MOLECULAR AND CELLULAR BIOLOGY, June 1997, p. 3116–3124 Vol. 17, No. 6
0270-7306/97/$04.00+0

Regulated Expression and RNA Processing of Transcripts from the *Srp20* Splicing Factor Gene during the Cell Cycle

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Received 7 October 1996/Returned for modification 6 December 1996/Accepted 25 March 1997

Eukaryotic splicing factors belonging to the SR family are essential splicing factors consisting of an N-terminal RNA-binding region and a C-terminal RS domain. They are believed to be involved in alternative splicing of numerous transcripts because their expression levels can influence splice site selection. We have characterized the structure and transcriptional regulation of the gene for the smallest member of the SR family, SRp20 (previously called X16). The mouse gene encoding SRp20, termed *Srp20*, consists of one alternative exon and six constitutive exons and was mapped to a 2-centimorgan interval on chromosome 17. When cells are transfected with SRp20 genomic DNA, both standard and alternatively spliced transcripts and corresponding proteins are produced. Interestingly, in starved (G_0) cells, the amount of SRp20 mRNA containing the alternative exon is large, whereas the amount of the standard SRp20 mRNA without the alternative exon is small. When starved cells are stimulated with serum, the alternative form is lost and the standard form is induced. These results suggest that splicing could be regulated during the cell cycle and that this could be, at least in part, due to regulated expression of SR proteins. Consistent with this, experiments with synchronized cells showed an induction of SRp20 transcripts in late G_1 or early S. We have also characterized the promoter of SRp20. It lies within a GC-rich CpG island and contains two consensus binding sites for E2F, a transcription factor thought to be involved in regulating the cell cycle. These motifs may be functional since reporter constructs with the SRp20 promoter can be stimulated by cotransfection with E2F expression plasmids.

The accurate selection of splice donor and acceptor sites in primary mRNA transcripts is crucial for the expression of most eukaryotic genes. This process involves the ordered assembly on the pre-mRNA of a multicomponent ribonucleoprotein complex called the spliceosome. The spliceosome consists of the small nuclear ribonucleoprotein particles (snRNP) U1, U2, and U4/U5/U6 as well as a growing number of non-snRNP proteins which are also essential for pre-mRNA splicing (16, 17, 29, 37). Several of these "accessory" proteins belong to a group of highly conserved serine- and arginine-rich nuclear phosphoproteins called the SR family (for recent reviews see references 12 and 32). Each member of the SR protein family has an N-terminal region consisting of one or two copies of an 80-amino acid (aa) motif known as the RNP-type RNA binding domain (RBD) (4) or RNA recognition motif (20). The RBD is a conserved structure found in a large number of RNA-binding proteins, including several snRNP and heterogeneous nuclear RNP (RNP) proteins. The C-terminal portions of the SR family members contain regions of alternating serines and arginines, hence the name SR. Although the role of the SR domain in splicing is still not fully clear, it may mediate protein-protein interactions with other splicing factors (1). In addition, it can target a heterologous protein to nuclear speckles (31), regions where several splicing components are preferentially localized. All members of the SR family can complement a splicing-deficient cytoplasmic S100 extract (9, 15, 22, 27, 28, 41, 56) and are thought to be involved in splice site selection (13, 14, 26, 57).

The SR proteins appear to function very early in the splicing reaction because they have been shown to promote binding of U1 snRNP to the 5′ splice site (11, 24). Since SR proteins can also promote U2AF and U1 snRNP association with the 3′ splice site (46) and can interact with one another, it has been proposed that the 5′ and 3′ splice sites are brought together in the earliest prespliceosomal complex via a network of protein-protein interactions involving U1 snRNP, U2AF, and SR proteins (1, 24, 46, 54).

While correct splicing depends on the recognition of the splice sites per se, the ability to produce variable protein isoforms by alternative splicing of mRNA transcripts is frequently used (33, 45, 47). It is also clear that alternative splicing is tissue specifically and developmentally regulated. Since the relative levels of the various splicing factors have been shown to influence splice site selection in vitro, the regulation of splice factor gene expression is probably crucial for cell-specific splicing.

We are studying a member of the SR gene family called SRp20 (previously named X16) in mice and have shown that it is differentially expressed in various mouse tissues (3). We show in this report that, in vivo, SRp20 mRNA levels are cell cycle regulated and that SRp20 itself is alternatively spliced, apparently in a cell cycle-specific manner. To gain insights into the structure, regulation of expression, and function of the SRp20 gene, we describe here the complete genomic organization, chromosomal localization and nucleotide sequence of the mouse SRp20 gene. Using reporter constructs, we have characterized the region of the SRp20 gene containing promoter activity and show that this promoter can be activated by cell cycle-regulated transcription factor E2F.

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MATERIALS AND METHODS

Isolation of genomic clones containing the murine SRp20 gene. The near-full-length SRp20 cDNA from a murine B-cell cDNA library (3) was labelled by

random priming and used to screen a genomic λGEM-12 phage library (a generous gift from J. Pelkonen) derived from DNA from a mouse T-cell line partially digested with *Mbo*I according to established procedures (38). Out of approximately 10^6 plaques screened, 71 positive clones were identified and plaque purified. Two clones, denoted gSRp20.17 and gSRp20.4 (described herein), were shown by Southern blot analysis and sequencing to contain the entire SRp20 gene.

Subcloning and sequencing. Genomic DNA fragments were subcloned into pTZ18 or pTZ19 (Pharmacia) by using established protocols (38). Templates were generated for both orientations and sequenced by the dideoxynucleotide chain termination method (39).

Chromosomal localization. DNA samples were prepared from mice of the C57BL/6 inbred strain; from mice of the SEG/Pas strain, a moderately inbred strain of the *Mus spretus* species maintained at the Institut Pasteur (7); and from several of their consomic derivatives, each carrying chromosomal segments of various lengths derived from *M. spretus* in an otherwise C57BL/6 background. This reduced DNA resource, as indicated below, permits a very rapid, although relatively imprecise chromosomal assignment. For a more accurate localization, we used a subset of samples from the EUCIB DNA resource (8). PCR were performed (30 cycles, each consisting of denaturation at 94°C for 50 s, annealing at 52°C for 30 s, and extension at 72°C for 50 s) with the following oligonucleotide primers: forward, 5′-GGACATGGTTCCCTATG-3′ and reverse, 5′-CCA AGGGACAGGAATCAC-3′, which are located at positions 5848 and 7034, respectively, in the SRp20 gene sequence. The resulting DNA product is 1,200 bp long with template DNA from the C57BL/6 strain and 1,300 bp long with DNA from the SEG/Pas (*M. spretus*) strain. This clear-cut length polymorphism, which can easily be resolved on a 1% agarose gel, was used for determining the chromosomal localization of the SRp20-encoding gene.

Mapping of the transcriptional start site. For primer extension, the X16PEX2 oligonucleotide (5′-CGAGATCCTGGGTTCAA-3′) was labelled at the 5′ end with ^{32}P by kinasing and was isolated from a 12% polyacrylamide gel containing 8 M urea. The purified oligonucleotide was annealed for 15 min at 50°C with 1 μg of poly(A)$^+$ RNA or 10 μg of total RNA from murine pre-B-cell line 33.1.1μ2. The extension reaction was performed with 200 U of murine leukemia virus reverse transcriptase for 1 h at 42°C. For S1 protection, aliquots of 3 to 15 μg of total RNA from murine B-cell lymphoma K46 were annealed overnight at 42°C to radiolabelled genomic probes spanning the region from −189 to +173 containing exon 1 (a *Sst*I-to-*Xho*I fragment; for numbering see Fig. 6) or from −290 to −470 (a *Kpn*I-to-*Pst*I fragment). In accordance with standard procedures (38), the probes were labelled by extending appropriate linearized plasmids with the Klenow fragment of DNA polymerase I in the presence of [^{32}P]dATP (Amersham Buchler), followed by purification from urea–5% acrylamide gels. The RNA-DNA hybrids were digested with S1 nuclease as recommended by the supplier (Ambion) and were precipitated. The products from both mapping procedures were electrophoresed on 6% denaturing polyacrylamide gel containing 8 M urea and sized by comparison with sequencing reactions run in adjacent lanes.

Cell culture and transient transfection assays. Cell lines were cultured in Iscove's modified Dulbecco medium (IMDM) (Gibco-BRL) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μM 2-mercaptoethanol at 37°C and 7.5% CO_2.

Transfection of the murine pre-B-cell line 33.1.1μ2 (19) and B-cell lymphoma K46 (21) was performed by electroporation with 10^7 cells and 10 μg of plasmid DNA at 225 V and 950 μF. Nonspecific plasmid DNA was added to the control transfections when pCMV-E2F-1 (18) was used. Transfection efficiency was determined by coelectroporation with 5 μg of pCMV-CAT (53) containing the chloramphenicol acetyltransferase (CAT) coding sequence downstream of the cytomegalovirus promoter.

K46 cells were synchronized by serum deprivation (0.5% FCS in IMDM for 48 h) and subsequently restimulated with 10% FCS in IMDM. X63Ag8 cells (23) (a murine hybridoma) and K46 cells were synchronized in S phase by using a double hydroxyurea block (40). Briefly, cells were grown in 200 μM hydroxyurea for 20 h, released by washing once in phosphate-buffered saline (PBS) and once in IMDM, and then grown in IMDM for 12 h. The cells were then blocked again by incubation in 200 μM hydroxyurea for 20 h before stimulation with medium and harvesting for RNA analysis. Synchronization of K46 and X63Ag8 cells with aphidicolin was by incubation at 5 μg/ml for 20 h. For the nocodazole block of X63Ag8, cells were incubated for 15 h in 0.1 μg of nocodazole per ml (58), collected by centrifugation, washed once in PBS, and resuspended in fresh medium at a density of 4×10^5 cells/ml.

For fluorescence-activated cell sorter (FACS) analyses of cellular DNA content, 10^5 to 10^6 cells were washed in PBS and resuspended in 1 ml of NH buffer (0.9% NaCl, 10 mM HEPES [pH 7.5]). Cells were then fixed by adding 3 ml of 70% EtOH dropwise with gentle mixing. After 30 min, cells were washed in PBS and resuspended in 0.2 ml of NH buffer. RNase A and propidium iodide were added to final concentrations of 1 mg/ml and 100 μg/ml, respectively, and the mixture was incubated for 30 min at room temperature. Cells were then washed once in PBS, and data were collected on a FACScan (Becton Dickinson) cytometer with CellQuest (Becton Dickinson) and ModFit (Verity Software House Inc, Topsham, Maine) software.

Western blot analyses. For expression of hemagglutinin (HA)-tagged SRp20, standard and alternative SRp20 cDNAs or genomic DNA was cloned into the pCGN expression vector (50) downstream of the HA tag. K46 cells were transfected, and cells were lysed 24 h later by resuspension at a concentration of approximately 3×10^4/μl in lysis buffer (0.5% Nonidet P-40, 10 mM Tris [pH 7.5], 1 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM KCl, 0.4 M NaCl). After 5 min on ice, the samples were centrifuged at 11,000 × *g* for 5 min. Protein in the supernatant was separated on sodium dodecyl sulfate–15% polyacrylamide gels (extract from 10^6 cells/lane) under reducing conditions and transferred to nitrocellulose membrane by standard procedures (38). After being blocked with 5% milk powder, the membrane was incubated, first with the primary antibody, anti-HA tag (monoclonal antibody 12CA5; Boehringer Mannheim) and then with peroxidase-coupled goat anti-mouse antibodies (Southern Biotechnology). The membrane was then developed by the enhanced chemiluminescence detection method (Amersham Buchler).

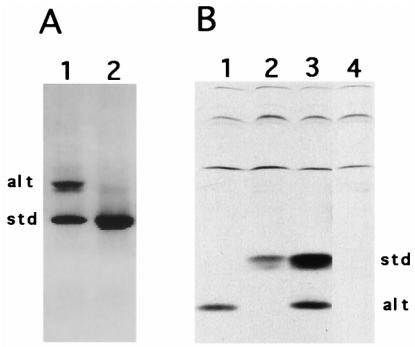

FIG. 1. SRp20 pre-mRNA is alternatively spliced and is translatable. (A) Total RNA extracted from K46 cells serum starved for 48 h (lane 1) and from the same cells 7 h after stimulation with serum (lane 2) was used for reverse transcriptase PCR with primers located at the start and stop codons of SRp20 mRNA. After agarose gel electrophoresis and blotting, the membrane was hybridized with a radioactively labelled SRp20 cDNA probe. Products derived from the alternative (alt; 966-bp) and standard (std; 519-bp) forms of SRp20 mRNA are indicated. (B) Sequences coding for the 12-aa HA peptide tag were fused to cDNA coding for both the alternative (lane 1) and standard (lane 2) forms of SRp20 and to SRp20 genomic DNA at the start ATG in exon 2 (lane 3). Following transfection and transient expression in the B-cell lymphoma K46, Western blots were made from whole-cell extracts and the tagged protein was revealed by incubation with anti-HA antibodies (see Materials and Methods). Lane 4 shows a control extract from cells transfected with the parental vector carrying no SRp20 sequences.

CAT and luciferase assays. Cells were harvested 20 h after electroporation, resuspended in 100 μl of Tris-HCl (pH 7.8), lysed by three freeze-thaw cycles, clarified by centrifugation in a microcentrifuge (10 min), and assayed for luciferase and CAT activity as described previously (52). Data shown are means ± standard deviations obtained after assay of the extracts from at least three separate experiments.

Northern blot analysis. For Northern blots, 10 μg of total cellular RNA, prepared as described by Chomczynski and Sacchi (10), was separated electrophoretically on 1% agarose-formaldehyde gels, was transferred to nylon membranes (GeneScreen [NEN] or Biodyne A [Pall]), and was hybridized with ^{32}P-labelled DNA probes by standard methods (38).

Nucleotide sequence accession number. The sequence reported here has been assigned GenBank accession no. X91656.

RESULTS

SRp20 mRNA is alternatively spliced in a regulated manner depending on serum stimulation. Northern analysis of SRp20 mRNA expression in various mouse tissues and cell lines shows a major 1.6-kb band and a second hybridizing band at about 1.9 kb (3). To determine whether the upper band is generated by alternative splicing of SRp20 mRNA, cytoplasmic RNA from the mouse K46 B-cell lymphoma cells was used as a template for cDNA synthesis. A subsequent PCR employing primers positioned at the start and stop codons of SRp20 mRNA and agarose gel electrophoresis showed a major band of 0.5 kb corresponding to the published SRp20 cDNA sequences as well as a second, less intense band of 0.9 kb (see Fig. 1). Subsequent cloning and sequencing showed that the larger band is derived from an alternatively spliced transcript con-

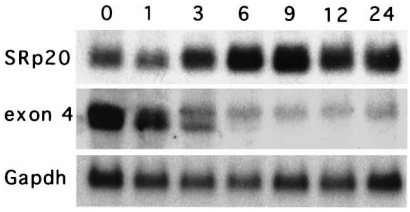

FIG. 2. Northern blot analysis of serum-starved K46 cells. Cells were incubated in medium with 0.5% serum for 48 h before being stimulated with medium containing 10% FCS. Total RNA extracted at different time points after stimulation (given in hours above each lane) was used for Northern blot analysis. The filter was hybridized consecutively with the indicated probes. The exon 4 probe is derived from the alternative exon of the mouse SRp20 gene.

taining exon 4 (see Fig. 4 and Table 1). Inclusion of this exon introduces an in-frame stop codon and would result in the translation of a truncated SRp20 protein containing the N-terminal RNA-binding domain but lacking the C-terminal SR domain.

Performing the same PCR with cDNA from serum-starved and serum-induced K46 B-cell lymphoma cells revealed that the alternative form is increased in G_0 cells and almost absent in stimulated cells (Fig. 1). Previous experiments with serum-starved fibroblasts showed that the expression of standard-form SRp20 mRNA was induced three- to fourfold 11 h after stimulation with fresh medium and serum (3). To measure the kinetics and extent of SRp20 mRNA induced by serum in more detail, K46 B-lymphoma cells were grown in low concentrations of serum (0.5% FCS) for 48 h and then stimulated with 10% FCS. Total RNA was extracted at different time points after serum addition and analyzed by Northern blotting for SRp20 mRNA levels (Fig. 2). Relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, the induction of standard-form SRp20 mRNA was maximal about 6 h after serum addition. Similar results were obtained with LTK$^-$ cells (data not shown). By using a probe for ASF, a second member of the SR protein family, hybridization signals were also stimulated with serum, but with a somewhat slower kinetics relative to that of SRp20 (data not shown). Rehybridization of the same filter with an exon 4 DNA probe showed that the 1.9-kb band seen with SRp20 cDNA corresponds to an alternatively spliced form of SRp20 (data not shown) and that the exon 4-containing or alternative-form mRNA is more prevalent in quiescent cells and decreases when proliferation is stimulated with serum (Fig. 2). The two bands in Fig. 2 recognized by the exon 4 probe probably represent the use of alternative poly(A) sites also seen in standard-form SRp20 mRNA (3).

The stimulation of standard-form SRp20 mRNA following serum addition is primarily the result of increased steady-state levels. Based on relative band intensities following Northern hybridization with SRp20 cDNA (data not shown), we estimate that the ratio of the alternative to the standard form is 10% in starved K46 cells and less than 1% in rapidly growing cells.

To test whether the alternative form of SRp20 mRNA can be translated into protein, we fused DNA coding for a 12-aa HA peptide tag (at the N terminus of SRp20) to both cDNA forms and to an SRp20 genomic fragment capable of expressing both forms of SRp20 protein. Following transient expression in K46 cells and Western analysis of total cellular extracts, proteins of the expected sizes for standard- and alternative-form SRp20 were detected (Fig. 1B, lanes 1 and 2). As expected, transfection with tagged genomic DNA resulted in expression of both forms from the same construct (Fig. 1B, lane 3).

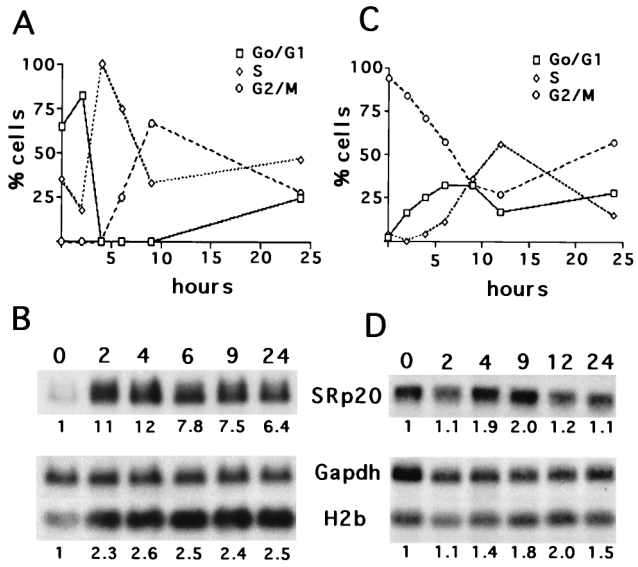

FIG. 3. Expression of SRp20 is cell cycle regulated. X63Ag8 cells were synchronized with a double hydroxyurea block (A and B) or nocodazole (C and D) as described in Materials and Methods. Aliquots of cells taken at the indicated times following release from the hydroxyurea block (A) or nocodazole (C) were stained with propidium iodide and then analyzed by FACS analysis (see Materials and Methods). Northern analysis was performed on total RNA extracted at the indicated times after removal of hydroxyurea (B) or nocodazole (D) (given in hours above each lane). The filter was hybridized consecutively with the indicated probes. SRp20 and H2b mRNA levels were quantitated by densitometry on the Northern blots and are listed below each Northern blot lane. The values are expressed relative to the amount at 0 h and are normalized to the respective GAPDH signal at each time point.

Cell cycle regulation of SRp20 transcripts. The regulated alternative splicing of SRp20 mRNA motivated us to investigate whether the alternative form of SRp20 mRNA is coupled to certain phases of the cell cycle other than G_0. Further, because of the presence of consensus E2F binding sites in the SRp20 promoter (see below) and the fact that a common feature of genes binding E2F is that their expression is correlated with cellular proliferation (30, 34), we wanted to test whether SRp20 expression is regulated during the cell cycle. To do this, the murine hybridoma line X63Ag8 was synchronized by either a double hydroxyurea block (40) or nocodazole (58). The distributions of the cells in the cell cycle at various times after release from the block were measured by FACS analysis of cellular DNA content (Fig. 3A and C). Hydroxyurea blocks cells late in G_1 or early in S (43). At 4 h after release from the hydroxyurea block, almost all cells were in S phase. By 9 h following release from the block, the majority of the cells were in the G_2/M phase. Total RNA was extracted at different time points after release from the hydroxyurea block and analyzed by Northern blotting for SRp20 expression. Probes for histone 2B (H2B), which is known to be induced in the S phase (48), and GAPDH were used as controls. Release from the cell cycle block resulted in an induction of both SRp20 and H2B mRNAs (Fig. 3), although SRp20 mRNA induction was more dramatic (10-fold). Induction of both mRNAs was seen primarily at 2 h after release, although the majority of the cells in the culture were in the S stage at 4 h. The expression of SRp20 was reduced twofold as the cells progressed further in the cell cycle. Similar results were obtained with aphidicolin-synchronized K46 and X63Ag8 cells (data not shown).

The induction of SRp20 and histone H2b mRNA in late G_1

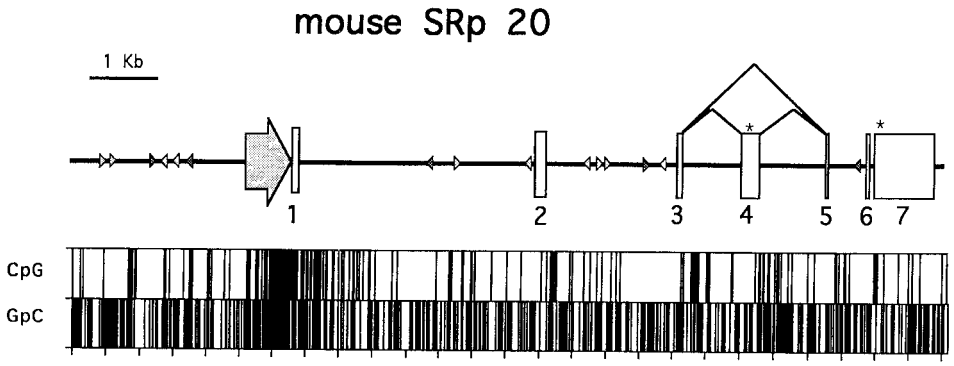

FIG. 4. Structure of mouse SRp20 gene. The exons, represented by open boxes, and introns are drawn to scale. Triangles indicate B1 repetitive elements, and the large arrow is the SRp20 promoter. The alternative splicing events involving exon 4 are shown. Asterisks mark the positions of the stop codons for the two splice forms. The lower part of the figure shows the positions of CG and GC dinucleotides.

or early S suggests that both are required in the preparation for entry into S. This conclusion is supported by results obtained in X63Ag8 cells blocked with nocodazole at G_2/M. As expected, following release, the majority of the cells reach the S phase later (maximum at 12 h) than when blocked with hydroxyurea (compare Fig. 3A with 3C). The peak induction of SRp20 mRNA is between 4 and 9 h after release, which is later than the induction seen after hydroxyurea release. The H2B stimulation also appears later. Hybridization with the exon 4 probe after hydroxyurea block (data not shown) did not lead to the detection of any differences in the amounts of alternative-form SRp20 mRNA at the different time points, suggesting either that the increase of the alternative form is limited to the G_0 phase or that the time points used were not appropriate for detecting the splicing regulation.

Isolation and characterization of mouse SRp20 genomic clones. In order to further study transcription and splicing regulation of SRp20, we characterized the SRp20 genomic organization. A mouse genomic lambda phage library was screened with near-full-length SRp20 cDNA, and multiple positive recombinants were isolated. (We adopt the name SRp20 for the cDNA previously called X16.) Two overlapping clones which contained the entire gene were characterized by restriction mapping, hybridization with cDNA subfragments, and sequencing. The resulting structure of the mouse SRp20 gene is summarized in Fig. 4.

Southern blots showed that all internal restriction fragments from the lambda clones which hybridized with SRp20 cDNA were also present in restriction enzyme-digested genomic DNA (data not shown), indicating that no structural alteration had occurred during the cloning of the SRp20 gene. The numerous bands seen in Southern blots when genomic DNA is hybridized with SRp20 cDNA (3) suggest that there is more than one SRp20 gene. Indeed, using genomic DNA as the template for PCR with oligonucleotide primers derived from the region of the cDNA corresponding to the translational start and stop codons, we cloned and sequenced this region from three additional SRp20 genes which appear to be intronless retroposons. The sequences differed from that of the cDNA by 1 to 3% (data not shown). Since none of these sequences have been found in numerous cDNA clones sequenced, we assume that they are nontranscribed pseudogenes.

Sequence analysis of the murine SRp20 gene. Several fragments of lambda clones gSRp20.17 and g2SRp20.4 were used to obtain the complete nucleotide sequence of the SRp20 gene. The gene spans about 10 kb and contains 7 exons. All exon-intron junction sequences conform to the canonical GT/AG rule (Table 1). Exon 4 is the previously uncharacterized alternative exon. The sequence of all remaining exons is identical to that of the cDNA (3), except for additional nucleotides at positions 12184 (G) and 12466 (T) in the 3′ untranslated region of the last exon. There are three potential poly(A) addition signals in the last exon, the last two of which have been reported to be used (3). There are nine B1 repetitive elements

TABLE 1. SRp20 exon-intron splice junctions

Exon	Size (bp)	Codon phase[a]	5′ splice donor[b]	Intron	Size (bp)	Splice acceptor	
						3′ sequence[b]	3-purine length[e] (bp)
1	114		GATCTCGgtaagg	1	3,454	ttttttaactacagAAATGC	17
2	208	II	ATGGAAGgtatg	2	2,041	gtcccttctctttagAACACT	18
3	135	II	GGCGCAGgtactt	3	829	gaaccccgttacagAGTCAC	10
4alt[c]	435		AAAACTAgtaagt	4	864	tgtttctgcttctagATCCCC	19
5	39	II	GGAGCAGgtaaat	5	392	tgctgtttgttttagGTCACT	13
6	87	II	CTCGTAGgtaagt	6	90	tgtttttgttttttagCCGATC	18
7	856						
Con.[d]			C/AAGgta/gagt			$(c/t)_n$nc/tagG	

[a] The position within the codon where splicing occurs.
[b] Exon sequences are in uppercase.
[c] Alternatively spliced (see text).
[d] Con., published consensus splice donor and acceptor.
[e] The maximum length of the sequence immediately flanking the splice acceptor which contains only three purines. The AG at the splice site is not counted.

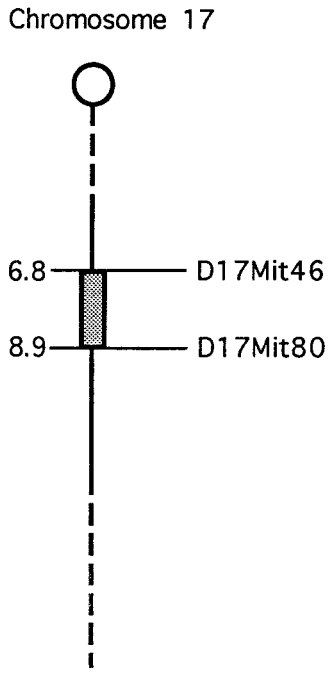

FIG. 5. Srp20 gene localization. The centromeric portion of mouse chromosome 17 is schematically represented. Distances from the centromere are indicated on the left. The boxed area indicates the region most likely to contain the Srp20 gene.

distributed within the gene but none in the introns flanking alternative exon 4. An additional cluster of six B1 elements is located immediately upstream of the promoter (Fig. 4).

Chromosomal localization of the SRp20 gene. To map the gene encoding SRp20 (suggested symbol, *Srp20*), we proceeded in two steps using a PCR-based screening protocol (see Materials and Methods). First, we used a panel of 16 DNA samples derived from a set of partially consomic strains with defined chromosomal fragments of *M. spretus* chromosomes on an otherwise C57BL/6 background (16a). By scoring the segregation of the PCR products for length polymorphism among these 16 informative DNA samples and matching our data to a reference chart of the already-known haplotype constitutions, as deduced from several previously scored molecular markers, we found that the *Srp20* locus mapped to mouse chromosome 17 between 0 and +32 centimorgans (cM) from the centromere. We then selected a highly informative subset of DNA samples from the EUCIB resource (8) for a second round of screening. We found that *Srp20* mapped within the 2-cM interval between the markers *D17Mit46* (+6.8) and *D17Mit80* (+8.9) (see Fig. 5).

Analysis of the SRp20 promoter region. Computer analysis demonstrated that the 5′ flanking region of the SRp20 gene has a high G+C content (about 65 to 70%; Fig. 4). This region also has a very high frequency of CpG dinucleotides and a CpG/GpC ratio of approximately 0.9, which is characteristic for CpG islands or *Hpa*II tiny fragments (HTF) (5). A search for potential transcription factor binding sites revealed that the region preceding exon 1 has a TATA box beginning at −31 (for numbering see Fig. 6 and below) and a CAAT box beginning at −71 in the reverse orientation. A second TATA box was observed beginning at −425. In addition, several other consensus elements, including two E2F sites, were identified by computer search and are illustrated in Fig. 6.

Identification of the SRp20 transcriptional initiation site. The 5′ end of the SRp20 mRNA was determined both by primer extension and S1 protection. For primer extension, an oligonucleotide primer complementary to the sequence located 43 bases downstream from the 5′ end of the previously isolated cDNA was annealed to both total RNA and poly(A)$^+$ RNA isolated from the pre-B-cell line 33.1.1μ2 and was extended with reverse transcriptase. Two major products were observed on a 6% polyacrylamide-urea gel (Fig. 7A). We interpret the shorter extension product (60 bases long) to be the result of a strong stop site for reverse transcriptase. Consistent with this, the predicted stop site corresponds to a region of 10 bases within which five independent SRp20 cDNAs begin (Fig. 6). The longer extension product (114 bases) probably corresponds to the authentic transcriptional start site initiating at a G residue 54 bases upstream from the 5′ end of the cDNA and 31 bases downstream from the first T in the TATA box (Fig. 6). This start site was confirmed by S1 protection analysis. By using a 360-bp genomic fragment containing exon 1 (nucleotides −189 to +173 in Fig. 6) as a probe and RNA from the B-cell lymphoma K46, a cluster of five bands was specifically protected; each differed in one nucleotide, with the middle band having a length of 114 bp (Fig. 7B, lanes 5 to 7).

Functional analysis of the SRp20 promoter with a luciferase reporter gene. To test whether sequences immediately upstream of the transcriptional start site exhibit promoter activity, restriction fragments from this region were inserted 5′ to the promoterless luciferase reporter gene of pAH1409 in sense and antisense orientations. The purified plasmids were electroporated into the murine B-cell lymphoma K46, and the promoter activities were monitored by a luciferase assay.

The 0.3-kb *Pst*I-*Bsa*I fragment (Fig. 8, construct b) from −289 to +19 showed 50-fold-higher transcriptional activity than the promoterless luciferase vector. Additional sequences extending upstream to the *Kpn*I or *Bgl*II site had no effect on the activity of the *Pst*I-*Bsa*I fragment. Surprisingly, the *Sst*I-*Bsa*I fragment from −189 to +19 containing the TATA box and the CAAT box (construct a) exhibits little or no promoter

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                                                                    -595
TATGCAAAAC CTTGCTCTCC TCCTGGGAAA GTGAGCCCTG TGACCGTTTG TCTTGTTTTT TTTTTTGTTT
                          E2F                              SP1/E2F      -525
TTTCATGCAG CCTCATCAAG TCGCATTTGC CGCGCGCGCG GTTTGGGCGA GGCGGGAAAG CGAAAGTTGA
                                                                KpnI    -455
GCATGAGCAA ACTTTGCAGG CTCTACCCTG CATCGGGGCG CAGACCGGGC ACCGTGGGGT ACCAGTTAAG
   CAAT                          TATA                               -385
TCGCCCAATC GCTGGAGCCT TAGAGCGAGC TATAAGGCTC CTTAGTCTGC GATGGATGCC CCACAACGAG
PEA3                    TRE                 PstI                    -315
GAAGCAAGCG TCCATGTTTC ACTCTGACCC GAGCCCGCGG CTGCAGCCGC CATCGCCCGG GATTAATGGC
                          PstI       E4Tf1/Ets1                       -245
GGCAGAGTCT GGACAGCTTT ATTCGCTGCA GCCTAGGCGG AAGTGGAGGA GAGGGGGCGT CTGCGCGGCT
                                                          SstI     RCE
CGCGGGGATG GCCGGGCCGA GCGCCGCATC TGCGGACAAG CCGGCACGCG GCGACGAGCT CGCGCCACCG
                    Ets1                                            -105
CGCGTACCCC ACCCACCTGA CGGAACGGCC GGCGCGCGCT CCCAGTAACC GCGGTTTCTC GCAGCGTTTG
        CREB/ATF                  CCAAT/AP1                         -35
TAGCGCTTAC CGTCACAGCC TGCGCCGCCT CTGATTGGGT GATCTGGTCA GGTCATGTGG CAGAGCAGGG
   TATA                              +1                Bsal          +36
TGCTATAAAT AGGGCGAGGG GACGGTGGTG GTCCGCCATT TCGTGACGCC GCGAGACCGA GAATCTGTAG
                start of cDNA                                       +106
GAGCAGAACC AGAGGGAAGC GGGTGGGCCT GTCGGAGCGT TAGGATTTGA GCTTGGGCCT TTTGAACCCA
                                                                    +176
GGATCTCGgt aggggaccgg cgaacggagg gaggggaggtg gaggggaggga ggtggagttc cgcagactcg
```

FIG. 6. Nucleotide sequence from the promoter region of the mouse SRp20 gene. The sequence is numbered by assigning the transcription start site +1. Underlined sequences indicate putative binding sites for transcription factors. Positions of the restriction endonuclease sites used for the construction of reporter plasmids are shown. The first nucleotide (start of cDNA) in the previously isolated cDNA corresponds to the smaller primer extension product shown in Fig. 7. The start of the first intron is in lowercase.

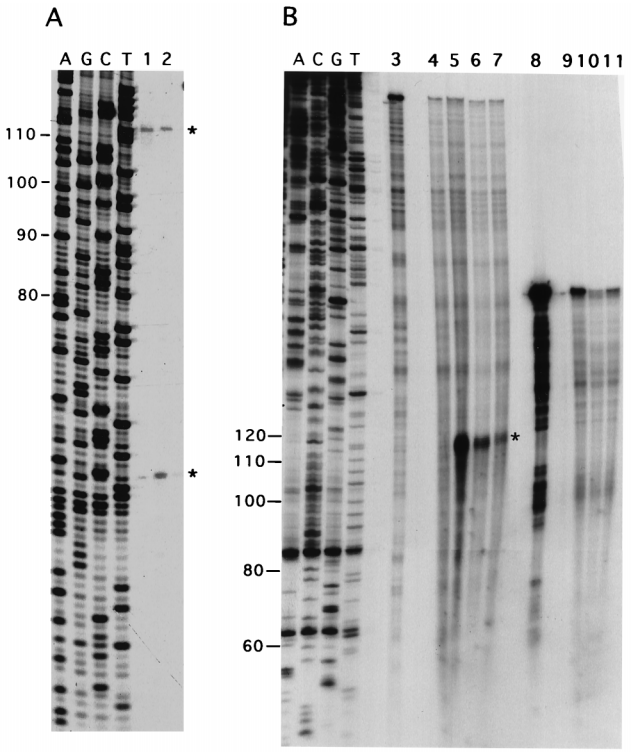

FIG. 7. Identification of the transcriptional initiation site. RNA was analyzed by primer extension (A) and S1 analysis (B). For primer extension, the 5′-end-labelled oligonucleotide X16PEX2 was annealed to 1 μg of poly(A)$^+$ or 10 μg of total RNA from 33.1.1μ2 pre-B cells (lanes 1 and 2, respectively). For S1 protection (B), total RNA from the B-cell lymphoma K46 was annealed to radiolabelled genomic probes spanning the region from −189 to +173 containing exon 1 (lanes 3 to 7) or from −290 to −470 (lanes 8 to 11). Lanes 3 and 8 contain undigested probe. Lanes 4 and 9 are controls of the respective probes annealed to yeast tRNA and then treated with S1. The amount of RNA used was 15 μg (lanes 5 and 10), 7 μg (lanes 6 and 11), or 3 μg (lane 7). For both panels, the products were separated by electrophoresis on 6% polyacrylamide—8 M urea gels and sized by comparison to sequencing reactions run in adjacent lanes (lanes A, G, C, and T). The extended or protected products obtained are marked by asterisks.

activity. Thus, at least in the context of SRp20 gene sequences surrounding the TATA box, the GC-rich region from −289 to −189 appears to be necessary for promoter function. Similar results were obtained in a pre-B-cell line (33.1.1μ2) (data not shown).

The *Bgl*II-*Pst*I fragment from −1052 to −346 (construct h) containing the upstream TATA box at −425 failed to induce significant promoter activity above the background level, confirming the importance of sequences between *Pst*I and *Bsa*I for constitutive promoter activity. When the antisense orientation of several active promoter fragments was tested, no significant promoter activity was detected. These results suggest that the basal SRp20 promoter is located within 289 bp upstream of the transcription start site. We also looked for transcripts derived from the sequences flanking the upstream TATA box in K46 cells by S1 protection and found none (Fig. 7B, lanes 9 to 11). We cannot exclude the possibility that the upstream TATA box is involved in the expression of SRp20 in other cell types or during development. To test whether the *Pst*I-*Sst*I fragment from −289 to −189 has enhancer activity, it was subcloned 5′ to the herpes simplex virus (HSV) tk promoter in the luciferase reporter gene vector pTK1409 (constructs j and k). The inserted fragment gave two- to threefold-higher levels (sense and antisense orientations) than the HSV tk promoter alone (Fig. 8) and thus appears to have only weak enhancer activity. A 60-bp *Nae*I deletion in the *Pst*I-*Bsa*I fragment, which included the *Sst*I site, had no effect on promoter activity (data not shown) showing that digestion with *Sst*I does not destroy an important *cis*-acting site. Thus, the 100-bp *Pst*I-*Sst*I fragment probably represents a crucial part of the SRp20 promoter rather than an enhancer.

Based on the observation that the SRp20 mRNA level is regulated during the cell cycle (Fig. 3) and on the presence of two E2F consensus sites located at −546 and at −571, a promoter fragment containing both these sites was tested for its ability to increase expression of the luciferase gene driven by the HSV tk promoter. The *Bgl*II-*Kpn*I fragment (−1052 to −469) was subcloned 5′ to the tk promoter in pTK1409 (construct m). Figure 8 shows that this fragment produces a threefold enhancement of luciferase activity (compare with construct l). To further investigate the role of the E2F consensus sites, cotransfection experiments were performed with an E2F-1 expression vector and various promoter constructs containing the E2F consensus sites. As shown in Fig. 8, cotransfection of an E2F-1 expression vector stimulated the luciferase activity in all constructs where the E2F sites were present, but to different extents depending on the construct used (up to 18-fold with construct g). This suggests that the E2F consensus sites are functional. As expected, a control cotransfection of the E2F-1 expression vector with a luciferase expression vector driven by the HSV tk promoter (construct l), which lacks an E2F consensus site, had no stimulatory effect.

DISCUSSION

SRp20 activity is probably tightly regulated in the cell. The results reported here indicate that the regulation of SRp20 expression includes both steady-state mRNA levels and RNA-processing components. In addition, in numerous attempts, we have been unable to generate cell transfectants or transgenic mice which stably overexpress SRp20. This suggests that overexpression of SRp20 may be lethal for the cell. Similar conclusions were drawn in a recent report on ASF function (51).

We have shown here that when serum was added to serum-starved K46 cells, the steady-state amount of SRp20 (as well as of ASF) mRNA increases with time, peaking (for SRp20) at 6 to 9 h after serum addition (Fig. 1 and 2). Interestingly, the alternative form of SRp20 mRNA containing exon 4 is dramatically increased in quiescent cells, whereas after serum addition, this form almost disappears. However, as estimated by Northern blotting, the amount of alternatively spliced SRp20 mRNA did not exceed 10% of the standard form. During the cell cycle, SRp20 mRNA is induced in late G_1 to S. Although there are numerous reports of changes in splicing accompanying growth stimulation, little is known about alternative splicing during the cell cycle. A variant splice form of tumor suppressor p53 which is more abundant in G_2 (55) and S phase expression of thymidylate synthase (2, 25) are rare examples. Given the fact that splice site selection can be influenced by the relative amounts of SRp20, ASF, and other SR proteins (13, 14, 26, 57), the results presented here suggest a mechanism for the regulation of alternative splicing during the cell cycle. Concerning the mechanism for the alternative splicing of the SRp20 transcript, both the splice donor and acceptor sites of exon 4 show suboptimal agreement with the respective consensus sequences (Table 1). The pyrimidine-rich stretch in the 3′ splice acceptor is much weaker in exon 4, and neither the A nor the G complementary to U1 snRNP at the 5′ splice donor junction is present.

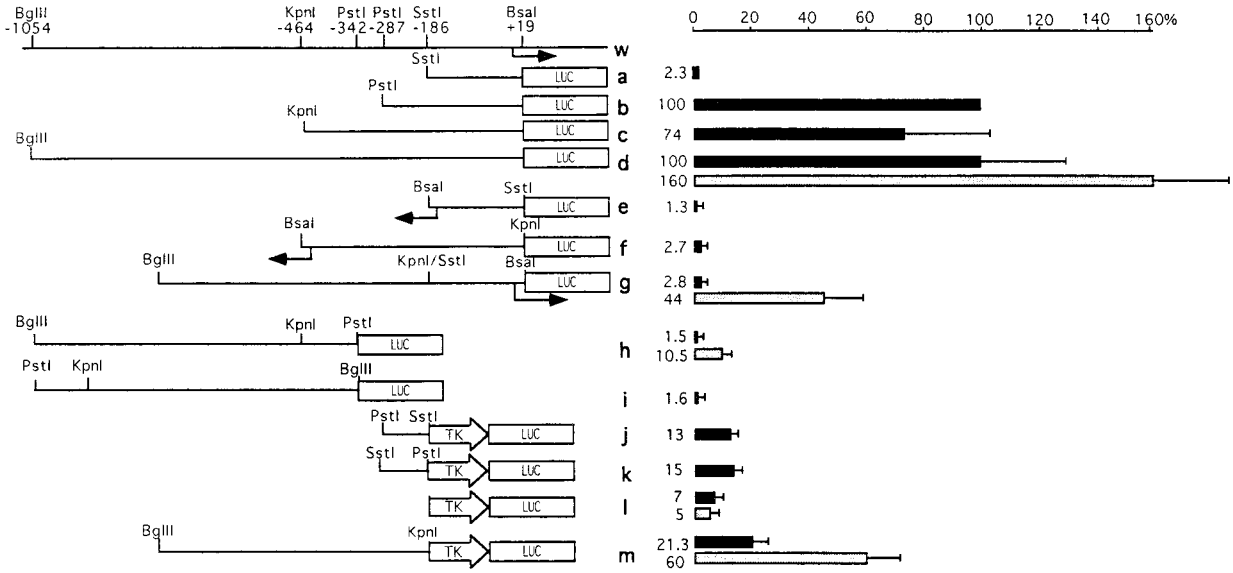

FIG. 8. Transient-transfection analysis of the mouse SRp20 promoter region. The *Bgl*II-*Bsa*I fragment (nucleotides −1052 to +19) was linked to the promoterless firefly luciferase gene (LUC) in pAH1409, and various deletions were performed as shown to give constructs a to d. Constructs e and f are the antisense orientations of a and c, respectively. The *Bgl*II-*Pst*I fragment (nucleotides −1052 to −346 containing the upstream TATA box) was tested in both orientations in constructs h and i. For constructs j and k, the *Pst*I-*Sst*I fragment (nucleotides −289 to −189) was tested for enhancer activity with the unrelated tk promoter. For construct m, the *Bgl*II-*Kpn*I fragment (nucleotides −1052 to −469 containing the E2F consensus sites) was tested. Shaded bars indicate that the E2F-1 expression vector pCMV-E2F-1 was coelectroporated with the corresponding construct. The luciferase values are the averages of at least three independent experiments and are expressed in percentages relative to that for construct b.

Suboptimal splice sites are often found in alternatively spliced transcripts, and it is thought that splicing factors binding to nearby splicing "enhancers" play an important role in regulating the use of alternative exons (for a review see reference 32). Interestingly, we have evidence that SRp20 can influence the splicing of exon 4 in its own mRNA (data not shown). An additional regulatory level may be introduced by considering that translation of the alternatively spliced SRp20 would lead to a protein with an RNA-binding motif but no SR domain. This truncated form of SRp20 could act as a dominant inhibitor of SRp20-mediated splicing.

It is tempting to speculate that regulation of splicing factors by alternative splicing may be a general phenomenon since, in addition to SRp20, other SR protein family members (15, 36, 41), as well as the *Drosophila melanogaster* sex determination genes *tra* and *tra2* (1, 6), have also been reported to be alternatively spliced.

We have also characterized the structural and promoter regions of the gene encoding the murine SRp20 splicing factor and determined the gene's chromosomal location. The gene, termed *Srp20*, is composed of one alternatively spliced exon and six constitutive exons, which span about 10 kb on mouse chromosome 17 (Fig. 4 and 5). The region around the first exon is GC rich with a high frequency of CpG dinucleotides, characteristic of the CpG or HTF islands seen in many "housekeeping" genes. The promoter region also contains a TATA box centered at 28 bp upstream of the transcriptional start site, as is often associated with tissue-specific, but less often with housekeeping, genes. Promoter activity, which was at least 10 times stronger than that of the HSV thymidine kinase promoter (Fig. 8), was observed in a 300-bp fragment (*Pst*I to *Bsa*I) immediately upstream of the cap site. Interestingly, a 200-bp subfragment from the 3′ part of this region is devoid of promoter activity, even though it contains consensus Ets1, CREB/ATF, CAAT, and TATA sequence motifs, as well as the cap site. Except for an E4TF1 site, the 5′ 100-bp region, which appears to be crucial for SRp20 promoter activity, does not contain recognizable transcription factor motifs. A more detailed functional analysis of this region could reveal novel transcription factors and/or their binding sites.

Computer-assisted analysis of the genomic sequence also revealed a second promoter-like region in the SRp20 gene containing a second TATA box, as well as two E2F consensus motifs. In transient transfection experiments, reporter constructs bearing this region, from −400 to −600 relative to the cap site characterized in this report, showed a low level of promoter activity on their own but were strongly activated by cotransfection with an E2F expression plasmid. The induction of the SRp20 promoter construct was not just a general effect of E2F on transcription since cotransfection of the E2F-1 expression vector with a reporter gene driven by the HSV tk promoter had no significant effect (Fig. 8) and since a reporter gene driven by the strong, ubiquitous SRα promoter (49) showed an approximately 10-fold reduction in activity when cotransfected with the E2F-1 expression vector (data not shown). However, no transcripts derived from this region of the gene were detected by S1 mapping (Fig. 7B) or reverse transcriptase PCR (data not shown) in a B-cell lymphoma. We cannot exclude, however, that transcripts originate from this region in other cell types or at other stages during differentiation.

The results presented here raise the possibility that the SRp20 promoter is regulated by E2F in vivo. E2F DNA binding sites have been found in the promoters of several genes (30, 35), including c-*myc*, B-*myb*, *cdc2*, and the genes for dihydrofolate reductase, thymidine kinase, and DNA polymerase α, which are required for cell cycle progression. These genes are transcribed in a periodic fashion during the cell cycle, usually being induced at the G_1/S boundary or during S phase. E2F-1 activity itself reaches a peak at the G_1/S boundary of the cell cycle (42, 44) and in this way is thought to participate in the transcriptional regulation of these genes. The data presented

in this paper suggest that some differential splicing may also be coupled to the cell cycle.

ACKNOWLEDGMENTS

We thank K. Helin and E. Harlow for their gift of pCMV-E2F-1, R. A. Sturm for the histone 2B plasmid p7AT, Jukka Pelkonen for the MRL genomic library, Barbara Lorenz, Tanna Franz, and Franck Bourgade for expert technical assistance, and Peter Shaw and Karl-Heinz Klempnauer for critical reading of the manuscript.

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