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An Intron Element Modulating 5′ Splice Site Selection in the hnRNP A1 Pre-mRNA Interacts with hnRNP A1

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The hnRNP A1 pre-mRNA is alternatively spliced to yield the A1 and A1b mRNAs, which encode proteins differing in their ability to modulate 5′ splice site selection. Sequencing a genomic portion of the murine A1 gene revealed that the intron separating exon 7 and the alternative exon 7B is highly conserved between mouse and human. In vitro splicing assays indicate that a conserved element (CE1) from the central portion of the intron shifts selection toward the distal donor site when positioned in between the 5′ splice sites of exon 7 and 7B. In vivo, the CE1 element promotes exon 7B skipping. A 17-nucleotide sequence within CE1 (CE1a) is sufficient to activate the distal 5′ splice site. RNase T_1 protection/immunoprecipitation assays indicate that hnRNP A1 binds to CE1a, which contains the sequence UAGAGU, a close match to the reported optimal A1 binding site, UAGGGU. Replacing CE1a by different oligonucleotides carrying the sequence UAGAGU or UAGGGU maintains the preference for the distal 5′ splice site. In contrast, mutations in the AUGAGU sequence activate the proximal 5′ splice site. In support of a direct role of the A1-CE1 interaction in 5′-splice-site selection, we observed that the amplitude of the shift correlates with the efficiency of A1 binding. Whereas addition of SR proteins abrogates the effect of CE1, the presence of CE1 does not modify U1 snRNP binding to competing 5′ splice sites, as judged by oligonucleotide-targeted RNase H protection assays. Our results suggest that hnRNP A1 modulates splice site selection on its own pre-mRNA without changing the binding of U1 snRNP to competing 5′ splice sites.

Most mammalian pre-mRNAs contain introns that must be removed through RNA splicing. Pre-mRNA splicing takes place in the spliceosome, which is a large, multicomponent complex that includes a set of the four snRNPs, U1, U2, U4/U6, and U5, and a number of non-snRNP proteins (reviewed in references 28, 29, and 46). Multiple snRNP-snRNP and snRNP–pre-mRNA interactions are crucial in specifying the efficiency and the precision of splicing (reviewed in references 49 and 54). One challenging aspect of RNA splicing is to understand how appropriate pairs of 5′ and 3′ splice sites are selected in pre-mRNAs containing multiple introns. Alternative splicing brings another dimension to the issue of splice site selection as it involves modulating the pairing of splices sites during development and differentiation.

The selection of splice sites is determined by several parameters including the proximity and strength of splicing signals (reviewed in reference 5). Although mutations in splicing signals often affect splice site selection, the fit of a splice site to consensus sequences is not sufficient to predict splice site utilization. Sequences lying outside splice sites appear to provide a context that influences splice site recognition. This notion of context has remained ill defined, but recent progress has revealed that the recognition of a weak 3′ splice site is improved by a downstream 5′ splice site through exon-bridging interactions (32, 52). A growing number of exon and intron sequences have been identified based on their ability to influence splice site selection. Splicing enhancers have been uncovered in a variety of mammalian exons (23, 37, 61, 65, 67, 70, 71, 73). Several exon enhancers are bound by SR proteins (37, 51, 58, 59), a family of related phosphoproteins essential for constitutive splicing (reviewed in reference 26). SR protein binding to exon enhancers stimulates U2AF and U2 snRNP binding to the upstream branch site/3′-splice-site region (17, 37, 69). In *Drosophila*, activation of the female-specific 3′ splice site in *dsx* pre-mRNA requires a downstream 13-nucleotide (nt) repeat bound by Tra and Tra-2 (30, 33, 53, 62). An interaction between SR proteins and a purine-rich sequence embedded within the *dsx* repeat elements stabilizes the formation of an enhancer complex which promotes the recognition of the suboptimal *dsx* 3′ splice site (31, 39, 63, 64). Intron enhancers have also been found (13, 20, 34, 45, 56). In the case of the neuron-specific exon of c-*src*, an intron element bound by hnRNP F is important for exon inclusion (45). Cell-specific inclusion of the fibronectin EIIIB exon requires a repeated UGCAUG motif located in the downstream intron (34), but the identity of the factors that recognize this and other intron elements is currently unknown.

Exon and intron sequences which repress the use of nearby splice sites have also been identified. Some of these sequences are involved in the formation of secondary structures that restrict the availability of splice sites (25, 57), while others are used as platforms for the assembly of aberrant splicing complexes (27, 48). A well-characterized example of negative regulation mediated by exon sequences is found in the *Drosophila* somatic P-element pre-mRNA. In this case, formation of an RNA-protein complex containing U1 snRNP, the soma-specific PSI protein, and the ubiquitous hrp48 protein prevents downstream 5′-splice-site recognition in somatic cells (55). In a manner that is reminiscent of the action of *Sxl* on the *Drosophila tra* pre-mRNA (66), the mammalian hnRNP I/PTB protein appears to interfere with U2AF binding to some 3′-splice-site sequences (38).

Several observations suggest that the mammalian hnRNP A/B proteins modulate splice site selection. In vitro and in vivo increases in the concentration of hnRNP A/B proteins antag-

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onize the activity of SR proteins by promoting exon skipping and distal shifts in 5′-splice-site utilization on some but not all pre-mRNAs (11, 41–43, 72). The variable efficiency with which A1 affects splice site selection suggests the existence of pre-mRNAs that may be more sensitive to A1 and hence more useful in understanding how A1 modulates alternative splicing. The A1 pre-mRNA is itself alternatively spliced to yield A1 and A1b, two proteins which display important differences in their ability to modulate splice site selection (43, 72). We undertook to characterize the elements involved in controlling the alternative splicing of the hnRNP A1 pre-mRNA. Our study reveals the existence of conserved sequences in the intron separating exon 7 and the A1b-specific exon 7B. Within these conserved sequences, we identified a binding site for the hnRNP A1 protein. Activation of the upstream 5′ splice site correlates with A1 binding but is not associated with a change in the ability of U1 snRNP to bind to either 5′ splice sites.

MATERIALS AND METHODS

Plasmids. pUC3-Fli-2 contains the hnRNP A1 genomic *Sal*I-*Eco*RI fragment from a BALB/c mouse (3). The alternative splicing unit was sequenced with oligonucleotides A1701 (CAATTTTGGTCGAGGAGGGA) and A1B3 (CCTC CGCCTCCGTTGTTATA). The sequence of intron 6, intron 7, and part of exon 7B was confirmed by sequencing with oligonucleotides A1E7 (TGCCAAATCC ATTATAGCCA), A1B2 (TTCCATAACCCTGTCCACCA), and A1B1 (TGG TGGACAGGGTTATGGAA), respectively.

(i) 553 series. The *Dsa*I-*Eco*RI fragment of pUC3-Fli-2 was subcloned into the *Sal*I and *Eco*RI sites in pBluescript KS+ (Stratagene) to yield pKgA1. pKgA1 was digested with *Bsu*36I and *Eco*RI and self-ligated to generate pK7A1. A fragment containing the 3′ portion of exon 7B extending to exon 8 was generated by PCR amplification with oligonucleotides A1B1 and A1893 (72) and cloned into the *Sma*I site of pBluescript KS+ to generate pK78A1. A *Stu*I-*Bam*HI fragment containing part of intron 7B and exon 8 sequences was replaced by a *Stu*I-*Bam*HI fragment containing the 3′ splice site of the adenovirus major late L2 exon to produce pK7BAd. The *Sal*I-*Pst*I fragment of pK7A1 was inserted in the *Sal*I and *Pst*I site of pK7BAd to produce pK77BAd. A 14-bp *Pst*I-*Sma*I-*Pst*I adapter was inserted in the *Pst*I site of pK77BAd to yield pS1. The *Bsu*36I-*Sph*I fragment corresponding to CE1 was cloned into the *Sal*I and *Sph*I sites of pUC19 to produce pUC109. The *Kpn*I-*Hin*dIII fragment was subcloned into pBluescript KS+ to produce pK109. The pK109 *Sma*I-*Eco*RV fragment containing CE1 was inserted in the *Sma*I site of pS1 to generate pS2 and pS3. pS4 was produced by inserting the *Sal*I-*Pvu*II fragment of pK7A1 into pK77BAd. pS5 and pS6 were produced by inserting the *Sma*I-*Eco*RV fragment of pK109 in the *Stu*I site of pS1. pS10 to pS15C were produced by inserting reannealed oligonucleotides at the *Sma*I site of pS1. The sequences of the sense oligonucleotides are indicated in the figures. The sequence and orientation of the insert were confirmed by sequencing with oligonucleotide A1B2.

(ii) E1A constructs. The *Sma*I-*Eco*RV fragment of pK109 was inserted at the *Sma*I site of plasmid pSP4-E1A (72) to produce pSP4-E2 and pSP4-E3.

Transcription and splicing assays. Plasmids pS1 to pS15C were digested with *Sca*I. Transcription with T3 RNA polymerase (Pharmacia Biotech), cap analog (Pharmacia Biotech), and [α-^{32}P]UTP (Amersham Life Sciences) and further RNA purification were as described previously (16). Adenovirus E1A pre-mRNAs E2 and E3 were produced following digestion with *Hin*dIII and transcription with SP6 RNA polymerase in the absence of labeled UTP. CE1 competitor RNA was produced by transcription of pK109 linearized with *Hin*dIII. The unrelated K RNA used as a control competitor was obtained by T3 transcription of pBluescript KS+ linearized at the *Sac*I site. RNA substrates used in immunoprecipitation assays were synthesized following digestion of pK109 with either *Hin*dIII, *Dde*I, *Rsa*I, or *Hin*fI. Shorter RNAs derived from S1, S2, S10, S13, and S14 RNAs were produced by transcription of the corresponding plasmids digested with *Bst*XI, which cuts in exon 7B.

Nuclear extracts were prepared (22) and splicing mixtures were set up and processed (35) as described previously. The identity of lariat products was confirmed by performing debranching reactions and estimating the size of the debranched molecules on acrylamide gels. Splicing mixtures incubated with E1A substrates were treated with DNase I, reverse transcribed, and amplified with *Taq* DNA polymerase as described by Yang et al. (72). Restriction enzyme analysis confirmed the identity of the amplified products. SR proteins were purified from calf thymus as described by Zahler et al. (75).

Transfection assays. The pCMV-A1 vector was generated by inserting an A1 genomic fragment containing exon 5 to exon 10 into a pCMVSV derivative (72). pCMV-A1ΔCE1 was produced by deleting the *Bsu*36I-*Sph*I fragment in intron 7. Transfection in HeLa cells was accomplished by the standard calcium phosphate precipitation technique. After 48 h, RNA was extracted by the guanidium-HCl method (16) and reverse transcription (RT)-PCR analysis was performed as previously described (72). The oligonucleotides used in the RT-PCR assay were A1893 (72) and CMV-1 (AGACGCCATCCACGCTGTTT). Amplification control experiments were performed on recombinant DNA molecules carrying the corresponding region of the A1 and A1b cDNAs inserted into pCMVSV.

Immunoprecipitation assays. Immunoprecipitation and RNase T_1 protection assays on CE1 RNA were performed essentially as described previously (18). Briefly, anti-A1 antibodies (9H10, kindly provided by G. Dreyfuss, HHMI, University of Pennsylvania) with or without RNase T_1 were added to splicing mixtures and kept on ice for 30 min. A 4-mg amount of protein A-Sepharose (Pharmacia Biotech) was added, and incubation was continued for 15 min. Each immunoprecipitate was washed four times with NET-2 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% [vol/vol] Nonidet P-40, 0.5 mM dithiothreitol). The percentage of recovery of input RNA with the anti-A1 antibody was calculated by measuring the number of counts per minute in each sample immediately after the addition of protein A-Sepharose and after all washes.

RNase H protection. HeLa extracts depleted in U1 snRNPs were produced by addition of RNase H (Pharmacia Biotech) and the oligonucleotide TCAGGTA AGTAT complementary to the 5′ end of U1 RNA (6). As a control, a mock-treated HeLa extract was obtained by incubation with RNase H and an unrelated oligonucleotide. RNase H and 75 pmol of oligonucleotides complementary to the 5′ splice sites of exons 7 and 7B (GAAACTTACCATC and GATACCTACCA CTA, respectively) were added to 10-μl splicing-reaction mixtures after incubation for the specified times (24). Incubation was continued for 15 min at 30°C.

Nucleotide sequence accession number. The GenBank accession number of the mouse hnRNP A1 alternative splicing unit is U65316.

RESULTS

Conserved hnRNP A1 intron sequences. As a first step toward identifying sequences involved in modulating the inclusion of the alternative exon 7B, we sequenced the genomic portion of the murine A1 gene from exon 6 to exon 7B by using a genomic copy of the murine A1 gene isolated previously (3). Comparing this sequence with the corresponding sequence of the human A1 gene (4) revealed that the murine I6 intron, which separates constitutive exons 6 and 7, was 38 nt larger than the corresponding human intron and shared little sequence conservation with the human I6 intron, except at the immediate splice junctions (Fig. 1). The poor degree of conservation between the mouse and human I6 introns is therefore comparable to that of most human and mouse constitutive introns. In contrast, the I7 intron, which separates exon 7 and the alternative exon 7B, was remarkably conserved between mouse and human: both introns were 204 nt long, and they displayed an overall similarity of 75% (Fig. 1). The striking conservation of the I7 intron suggests that it may contain elements that modulate the alternative splicing of exon 7B.

A conserved element in intron 7 affects 5′-splice-site selection. We carried out in vitro splicing reactions in HeLa nuclear extracts to test the effect of some of the conserved sequences of I7 on splice site selection. As a model pre-mRNA substrate, we used a hybrid RNA that contained the 5′ splice sites of exons 7 and 7B in competition for the 3′ splice site of the adenovirus L2 exon (S1 RNA) (Fig. 2). A similar construct carrying the 3′ splice site of exon 8 was spliced with extremely low efficiency (data not shown). Splicing of S1 RNA occurred predominantly to the proximal 5′ splice site of exon 7B (Fig. 2, lanes 2 and 4). When a 150-nt insert containing the central portion of intron I7 (underlined region in Fig. 1 and herein referred to as CE1) was included between the two 5′ splice sites to produce S2 RNA, splicing to the distal 5′ splice site of exon 7 occurred preferentially (lanes 1 and 6). A control pre-mRNA, which contained CE1 in the reverse orientation (S3 RNA), was spliced to each 5′ splice site with approximately equal efficiency (lane 5). Within S3 RNA, a pseudo-5′ splice site in the downstream portion of the insert was also used. A pre-mRNA containing a filler sequence of 175 nt (S4 RNA) was spliced exclusively to the internal 5′ splice site (lane 3). Thus, the presence of CE1 in its natural position, in between alternative donor sites, shifted selection toward the distal site. When CE1 was inserted downstream of the internal 5′ splice site (S5 RNA), the selection of the distal 5′ splice site was not stimu-

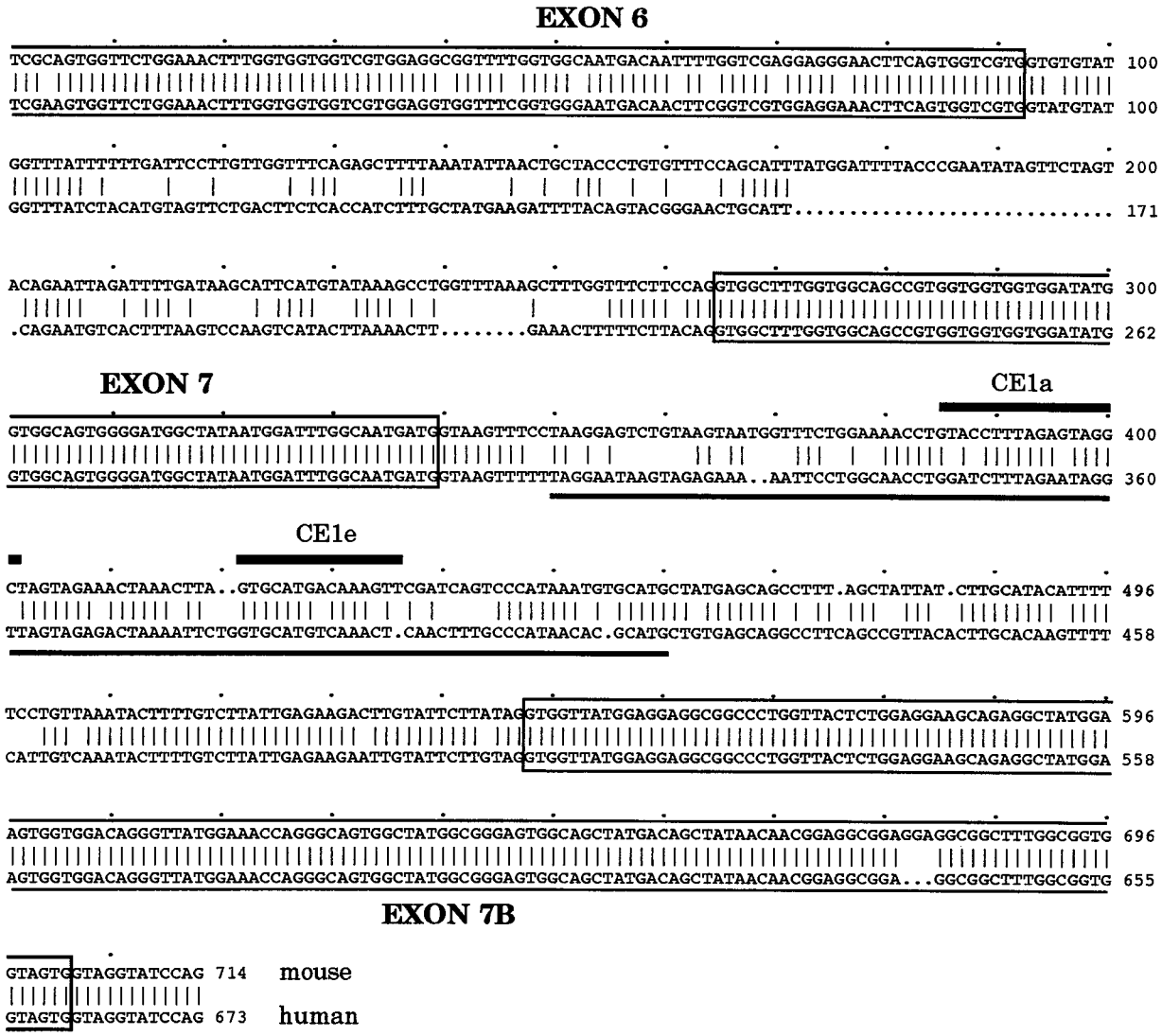

FIG. 1. Sequence of the mouse and human hnRNP A1 alternative splicing unit. A portion of the mouse hnRNP A1 gene (top) extending from exon 6 to 12 nt downstream of alternative exon 7B was sequenced and compared to the corresponding region of the human A1 gene (bottom [4]). Exon sequences are boxed. The alignment reveals that intron 7 (I7), which separates exon 7 from the alternative exon 7B, is very similar between mouse and human. Within I7, the mouse *Bsu*36I-*Sph*I fragment corresponds to CE1 (underlined). The sequences corresponding to CE1a and CE1e are indicated. Note that the murine exon 7B contains an additional GGA codon.

lated in comparison to a control substrate containing the complementary sequence of CE1 inserted at the same position (S6 RNA) (lanes 7 and 8).

The effect of CE1 on 3′-splice-site selection was addressed by using a substrate containing the 5′ splice site of exon 7, which can be spliced to either the proximal 3′ splice site of exon 7B or the distal 3′ splice site of the adenovirus L2 exon. The presence of CE1 either between 3′ splice sites or upstream of the internal 3′ splice site did not affect 3′-splice-site selection (data not shown).

To verify whether CE1 could act on heterologous 5′ splice sites, CE1 was inserted in both orientation in the adenovirus E1A gene downstream of the 12S 5′ splice site. E1A pre-mRNA splicing in a HeLa extract was monitored by RT-PCR with primers that map downstream of the major 3′ splice site and upstream of the distal 9S 5′ splice site, as described previously (72) (Fig. 3). The control E1A pre-mRNA containing the complementary sequence of CE1 preferentially led to the amplification of 13S products (Fig. 3, lane 2). In contrast, insertion of CE1 in the correct orientation eliminated the appearance of 13S products and favored the use of the 9S 5′ splice site (lane 1). Thus, CE1 promoted the selection of the distal 5′ splice site in an heterologous pre-mRNA.

CE1 promotes exon 7B skipping in vivo. Our results suggest that CE1 may contribute to the alternative splicing of the natural hnRNP A1 pre-mRNA. To test the effect of CE1 in its natural context in vivo, we constructed an expression vector carrying a genomic portion of the mouse A1 gene under the control of the CMV promoter (pCMV-A1). Following transient expression of this minigene in HeLa cells, RT-PCR analysis revealed that the pre-mRNAs were spliced preferentially to yield mRNAs that lacked exon 7B (Fig. 4, lane 4). This splicing profile corresponds to the profile of the endogenous A1 pre-mRNA in HeLa cells, where less than 5% of A1 transcripts contains exon 7B (data not shown). A derivative of the A1 minigene, pCMV-A1ΔCE1, was produced by deleting the

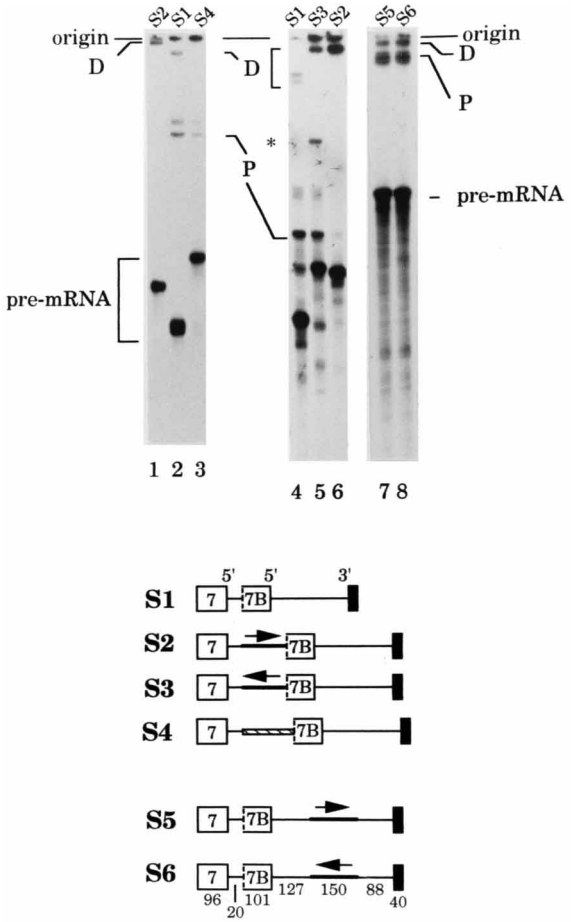

FIG. 2. CE1 affects 5′ splice site selection. RNA substrates were synthesized from plasmids lacking CE1 (S1 and S4 RNAs) or containing CE1 in the sense (S2 and S5 RNAs) or antisense (S3 and S6 RNAs) orientation. Following incubation in HeLa extracts, the splicing products were loaded onto 8% acrylamide–7 M urea gels. The relative frequency of distal and proximal splice site utilization can be evaluated by comparing the intensity of the bands derived from proximal (P) and distal (D) lariat intermediates and products which migrate above the pre-mRNA. To improve resolution of lariat species, gels were run extensively, accounting for the absence of the mRNAs and 5′ exons. Because of the large size of the intron in distal lariat intermediates and products, these species migrated close to the origin of the gel and did not resolve well. The individual lariat species were detected in gels run for a longer period (not shown). Note that a pseudo-5′ splice site located in the 3′ portion of the insert in S3 RNA was also used; the lariat molecule derived from the use of this site is indicated by an asterisk. The structure and size (in nucleotides) of various portions of the pre-mRNAs are indicated at the bottom.

CE1 element in intron 7. Transient expression of pCMV-A1ΔCE1 led to a dramatic increase in the inclusion of exon 7B (lane 5). This result indicates that the CE1 element plays a role in promoting efficient exon 7B skipping in vivo.

A 17-nt sequence within CE1 is sufficient to shift 5′ splice site selection. To identify sequences within CE1 which are responsible for activating the distal 5′ splice site, portions of CE1 were inserted between the two competing 5′ splice sites. Strikingly, insertion of a sequence derived from the conserved 5′ portion of CE1 (CE1a; Fig. 1) activated the distal 5′ splice site (S10 RNA; Fig. 5, lanes 2 and 5). Insertion of various control oligonucleotides did not affect 5′-splice-site selection (lane 6; data not shown). An oligonucleotide containing conserved sequences downstream from CE1a (CE1e; Fig. 1) also had no effect on splice site selection (S12 RNA; Fig. 5, lane 7). Insertion of CE1a upstream of the CE1 element in S2 RNA

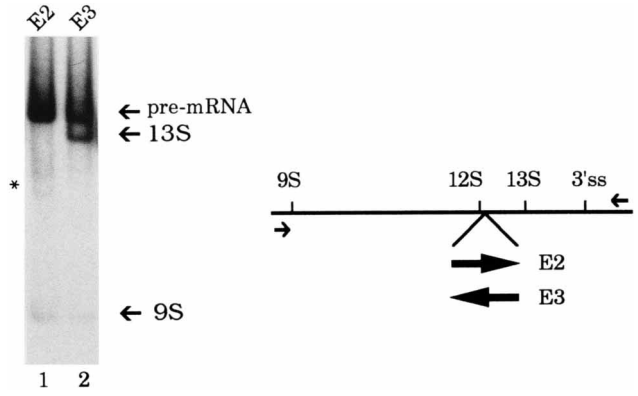

FIG. 3. CE1 acts in a heterologous fashion. The CE1 element was inserted in the sense (E2 RNA) and antisense (E3 RNA) orientations downstream from the 12S 5′ splice site in the adenovirus E1A gene. Unlabeled E2 and E3 RNAs were incubated for 2 h in HeLa nuclear extracts. Splicing was monitored by RT-PCR with [α-^{32}P]dCTP and previously described oligonucleotides (right) (72). Amplified products were loaded onto a 5% nondenaturing acrylamide gel. The identity of the pre-mRNA, 13S, and 9S products was confirmed by restriction enzyme analysis (not shown). The identity of the bands indicated by an asterisk in lane 1 has not been investigated. One of the bands may correspond to the 12S product, a result that would be consistent with the CE1-mediated activation of distal 5′ splice sites. ss, splice site.

further enhanced the use of the distal 5′ splice site (data not shown).

hnRNP A1 binds to CE1a. To address whether CE1 mediates distal 5′-splice-site selection through the interaction with factors present in HeLa extracts, splicing of S2 RNA was performed in the presence of excess amounts of an RNA made up mostly of CE1 sequences (CE1 RNA). Preincubation with a 1,000-fold molar excess of CE1 RNA shifted 5′-splice-site selection toward the proximal 5′ splice site (Fig. 6A, lane 5). Lower concentrations of CE1 RNA did not affect 5′-splice-site selection (data not shown). Addition of a 1,000-fold excess of an unrelated RNA (K RNA) had no effect (lane 6). A 1,000-fold excess of CE1 RNA also favored proximal 5′-splice-site selection on transcripts that lacked the CE1 element (lane 2).

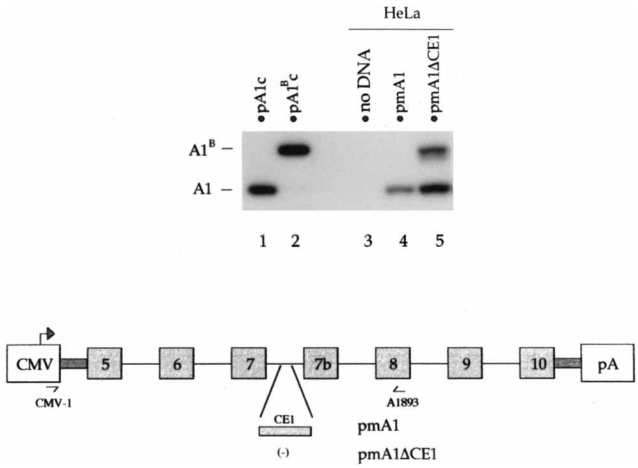

FIG. 4. CE1 promotes exon 7B skipping in vivo. A genomic portion of the mouse A1 gene (exons 5 to 10) was inserted into an expression vector containing the CMV promoter (72). The pmA1 expression vector and the pmA1ΔCE1 derivative were transfected into HeLa cells. At 48 h posttransfection, total RNA was analyzed by RT-PCR. Reconstructed A1 and A1b cDNAs were used as controls (lanes 1 and 2). A schematic diagram of the vectors and the position of the oligonucleotides used as primers are indicated at the bottom.

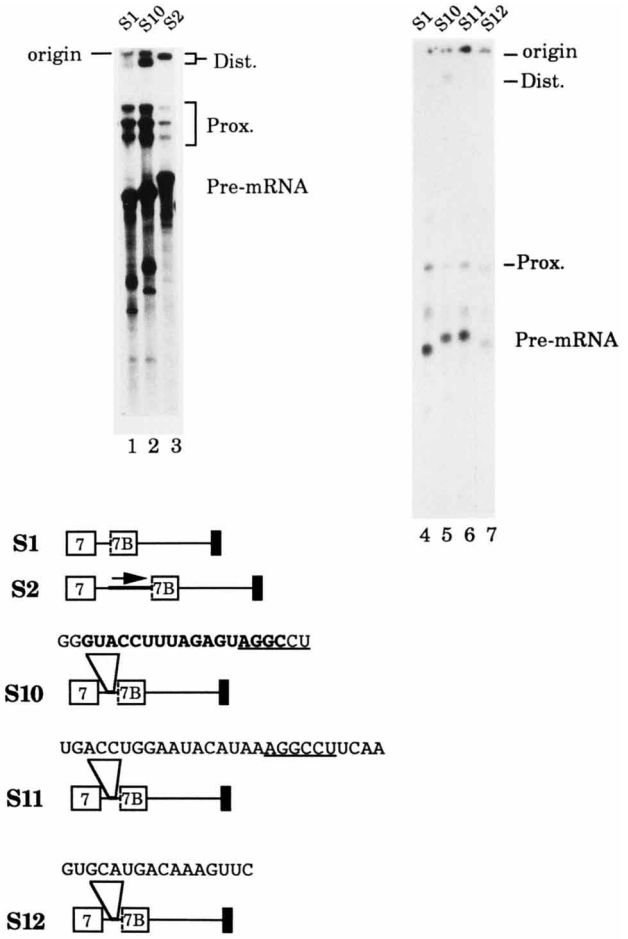

FIG. 5. A 17-nt region within CE1 activates the upstream 5′ splice site. An oligonucleotide containing the CE1a sequence (boldface type in S10 [Fig. 1]) was inserted between the two competing 5′ splice sites, and the resulting RNA was tested for splicing by comparison to S1 and S2 RNAs (lanes 1 to 3). Two other oligonucleotides were used as controls (S11 and S12 RNAs). The inserts in S10 and S11 share the sequence AGGCCU corresponding to a *Stu*I site (underlined). The oligonucleotide inserted in S12 corresponds to the CE1e sequence (Fig. 1). Splicing products were separated on an 8% acrylamide–7 M urea gel. The positions of the pre-mRNAs as well as lariat molecules derived from proximal or distal 5′-splice-site utilization are indicated. Note that in lanes 1 to 3 (see also Fig. 8), three types of proximal lariat molecules are observed. Based on debranching assays, the top band corresponds to the lariat intermediate and the middle band corresponds to the lariat product. The faster-migrating band corresponds to lariat products that have lost the 3′ tail, possibly due to an increased activity of exonucleases in some but not all HeLa extracts.

These results suggest that an excess of CE1 RNA sequesters a factor(s) that generally encourages the selection of distal 5′ splice sites.

Because hnRNP A1 proteins promote distal 5′-splice-site selection on a variety of transcripts (42, 43, 72), it was of interest to address whether hnRNP A1 interacts with CE1. We performed immunoprecipitation assays with a monoclonal antibody against A1 proteins (9H10, kindly provided by G. Dreyfuss, Howard Hughes Medical Institute, University of Pennsylvania). Labeled CE1 RNA was added to a HeLa extract, and the A1-bound RNA was recovered with the anti-A1 antibody. 9H10 immunoprecipitated 35% of input CE1 RNA compared to 6% when we used a control RNA lacking the CE1 sequences (Fig. 6B). An RNA containing the first 68 nt of CE1 was also efficiently immunoprecipitated (21% of input RNA), whereas an RNA containing only the first nonconserved 37 nt of CE1 was recovered at background level. A control RNA containing the full complementary CE1 sequence was recovered with an efficiency inferior to 10% (data not shown). Thus, the conserved region located between positions +34 and +63 in CE1 contains sequences that appear to be important for hnRNP A1 binding. Notably, when the immunoprecipitation assay was performed with monoclonal antibodies against hnRNP C or hnRNP A2, A1 proteins were immunoprecipitated but less than 5% of input CE1 RNA was recovered (data not shown). This result suggests that the A1 molecules binding to CE1 at 0°C are not associated with the hnRNP C or A2 proteins. Further investigations will examine whether binding of A1 to CE1 drives the assembly of core hnRNP particles.

RNase T_1 protection/immunoprecipitation assays were next performed to map with more precision the site of interaction of hnRNP A1 with CE1. CE1 RNA was incubated in a HeLa extract, and the monoclonal antibody 9H10 was used to recover four protected fragments ranging in size between 14 and 22 nt (Fig. 6C, lane 2). Secondary RNase T_1 analysis indicated that all four fragments contained a 9-nt T1 oligomer (Fig. 6D). Fragments 1 and 2 contained an additional 4-nt oligomer. Inspection of the 160-nt CE1 RNA indicates that only one region within CE1 contained adjacent 9- and 4-nt T1 oligomers within a 22-nt stretch. This region corresponds to the middle of the CE1 element, precisely in the region previously shown to be required for anti-A1 immunoprecipitability (Fig. 6B). From this analysis, we deduced that protected fragments 3 and 4 are 14 and 15 nt, respectively. Fragment 2 is 19 nt and includes an additional downstream 4 nt, while fragment 1 is 22 nt and contains an additional downstream 3-nt oligomer which would not be labeled since RNase T_1 cuts 3′ to the phosphate of G nucleotides. The sequence of the smallest protected fragment is therefore UACCUUUAGAGUAG; the underlined sequence is related to the optimal A1 binding site consensus sequence UAGGG(A/U), as determined by selection and amplification from pools of random RNA sequences (8). It is notable that the two smallest A1-protected fragments are contained within CE1a, the portion of CE1 which activates the distal 5′ splice site of exon 7 (Fig. 6B, CE1a sequence in boldface type).

Activation of the distal 5′ splice site correlates with A1 binding. Our results indicate that hnRNP A1 binds to the CE1a sequence, suggesting that the A1-CE1a interaction is required for the shift in 5′-splice-site selection. This possibility would be consistent with the demonstrated ability of hnRNP A1 proteins to displace 5′-splice-site selection toward distal donor sites in vitro and in vivo (42, 43, 72). Moreover, this would explain why addition of an excess competitor CE1 RNA led to proximal 5′-splice-site selection even on a pre-mRNA that lacks the CE1 element. If the A1-CE1 interaction is indeed responsible for promoting distal 5′-splice-site selection, differences in shifting efficiency should correlate with differences in A1 binding. To address this issue, we tested several sequences (Fig. 7A) for their ability to activate the distal 5′ splice site. Pre-mRNAs containing the putative A1 binding sequence UAGAGU or UAGGGU flanked by completely different sequences (S13 and S14, RNAs, respectively) activated the distal 5′ splice site with efficiencies equal to or greater than CE1a (S10 RNA) (Fig. 7B, lanes 2 to 4). Deletion of the GAG sequence in UAGAGU to produce S10Δ RNA restored preferential splicing to the proximal 5′ splice site (lane 6). Compared to S15 RNA, which carries the UAGAGU sequence (lane 7), the G-to-A mutation at position +5 (S15A RNA; lane 8) had no effect on splice site selection while the G-to-C mutation at the same position (S15C RNA; lane 9) considerably reduced distal 5′-splice-site selection. These results indicate

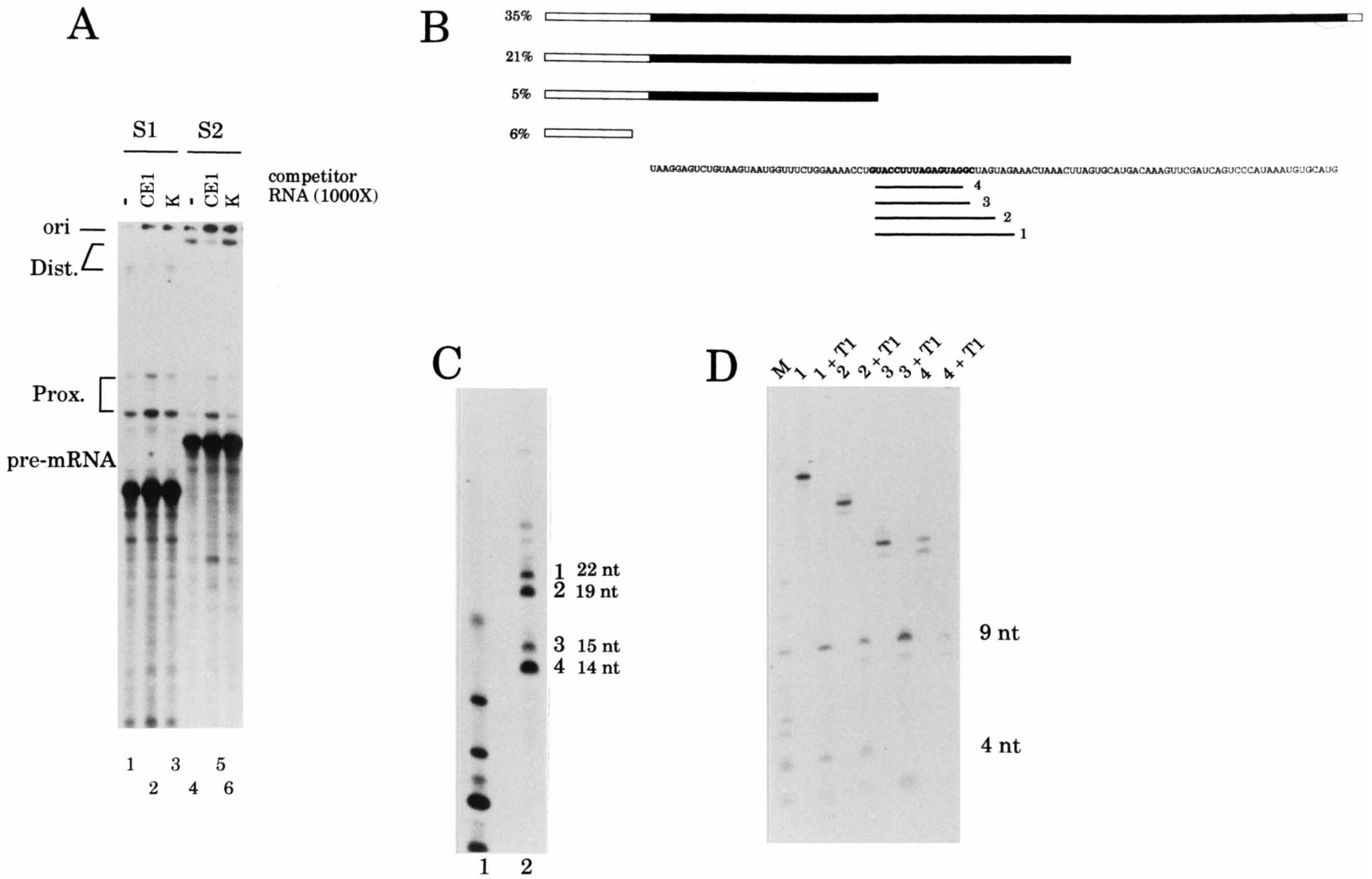

FIG. 6. hnRNP A1 binds to CE1a. (A) Distal 5′-splice-site selection is affected by an excess of CE1 RNA. A HeLa extract was preincubated with either no RNA (lanes 1 and 4) or a 1,000-fold molar excess of CE1 RNA (lanes 2 and 5) or unrelated K RNA (lanes 3 and 6). CE1 RNA contains the sequence underlined in Fig. 1 plus 39 nt at the 5′ end and 12 nt at the 3′ end derived from the transcription template. Following preincubation with the competitor RNA for 15 min at 30°C, S1 or S2 RNA was added and incubation was continued for 2 h. Splicing products were fractionated as described previously. The position of lariat intermediates and products derived from proximal and distal 5′-splice-site utilization is indicated. (B) Immunoprecipitation of CE1 RNA and shortened derivatives by the anti-A1 antibody. Labeled RNAs produced by runoff transcription were added to HeLa extracts and immunoprecipitated with an anti-A1 monoclonal antibody (9H10; provided by G. Dreyfuss). The recovery of input RNA following four washes is indicated as a percentage and represents an average of four independent experiments. The identity of the RNAs relative to the sequence of CE1 is shown, and the sequence of CE1a is in boldface type (C) RNase T_1 protection/immunoprecipitation assay. The procedure was identical to that in panel B, except that RNase T_1 was added along with the anti-A1 antibody (lane 2). Protected fragments were resolved on a 15% acrylamide–7 M urea gel. The size of each protected fragment is indicated. Lane 1 represents a complete RNase T_1 digestion of CE1 RNA. (D) Each protected fragment obtained in panel C (lane 2) was isolated and purified, and a portion was digested with RNase T_1. Digested and undigested samples were loaded onto a 20% acrylamide–7 M urea gel along with a complete RNase T_1 digestion of CE1 RNA (M). The positions of the four protected fragments relative to the CE1 sequence are indicated in panel B.

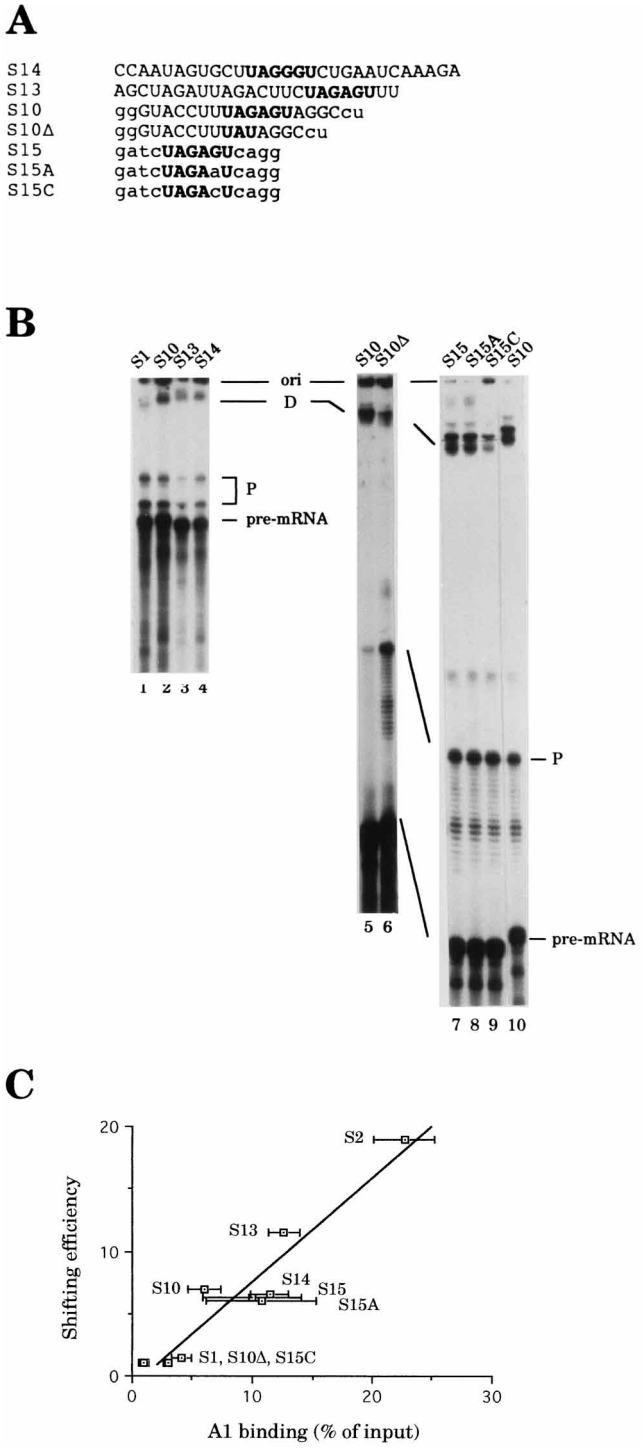

FIG. 7. A1 binding and 5′-splice-site selection. (A) Sequences tested for their ability to promote distal 5′-splice-site selection (B) and to be bound by hnRNP A1 in nuclear extracts (C). The UAGRGU sequence is shown in boldface type. Lowercase letters indicate that these nucleotides are not part of the CE1a sequence. (B) Splicing assay of pre-mRNAs containing the sequences shown in panel A, inserted between the two 5′ splice sites of S1 RNA. Incubation in HeLa extracts was carried out for 2 h, and splicing products were resolved as described previously. The position of the lariat molecules derived from proximal (P) and distal (D) 5′-splice-site utilization is indicated. (C) Graph representing the shifting efficiency (abscissa) of the inserted sequences relative to the percentage of RNA recovered by immunoprecipitation (ordinate). Included in this representation are values obtained without inserted nucleotides (S1 RNA) and with the complete CE1 element (S2 RNA). Immunoprecipitation assays were performed with shorter versions of each RNA (i.e., terminating in exon 7B).

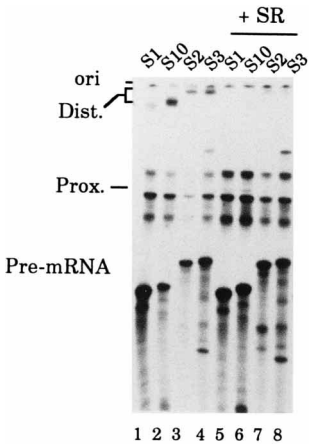

FIG. 8. SR proteins neutralize the effect of CE1. Splicing assays in HeLa extracts were performed in the absence (lanes 1 to 4) or in the presence (lanes 5 to 8) of purified calf thymus SR proteins. Incubation was carried out for 2 h at 30°C.

that the UAGAGU sequence plays a role in modulating 5′-splice-site selection. Next, immunoprecipitation assays in nuclear extracts were performed with RNAs terminating approximately 30 nt after the inserted sequences (corresponding to a *Bst*XI site in exon 7B). Short RNAs derived from S13, S14, S15, and S15A were bound by A1 with efficiencies equal to or greater than those of short RNAs derived from S10 (Fig. 7C). RNAs lacking the UAGAGU sequence (S1 and S10Δ) or carrying the UAGACU mutation were bound least efficiently by hnRNP A1. A short RNA derived from S2 was recovered with the highest efficiency with anti-A1 antibodies. Thus, a correlation between A1 binding and the amplitude of the shift suggests that A1 binding favors distal 5′-splice-site utilization.

SR proteins abrogate the effect of CE1. In other pre-mRNAs, SR proteins promote proximal 5′-splice-site selection and antagonize the action of hnRNP A1 (42). To verify whether the CE1-mediated shift in 5′-splice-site selection was sensitive to increased levels of SR proteins, a mixture of SR proteins purified from calf thymus was added to HeLa splicing-reaction mixtures containing S1, S2, S3 and S10 RNAs. The highest concentration of SR proteins almost completely eliminated distal 5′-splice-site utilization on all transcripts to favor proximal 5′-splice-site selection (Fig. 8, lanes 5 to 8). Under these conditions, the ratio of proximal to distal 5′-splice-site selection was identical for S2 and S3 RNAs. Thus, addition of SR proteins neutralized the effect of CE1 on 5′-splice-site selection. Gradual reductions in the amounts of SR proteins added to extracts allowed gradual restoration of normal splicing patterns without evidence for CE1 to offer more resistance to the action of SR proteins (data not shown). This result may indicate that A1 and SR proteins influence different events associated with splice site selection.

CE1 does not affect U1 snRNP binding to competing 5′ splice sites. To gain insights into the mechanism of action of CE1, we performed an oligonucleotide-targeted RNase H cleavage assay to monitor U1 snRNP binding to the alternative 5′ splice sites (24). Following various periods of incubation (0, 5, and 20 min at 30°C), oligonucleotides complementary to the 5′ splice sites of exons 7 and 7B were added to splicing mixtures and RNase H digestion was carried out. The protection profiles obtained with S2 and S3 RNA were identical (Fig. 9, lanes 1 to 3 and 7 to 9, respectively). A similar result was obtained when the protection profiles of S1 and S10 RNAs

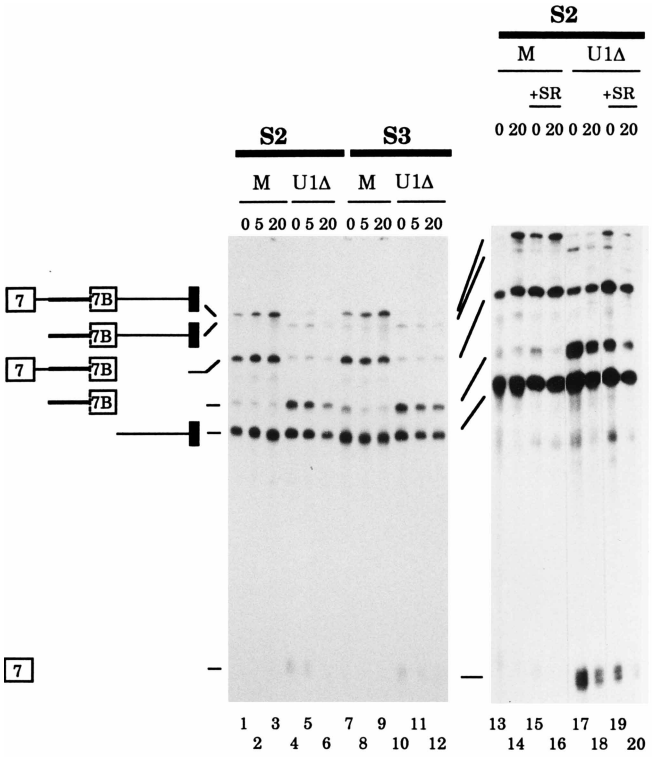

FIG. 9. Monitoring U1 snRNP binding to competing 5′ splice sites. S2 and S3 RNAs were incubated at 30°C for the indicated times (in minutes) in mock-treated (M) or U1 snRNP-depleted (U1Δ) HeLa nuclear extracts. In lanes 15, 16, 19, and 20, splicing mixtures were supplemented with SR proteins purified from calf thymus before addition of S2 RNA. Following incubation, oligonucleotides complementary to the 5′ splice sites of exons 7 and 7B were added along with RNase H. The positions of fully protected pre-mRNAs or molecules derived from the cleavage at either the downstream, the upstream, or both 5′ splice site are shown.

were compared (data not shown). The intensity of the bands corresponding to protected molecules was reduced considerably when the assay was performed in an extract pretreated with an oligonucleotide against the 5′ end of U1 RNA, confirming that U1 snRNP binding is responsible for the protection (lanes 4 to 6 and 10 to 12). The residual protection is probably due to a low level of intact U1 snRNAs since the U1-decapitated extract was still active and spliced S2 and S3 RNAs to the distal 5′ splice site exclusively (data not shown), as expected from the results of Eperon et al. (24). For both transcripts incubated for 0 min in a mock-treated extract, approximately 80% of the pre-mRNA molecules had U1 snRNP bound to the upstream 5′ splice site only, less than 5% had U1 snRNP bound to the downstream site only, while close to 20% of input RNA carried U1 snRNP bound to both sites. When purified SR proteins were added to either mock-treated or U1 snRNP-depleted extracts, the proportion of doubly protected S2 RNA molecules increased at 0 min of incubation (compare lanes 13 and 17 with lanes 15 and 19, respectively) and splicing shifted to the proximal 5′ splice site (Fig. 8 and data not shown), as predicted from the results of Eperon et al. (24). Thus, although our assay is sensitive enough to detect changes in U1 snRNP binding associated with shifts in 5′-splice-site utilization mediated by U1 snRNP degradation and SR protein addition, the presence of the CE1 did not affect U1 snRNP binding to either 5′ splice site. Our results suggest that the A1-CE1 interaction affects a different step in the assembly of splicing complexes.

DISCUSSION

A novel intron element that promotes distal 5′-splice-site selection. The mouse and human intron sequences separating exon 7 from the alternative exon 7B in the pre-mRNA of hnRNP A1 display an unusually high degree of conservation, suggesting that conserved intron sequences might play a role in modulating the frequency of inclusion of exon 7B. In its natural position, between two competing 5′ splice sites, a region (CE1) containing a portion of the conserved intron sequences shifted splice site selection toward the distal 5′ splice site of exon 7. In contrast, when CE1 was inserted downstream of the two competing 5′ splice sites, no effect on 5′-splice-site selection was observed. Thus, the position of CE1 relative to the competing 5′ splice sites is an important parameter that affects its activity. Dissecting CE1 into smaller regions revealed that a 17-nt portion located at the 5′ end of the conserved region in CE1 (CE1a) possessed the information required to activate the distal 5′ splice site. As the amplitude of the shift obtained with a single CE1a element was not as important as the full CE1 region, other conserved sequences within CE1 may cooperate with CE1a to favor the use of the distal 5′ splice site. Experiments are in progress to identify other sequences within CE1 that contribute to splice site selection.

The hnRNP A1-CE1 interaction modulates 5′-splice-site selection. Two sets of results suggest that the effect of CE1 is mediated by a *trans*-acting factor. First, CE1 promoted distal 5′-splice-site selection when present in a heterologous pre-mRNA. Second, an excess of CE1 RNA restored selection to the proximal 5′ splice site. Several results demonstrate that the binding of hnRNP A1 to CE1 is directly involved in promoting distal 5′-splice-site selection. First, the 17-nt CE1a region harbors the sequence UAGAGU, which resembles the optimal A1 binding site UAGGGU (8). Insertion of different oligonucleotides carrying the sequence UAGAGU or UAGGGU promoted a distal shift in 5′-splice-site selection. Most importantly, deleting the GAG nucleotides from the UAGAGU sequence or mutating the G at position +5 into a C restored the preferential selection of the proximal 5′ splice site. Second, immunoprecipitation assays with a monoclonal antibody specific for hnRNP A1 proteins showed that A1 interacts with CE1, in particular with the CE1a sequence, which was shown to be sufficient to activate the distal 5′ splice site. Although the sequence UAGAGU is not perfectly conserved in the human I7 intron, the related sequence UAGAGA is found nearby within the human CE1 element (Fig. 1). Third, more efficient binding of A1 was accompanied by a more important shift to the distal 5′ splice site. Our results therefore suggest that the interaction between hnRNP A1 and CE1a promotes the shift in distal 5′-splice-site utilization in HeLa extracts. Because deletion of CE1 stimulates exon 7B inclusion in vivo, the A1-CE1 interaction probably modulates exon 7B inclusion in the natural A1 pre-mRNA. At this stage, we cannot rule out that factors other than A1 bind to CE1a or that A1 recruits factors to affect splice site selection.

Molecular mechanism of 5′-splice-site selection by hnRNP A1. Addition of SR proteins counteracted the effect of CE1 by promoting proximal 5′-splice-site selection. SR proteins indiscriminately encourages U1 snRNP binding to all 5′ splice sites (24). Because more pre-mRNA molecules have U1 snRNP bound to all 5′ splice sites, preferential selection of the internal 5′ splice site is dictated by its proximity to the 3′ splice site (24). As hnRNP A1 antagonizes the action of SR proteins, it was

proposed that A1 could interfere with U1 snRNP binding (24). Consistent with this view, A1 was shown to reduce the affinity of U1 snRNP for the 5′ splice site of β-globin pre-mRNA (19). However, our oligonucleotide-targeted RNase H assays have failed to reveal an effect of CE1 on U1 snRNP binding, suggesting that the A1-CE1 interaction affects a different step in the selection of 5′ splice sites.

The mechanism by which the A1-CE1 interaction promotes distal 5′-splice-site selection remains puzzling. We have attempted to determine whether the A1-CE1 interaction stimulates splicing to the 5′ splice site of exon 7 and/or represses splicing to the 5′ splice site of exon 7B. Because the splicing of simple pre-mRNAs was not affected by the presence of CE1 (data not shown), the modulating activity of CE1 on splicing apparently requires competing 5′ splice sites. Thus, we cannot rule out that the A1-CE1 interaction promotes a subtle shift in the rate of spliceosome assembly to favor complexes assembled with the distal 5′ splice site. Alternatively, the A1-CE1 interaction may change the conformation of the pre-mRNA to increase the competitive advantage of the distal 5′ splice site. Given that A1 molecules have the property to self-associate (14, 15, 50), a change in conformation may be achieved if the A1 protein bound to CE1 interacts with another A1 protein bound downstream of exon 7B. This interaction would loop out the 5′ splice site of exon 7B, bringing the distal 5′ splice site closer to the 3′ splice site to increase the frequency of its selection. Current efforts are devoted toward identifying A1 binding sites downstream of exon 7B. Previous studies have shown that A1 can interact with 3′-splice-site sequences, including the adenovirus 3′ splice site used in our assays (10, 60). However, the functional relevance of this latter interaction remains to be demonstrated, and a recent study (1) has shown that the affinity of A1 for 3′-splice-site sequences is more than 100-fold lower than for the Burd and Dreyfuss (8) "winner" sequence. Interestingly, a UAGAGU motif is part of a conserved element ≈50 nt downstream of exon 7B (7). If A1 binds to this element and/or to the adenovirus 3′-splice-site region, cooperative protein interactions between A1 molecules bound on each side of the alternative exon may favorably position the 5′ splice site of exon 7 prior to commitment complex formation. This structural model is attractive because it helps reconcile the results of several experiments. First, it provides an explanation for the failure of CE1 to affect splice site selection when positioned downstream of two competing 5′ splice sites. Second, it does not require a change in the binding of U1 snRNP to competing 5′ splice sites, consistent with the results of our RNase H protections. A prediction from this model is that inefficient U1 snRNP binding to the 5′ splice site of exon 7B may be required to kinetically favor the A1-A1 interaction over the assembly of commitment complexes involving the proximal 5′ splice site. Consistent with this view, at 0°C, U1 snRNP was bound to the proximal donor site approximately four times less efficiently than to the upstream 5′ splice site and the SR-mediated increase in U1 snRNP binding to the proximal site completely neutralized the effect of CE1. Further experiments will be designed to explore the validity of this model and its relevance to other pre-mRNAs which are sensitive to variations in hnRNP A1 levels.

It is striking that of the five TAGRG(A/T) motifs found in the human A1 gene (more than 5 kb), three reside within a 500-bp region in the introns adjacent to the alternative exon 7B. The position of these three A1 binding motifs is conserved in the regions flanking the mouse alternative exon 7B. Likewise, the rat skeletal troponin T gene (≈17 kb) contains 18 TAGRG(A/T) motifs, four of them clustered within a 600-bp region around alternative exon 17. Because the binding of hnRNP proteins to 700 nt of RNA has been associated with the assembly of a 40S monoparticle (44), a change in the conformation of the pre-mRNA mediated by closely positioned A1 binding sites flanking an alternative exon may trigger the assembly of such a complex and could promote exon skipping.

It is also of interest that (A/U)GGG repeats in the intron downstream of an alternative β-tropomyosin exon increase in vitro splicing efficiency (56) while a UAGG sequence in one of the alternative exon of the fibroblast growth factor receptor-2 gene inhibits splicing (21). Verification of whether A1 plays a role in modulating splice site selection in these and other alternative splicing units will be worth investigating.

Regulation of hnRNP A1 pre-mRNA splicing. Vertebrate cells express both the A1 and A1b spliced isoforms, with the latter generally representing less than 5% of the total amount of A1 proteins (9). A1b shares with A1 an RNA-binding domain but differs by containing an extended glycine-rich domain encoded by exon 7B (9). Because the hnRNP A1-CE1 interaction modulates the alternative splicing of a model A1 pre-mRNA in vitro, hnRNP A1 may modulate the splicing of its own pre-mRNA by promoting exon 7B skipping. Autoregulation of splicing has been reported in the case of the *Xenopus* rpL1, the yeast rpL32, and the *Drosophila* Sxl, tra-2, and SWAP proteins (2, 12, 40, 68, 74). Although A1 and A1b display an equivalent ability to bind to CE1 (36), we do not yet know whether A1 and A1b differ in their ability to promote distal 5′-splice-site selection on our model pre-mRNAs. Given that A1b is less efficient than A1 at activating distal 5′ splice sites on other pre-mRNA substrates (43), A1 may be used to ensure that mostly A1 mRNAs are produced, with little contribution of A1b to this process.

Although our study has revealed the participation of a novel element in splice site selection, it is unlikely that the A1-CE1a interaction is the only contributor in setting the frequency of exon 7B inclusion. The role of additional conserved nucleotides within CE1 and in the 3′ portion of intron 7 remains to be investigated. Moreover, exon 7B is also extremely conserved and contains several stretches of purine-rich sequences bound by SR proteins (47). Thus, the skipping potential of the A1-CE1 interaction may be antagonized by SR proteins bound to exon 7B. Lastly, intron 7B, which separates exons 7B and 8, also contains several regions which are highly conserved between mouse and human (7). One conserved element (not present in our model pre-mRNAs) forms stable base pairs with complementary sequences in exon 7B (7). Thus, the high density of conserved sequences in the alternative splicing unit of hnRNP A1 suggests that regulation of exon 7B inclusion is complex. An exhaustive study will be required to evaluate the contribution of each conserved domain and each associated factor and the cooperative or antagonistic interactions between factors bound at different sites.

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