISOGEN (Nippongene, Tokyo, Japan) was reverse-transcribed to cDNA with a SuperScript Preamplification System for First Strand cDNA Synthesis kit (Gibco BRL, Rockville, MD) using an oligo-dT primer. Using the first-strand cDNA as a template, nested PCR was performed. For the first PCR, a gene-specific primer (5'GTGCAGTGGATTGGACAG3') and a (dT)₁₇-adaptor primer (5'GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT3') were used. For the second PCR, a gene-specific primer (5'ATTGGACAGCAGAGTGTAC3') and an adaptor primer (5'CCGACTCGAGTCGACATCG3') were used. PCR conditions used in this study were as follows: initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min, and the final extension at 72°C for 10 min.

5' RACE. Total RNA (5 μ g) extracted from MIN6 cells was reverse-transcribed to the first-strand cDNA with a gene-specific primer (5'GTTGAAGTGGATCGCGATGT3'). After ethanol precipitation, the pellet was dissolved in 30 μ l of H₂O and denatured at 95°C for 3 min. To extend poly(dA) from 3' end of the first-strand cDNA, it was incubated in 100 mM sodium cacodylate buffer (pH 7.2) containing 1 mM CoCl₂, 0.1 mM DTT, 2 mM dATP and 10 U of terminal deoxynucleotidyl transferase (TdT) (Takara, Tokyo, Japan)

at 37°C for 15 min, and the reaction was terminated at 70°C for 15 min. Using the first-strand cDNA accompanied by poly(dA), nested PCR was performed. For the first PCR, the sense (dT)₁₇-adaptor primer described above and a gene-specific antisense primer (5'GTTGAAGTGGATCGCGATGT3') were used; for the second PCR, the sense (dT)₁₇-adaptor primer and a gene-specific antisense primer (5'TTGTTGGCCCTGGGATCTGGAG3') were used; and for the third PCR, a sense (dT)₃-adaptor primer (5'ATCGACTCGAGTCGACATCGATT3') and a gene-specific antisense primer (5'TGCTTGCTTCTCCTCAAC3') were used. To ascertain the continuity of cDNA fragments obtained from MIN6 cDNA library and obtained with 3' and 5' RACE, the full-length mouse SR-25 cDNA was amplified at a stretch by PCR using the first-strand cDNA as a template with a sense primer (5'CCTGCTGTGGGCTAATGGTG3') and an antisense primer (5'TCCAATCCACTGCACCCAAG3').

Cloning of human SR-25 cDNA. The cloning strategy is shown in Fig. 1. At first, 3' RACE was carried out. Total RNA (5 µg) purified from a human insulinoma cell line was reverse-transcribed to first-strand cDNA with an oligo-dT primer. The first PCR of nested PCR was performed with a primer for mouse SR-25 cDNA (P1: 5'GCCTTCCAGATCGCAACAGG3') and the (dT)₁₇-adaptor primer (Pa: 5'GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTTT3'); the second PCR was performed with a primer for mouse SR-25 cDNA (P2: 5'CGACAGAGCGTTATTCGAAG3') and the adaptor primer (Pb: 5'CCGACTCGAGTCGACATCG3'); and the third PCR was performed with a primer for mouse SR-25 cDNA (P3: 5'CGTAACCAAGAACGACACA3') and the (dT)₃-adaptor primer (Pc: 5'ATCGACTCGAGTCGACATCGATT3'). The obtained 3' fragment of human SR-25 cDNA was sequenced and two antisense primers specific to this sequence (P5: 5'GAGAAGAGACTGGGCTGGG3' and P6: 5'AGACCAATCCACGGGGGAAA3') were prepared. Using these antisense primers and a sense primer for mouse SR-25 cDNA (P4: 5'TCCAGATCCAGGGCCACAA3'), semi-nested PCR was carried out.

The obtained human SR-25 cDNA fragment (approximately 500 bp) was sequenced and four antisense primers specific to this sequence (P7: 5'TGCTGTGCTTCCGCTTCTC3', P8: 5'AGGAGCTA-GAAGAACTGGAAG3', P9: 5'GCAGTTCCTCGAGGTGTCTTTC3', and P10: 5'TTTCTTTTTCCGACCCCG3') were prepared for 5' RACE. Total RNA (5 µg) extracted from human insulinoma cell line was reverse-transcribed with P7, and poly(dA) was added to 3' end of first-strand cDNA by TdT as well as 5' RACE for mouse SR-25 cDNA. Using the first-strand cDNA with poly(dA), nested PCR was performed. For the first, second, and third PCR, the following primer sets were used respectively: Pa vs. P8, Pb vs. P9, and Pc vs. P10. To ascertain the continuity of obtained cDNA fragments, the full-length human SR-25 cDNA was amplified at a stretch by PCR using the first-strand cDNA as a template with a primer set specific to human SR-25 cDNA (5'CCTGCTGTGGGCTAATGGTG3' and 5'TCCAATCCACTGCACCCAAG3').

Sequence analyses. Nucleotide sequence was determined by dye primer or dye terminator cycle sequencing using a Model 377 DNA sequencer (PE Biosystems, Foster City, CA). Sequence homology and hydrophilicity of mouse and human SR-25 were analyzed by "Genetyx ver 8.0" (Software Development Co., Ltd., Tokyo, Japan). The open reading frame (ORF) of SR-25 cDNA was detected by "ORF finder" which is a computer program available at <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>. A homology search of the deduced amino acid sequence was analyzed by "FASTA" at <http://crick.genes.nig.ac.jp/homology/fasta-e.html>. To detect sorting signals of SR-25 and to predict its subcellular localization, "Prediction of the Subcellular Location of Proteins by Neural Networks" at <http://predict.sanger.ac.uk/npsl/> (3) and "PSORT" at <http://psort.nibb.ac.jp:8800/> (4) were used.

Southern blot analysis. Mouse genomic DNA fragments (5 µg) digested with restriction enzymes were separated by electrophoresis on a 1% agarose gel, and transferred onto a nylon membrane (Gene-

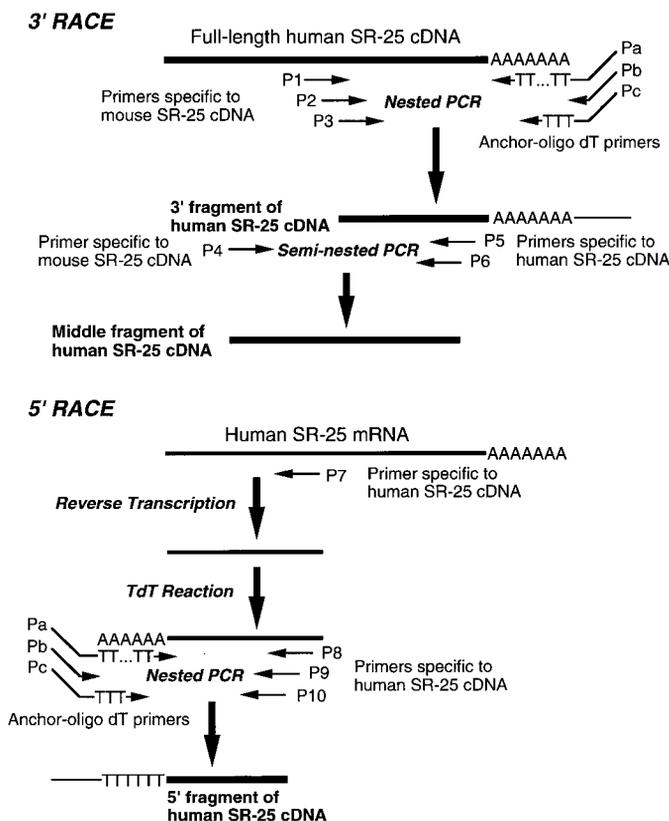


FIG. 1. Cloning strategy for human SR-25 cDNA.

Screen Plus; NEN Research Products, Boston, MA). Mouse full-length SR-25 cDNA (1,100 bp) was radiolabeled with [α -³²P]-dCTP (3,000 Ci/mmol, Amersham, Buckinghamshire, U.K.) using a Megaprime DNA Labeling System (Amersham), and used as a probe. DNA on the membrane was hybridized to the probe in a mixture of 50% formamide, 10% dextran sulfate, 1% SDS, and 100 µg/ml salmon sperm DNA at 42°C for 16 h. The membrane was washed three times with 0.2 × SSC at 50°C for 30 min.

Northern blot analysis. Total RNA isolated with ISOGEN (Nippongene) was electrophoresed on a 1.0% agarose gel with 2% formaldehyde, and transferred onto nylon membrane. The same probe as Southern blot analysis was used. Hybridization was carried out in 5 × SSPE containing 50% formamide, 0.1% SDS, 100 µg/ml salmon sperm DNA, and a radiolabeled probe at 42°C for 16 h. The membrane was washed three times with 2 × SSPE, 1 × SSPE, and 0.1 × SSPE + 0.1% SDS, in turn, at 65°C for 30 min.

Cell cycle analysis. To examine whether SR-25 expression is cell cycle-dependent, TIG-1 fibroblasts synchronized in the G₀ phase by serum deprivation (0.5% FBS) for 48 h were released with 10% FBS from cell arrest, and total RNA was extracted from TIG-1 cells at 0, 12, 18, 24, 30, and 36 h after serum stimulation for Northern blot analysis.

RESULTS AND DISCUSSION

Nucleotide Sequences of Mouse and Human SR-25 cDNAs

Full-length sequences of mouse and human SR-25 cDNAs are shown in Figs. 2 and 3, respectively. Their

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TCCCGCAAAGGCCTGCTGTGGGCTAATGGTGGATGTGGGGACCCAGCAGCTCGGGACG 60
CGTCTCCGGCGACCCACGCGGTCTATTGCAGCTGAGGAAAGCTGCAGCGTTCCCTCCCT 120
GGGCCACCAAGTCCAGTGCAGGCTTGAGGGCGGGCGGGAGTCGGAGGAGGTGCTGGG 180
ATCGCTAAGCCCCGGGCTCGCTCCGGCAGGAGTGGGCACTGGGCGCCCCGAGGATCGGG 240
TCCCGTTGGCGCTGCGTTTGGATTCGCCTCCGAGGACACGCTCACGAAGAGTCGAGGCT 300
AAGCGCTTTCCTTCCGGTCCCTAGCCCCCTCGCTGATGGCTCACGTTGGCTCTCGAAAG 360
                                     M A H V G S R K

CGCTCGAGAAGTCGCAGTCGGTCCCCTAGTGGTGGCGAGGGTCAGAAAAGCGAAGTAAG 420
R S R S R S R S R S G R R G S E K R S K
= = = = = = = = = =

AGGAGTAGCAAGGATGCCTCGAGGAACGTTCAGCCTCCAGATCCCAGGGCCACAAGGCT 480
R S S K D A S R N C S A S R S Q G H K A

GGCAGCGCCTCCGGGTTGAGGAGAGAAGCAAGCACAAAGCCCAGAGGACATCGCGATCC 540
G S A S G V E E R S K H K A Q R T S R S

AGTTCAACCTCCTCCTCTTCCAGTTCTTCTAGCTCCGCCTCGTCCTCATCTCCAGTGAT 600
S S T S S S S S S S S A S S S S S S D

GGCCGAAGAAGCGAGCGAAGCACAAAGGAGAAGAAGAGGAAGAAGAAGAAGAAACGG 660
G R K K R A K H K E K K R K K K K K R

AAGAAGAAGTTGAAGAAGAGAGTCAAGGAGAAGGCAGTGGCGGTGCACCAGGCCGAGGCT 720
K K K L K K R V K E K A V A V H Q A E A

CTGCCCCGCCCTCACTGGATCAGTGGCACAGATCAGCTGGGGAGGACAATGATGGCCCA 780
L P G P S L D Q W H R S A G E D N D G P

GTCCTGACAGATGAGCAGAAGTCTCGTATCCAGGCCATGAAACCCATGACAAAGGAGGAG 840
V L T D E Q K S R I Q A M K P M T K E E

TGGGATGCCCGACAGAGCGTTATTTCGAAAGGTGGTGGACCCAGAGACAGGACGCACAAGG 900
W D A R Q S V I R K V V D P E T G R T R

CTCATCAAGGGAGACGGCGAGGTTTTAGAGGAAATCGTAACCAAGAACGACACAGAGAG 960
L I K G D G E V L E E I V T K E R H R E

ATCAACAAGCAAGCCACCCGAGGGGATGGGCTGGCCTTCCAGATGCGAACAGGCC'TACTT 1020
I N K Q A T R G D G L A F Q M R T G L L

CCCTGAGGGCTCAGCCATCCCAGGTTTGTGAACTGCTGTTGGTGACTTGGAGACTAATAG 1080
P *

GTCCAGGTGCCTTTCCTACAGCCAGCATCCTCTTGGGTGCAGTGGATTGGACAGCAAAT 1140
TGGACAGCAGAGTGTCACTATTAAATGATCTTGGTCACAG 1180

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FIG. 2. The cDNA sequence and deduced amino acid sequence of mouse SR-25. A polyadenylation signal is indicated in bold style. = = =, SR repeat; - - -, serine cluster; —, basic cluster; and *, stop codon.

ORFs were considered to be from nucleotide number 339 to 1,028 for mouse SR-25 cDNA (Fig. 2), and from nucleotide number 63 to 719 for human SR-25 cDNA (Fig. 3), respectively. The reasons why these ORFs were determined are as follows: Peptide chains translated in other frames were too short to function; "ORF finder" indicated that these ORFs were most feasible; Nucleotide sequence identity between mouse and human SR-25 cDNAs was higher in these ORFs (79.0%) than those in both 5' (50.0%) and 3' (54.7%) flanking regions, which are putative non-coding regions; and the sequence around the ATG initiator codon of human

SR-25 cDNA (GCCATGG) was compatible with Kozak sequence for efficient ribosome binding and protein translation (5). Kozak reported that a purine nucleotide (A or G) in position -3, *i.e.*, 3 nucleotides upstream from the ATG codon, has a dominant effect for initiation by eukaryotic ribosomes. Although the sequence surrounding the ATG start codon of mouse SR-25 cDNA (CTGATGG) does not have the purine nucleotide in position -3, it has G in position +4 as well as human SR-25 cDNA. The G at +4 was also reported to be important for the efficient ribosome binding next to the purine nucleotide at -3 (5).

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GTCTCTCGAGAAGGCCGCGGGCGGGCTGTCCGGCCCGCAGGGCGGTCGAGCCCGCGGGC 60
CCATGGCTCACGTCGGCTCCGGCAAGCGCTCGAGGAGTCGCAGCCGGTCCCGGGACGGG 120
  M A H V G S R K R S R S R S R S R G R G
      = = = = = = = = =
GGTCGGAAAAGAGAAAAGAAGAAGAGCAGGAAAGACACCTCGAGGAAGTCTCGGCTCCA 180
  S E K R K K K S R K D T S R N C S A S T
CATCCCAAGAGACAAGCAAGCAGAAGGCCCGGAGGAGAACAAGATCCAGCTCCTCCTCCT 240
  S Q E R S K Q K A R R R T R S S S S S S
CTTCTTCCAGTCTCTTAGCTCCTCTTCTTCTCCTCCTCGTCTCCTCCTCTTCTCCTCAGTG 300
S S S S S S S S S S S S S S S S S S S S S S D
ATGGCCGGAAGAAGCGGGGAAGTACAAGGACAAGAGGAGGAAGAAGAAGAAGAGGA 360
  G R K K R G K Y K D K R R K K K K K R K
AGAAGCTGAAGAAGAAGGCCAAGGAGAAGGCCAAGCACAGCAGGTGGAGGCTCTGCCGG 420
K L K K K G K E K A E A Q Q V E A L P G
GCCCTCGCTGGACCAGTGGCACCGATCAGCTGGGGAGGAAGAGGATGGCCAGTCTCTGA 480
  P S L D Q W H R S A G E E E D G P V L T
CGGATGAGCAGAAGTCCCGAATCCAGGCCATGAAGCCATGACCAAGGAGGAGTGGGATG 540
  D E Q K S R I Q A M K P M T K E E W D A
CCCGGCAGAGCATCATCCGAAGGTGGTGGACCCTGAGACGGGGCGCACCAGGCTTATTA 600
  R Q S I I R K V V D P E T G R T R L I K
AGGGAGATGGCGAGGTCTAGAGGAAATCGTAACCAAAGAACGACACAGAGAGATCAACA 660
  G D G E V L E E I V T K E R H R E I N K
AGCAAGCCACCCGAGGGGACTGCCTGGCCTTCCAGATGCGAGCTGGGTTGCTTCCCTGAG 720
  Q A T R G D C L A F Q M R A G L L P *
GGCCCCGCTGGCCAAGGCCTGTGGACGACGCTGGCGGCCAGCCTGGGCAGGTTTCAGG 780
GTGCCAGTGGGAAGCCTGATGGGTGCTGGTGGCCTTTCCCCGTGGATPGGTCTCTGGCC 840
CAGCCAGTCTCTTCTCAGGGGAGGGGTGGAGGTTGGGGTACCAGCCTGCCTTGGCAC 900
CCCCATCTGAAAGAGCAGCACTTCTCAGCTATTAAGGCCCCCTGGATAGAC 952

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FIG. 3. The cDNA sequence and deduced amino acid sequence of human SR-25. Marks are the same as in Fig. 2.

Amino Acid Sequences of Mouse SR-25 and Human SR-25

Deduced amino acid sequences of mouse SR-25 and human SR-25 showed 77.7% identity (Fig. 4). Both proteins have three kinds of highly conserved amino acid motifs, *i.e.* a serine/arginine repeat (SR repeat), a serine cluster, and a basic cluster. Hydrophilicity analysis indicated that SR-25 is a highly hydrophilic protein, and its pattern for mouse SR-25 was very similar to that for human SR-25 (Fig. 5). In both mouse SR-25 and human SR-25, 28% of amino acid composition was basic amino acids (lysine and arginine) with calculated pI of 11.7 for mouse SR-25, and 11.4 for human SR-25. Molecular weights of mouse and human SR-25 were 25,525 Da and 24,629 Da, respectively.

By analysis with "Prediction of the Subcellular Location of Proteins by Neural Networks", the probability

that SR-25 is localized in the nucleus was estimated at 81.9% for both mouse and human SR-25. Similarly, "PSORT" predicted that SR-25 is a nuclear protein at the probabilities of 82.6% for mouse SR-25 and 78.3% for human SR-25. According to these two computer programs, basic clusters (RKKRAKHKE-KKRKKKKKKRKKKLKKRVKEK for mouse SR-25 and RKKRGKYKDKRRKKKKRKKLKKKGKEK for human SR-25) are putative nuclear targeting signals. Indeed, a cluster of several basic amino acid residues in proteins has been shown to be a signal required for nuclear import by an interaction with positively-charged portions of the nuclear pore complex: for example, KRPRP for adenovirus E1a protein (6) and PKKKRKY for SV40 virus large T antigen (7). However, the basic clusters of mouse SR-25 and human SR-25 appear to be too long for only nuclear transport.

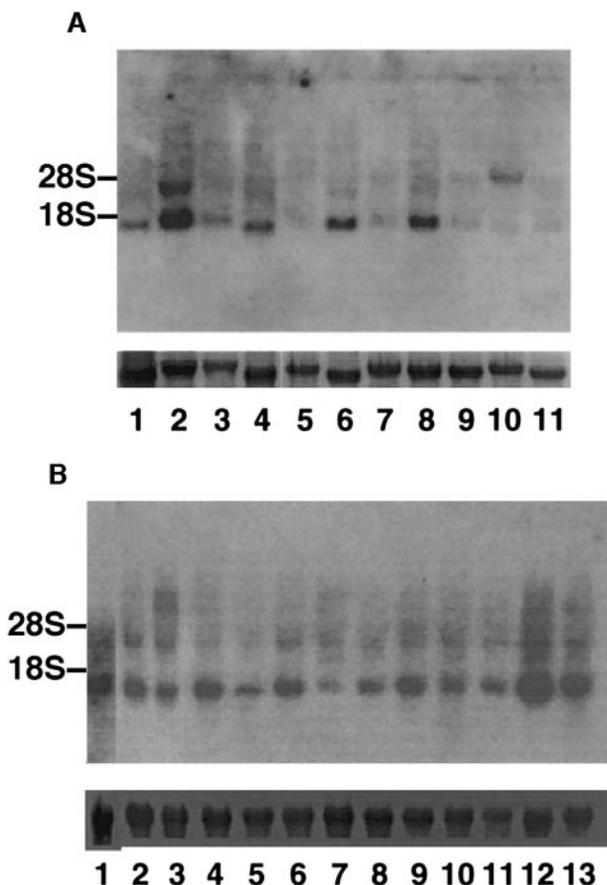


FIG. 7. Northern blot analysis. (A) Cell lines. Lane 1, pancreatic islet; lane 2, MIN 6; lane 3, NIT-1; lane 4, α -TC1; lane 5, AR42J; lane 6, AtT-20; lane 7, GH3; lane 8, NIH/3T3; lane 9, PC12; lane 10, QGP-1; and lane 11, α -TSH. (B) Mouse organs. Lane 1, pancreas; lane 2, MIN6; lane 3, brain; lane 4, heart; lane 5, intestine; lane 6, kidney; lane 7, liver; lane 8, muscle; lane 9, ovary; lane 10, spleen; lane 11, stomach; lane 12, testis; and lane 13, thymus.

Single bands observed in other lanes indicate that SR-25 gene exists as a single copy per haploid in the mouse genome.

Northern Blot Analysis

Because SR-25 cDNA was originally cloned from a mouse insulinoma cell line (MIN6), SR-25 mRNA expression was examined mainly in several endocrine cells (Fig. 7A). The major transcript for SR-25 was detected at 1.5 kb and several minor transcripts were around 5.0 kb. SR-25 mRNA was relatively abundant in pancreatic islets, MIN6, NIT-1, α -TC1, AtT-20, and NIH/3T3, whereas its level was relatively low in GH3, PC12, QGP-1, and α -TSH. Only in AR42J cells, SR-25 mRNA expression was not detected.

Organ distribution of SR-25 mRNA was also examined (Fig. 7B). It was expressed in all organs tested, and relatively abundant in testis and thymus. X16/SRp20, which is a splicing factor, is also strongly ex-

pressed in thymus and testis (28). These results suggest that SR-25 expression is associated with X16/SRp20 expression, and that it may be correlated with cell proliferation/differentiation (29–31). Thus, whether the level of SR-25 expression changes in a cell cycle-dependent manner was examined using TIG-1 fibroblasts, which have been used for cell cycle analysis. The level of SR-25 mRNA standardized for 28S rRNA did not obviously change in TIG-1 cells (data not shown).

Taken together, this novel protein, SR-25, is strongly suggested to be localized in the nucleus and may interact with RNA splicing factors.

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