Distinct Sets of Adjacent Heterogeneous Nuclear Ribonucleoprotein (hnRNP) A1/A2 Binding Sites Control 5’ Splice Site Selection in the hnRNP A1 mRNA Precursor

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The alternative splicing of mRNA precursors (pre-mRNAs) is a major contributor to the diversity of the mammalian pro-
tome (1–3). The control of splice site selection therefore has profound implications in the production of protein isoforms with different functions. Recent progress in uncovering the molecular strategies that control alternative splicing has led to the identification of many types of sequence elements that influence either positively or negatively the selection of the alternative splice sites. Exonic splicing enhancers are bound by specific members of the SR protein family that can enforce the use of weak 5’ and 3’ splice sites (reviewed in Ref. 4). Enhancer elements have also been described in the introns flanking some alternative exons (5–9). Other types of proteins, including members of the hnRNP F/H family of proteins, can bind specifically to intron or exon control elements and hence can contribute to enhancer activity (10–14).

Elements that reduce the use of a neighboring splice site are also important in the control of splice site selection. In many cases, the activity of splicing silencers can be mediated by proteins that inhibit specific steps of splice site recognition or spliceosome assembly. A frequent example of this kind of splicing control in mammals involves the polypyrimidine tract-bind-
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1 The abbreviations used are: pre-mRNA, mRNA precursor; hnRNP, heterogeneous nuclear ribonucleoprotein; nt, nucleotide(s); GST, glutathione S-transferase; rA1, rA2, and rA1B, recombinant A1, A2, and A1B, respectively.

Members of the family of core hnRNP A/B proteins have also been identified as factors involved in the modulation of splice site selection. Using model pre-mRNAs carrying competing 5’ splice sites, important shifts toward distal 5’ splice sites can be obtained by the addition of purified or recombinant hnRNP A1 to a HeLa nuclear extract (23, 24). The Drosophila hrp48 protein, which is similar to hnRNP A1, is required in collaboration with P-element somatic inhibitor and the U1 snRNP to repress splicing of the P-element pre-mRNA in somatic tissues (25). hnRNP A1 can elicit exon skipping, but not all pre-mRNAs are responsive to variations in the concentration of hnRNP A1 in vitro (26). A1 can also negatively affect the use of 3’ splice sites in a variety of exons including the alternative exon of fibroblast growth factor receptor 2 gene (27), the tat and vpr exons of the human immunodeficiency virus (25–30), and the V6 exon of the human CD44 gene (31, 32). Consistent with the finding that A1 can recognize specific RNA elements (33), most of the functions that have been attributed to hnRNP A1 in natural pre-mRNAs are based on its ability to interact with specific sequences. In all cases examined to date, the hnRNP A1 splice isoform A1B,
the A2 protein, and its splicing variant B1 can functionally replace A1 in 5′ splice site and 3′ splice site selection assays in vitro (24, 28, 29).

We have shown previously that hnRNP A1 can modulate the alternative splicing of its own pre-mRNA through binding to sequences in the introns flanking alternative exon 7B. The 17-nt-long CE1a element downstream of exon 7 and the 24-nt-long CE4 sequence downstream of alternative exon 7B can promote distant 5′ splice site selection in an A1-dependent manner (34, 35). Both CE1a and CE4 contain the sequence UAAGGU, which closely resembles the “winner” A1 binding site UAAGGU obtained by selection of amplified pools of RNA sequences (33). Whereas these high affinity A1 binding sites promote strong shifts in 5′ splice site utilization in vitro, their effect on splicing is not associated with equivalent changes in U1 small nuclear ribonucleoprotein particle binding to the competing 5′ splice sites (34). We have proposed that the mechanism by which hnRNP A1 controls 5′ splice site selection in this system involves an interaction between bound A1 molecules, an event that would place the proximal 5′ splice site in a loop and would bring in closer proximity the most distant pair of splice sites (35-37). Here, we have uncovered additional elements capable of promoting distant 5′ splice site selection in vitro. Notably, we find that sequences flanking CE1a and different portions of CE4 can individually interact with hnRNP A1. Our results indicate that control elements promoting distant 5′ splice site utilization in the hnRNP A1 pre-mRNA are organized in groups of adjacent A1 binding sites. This organization may facilitate the recruitment of hnRNP A1/A2 proteins and may stimulate or stabilize the proposed change in pre-mRNA conformation.

MATERIALS AND METHODS

Plasmid Constructs—The ps1 plasmid was described previously (34). pSCE1 and pSCE1a correspond to the ps2 and ps10 plasmids described in Ref. 34, pSCE1z, pSCE1z31, pSCE1z35, pSCE1e, pSCE1d, and pSCE1ad were produced by inserting reannealed oligonucleotides corresponding to the mouse sequences of various portions of CE1 at the Smal site of ps1. pC3′-ps1, pC3′-ps1/M6, and pC3′-ps1/M7 were used to insert CE1a and CE4a into the XmaI site of p45, following Klenow treatment. The reannealed CE1a, CE1d, CE1dM6, and CE1dM7 oligonucleotides were inserted into the HinClI site of pBlueScript KS+ to produce pKCE1e, pKCE1d, pKCE1dM6, and pKCE1dM7. pKCE4m and pKCE1a were described previously (35).

Transcription and Splicing Assays—Splicing substrates were produced from plasmids linearized with ScaI, except for p45-M5 which was obtained by cutting with EarI, and transcribed with T3 RNA polymerase (Amersham Biosciences) in the presence of cap analog and [α-32P]UTP (Amersham Biosciences). CE1e, CE1d, M6, and M7 RNAs were produced from pKCE1e, pKCE1d, pKCE1dM6, and pKCE1dM7 linearized with HindIII, and CE1a and CE4m were produced from pKCE1a and pKCE4m linearized with EcoRI and transcribed as above. RNA purification was performed as described in Ref. 38. HeLa, CB3C7, and CB3C7-20 nuclear extracts were prepared (39) and used in splicing reactions as described previously (34). Creatine kinase was added to HeLa, CB3C7, and CB3C7-20 extracts at a final concentration of 1 unit/25 μl. In all splicing gels, we used lariat molecules (intermediates and products) to monitor splicing at each 5′ splice site. The fact that these molecules usually migrate above the pre-mRNA shows a measure of splicing efficiency, particularly when pre-mRNA degradation obscures mRNA production. Furthermore, adequate separation between lariat products requires longer runs or the use of lower percentage acrylamide gels, which cause the distal 5′ exon and the distal mRNA to run out the gel.

Purification of Recombinant Proteins—Recombinant GST-A1, A2, and A1B proteins were purified using a glutathione-Sepharose column (Amersham Biosciences) following procedure II as described in Fiset and Chabot (40). Bacterial lysis was performed in buffer A (50 mM piperazone-HCl, pH 9.8, 0.5 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol, 20 μg/ml bacitracin, 1 mM benzamidase, 0.5 mM phenylmethylsulfonyl fluoride) in the presence of 3 mg/ml lysozyme and 1% Triton X-100. The lysates were mixed with glutathione-Sepharose-4B resin following the manufacturer’s protocol (Amersham Biosciences). The resin was washed twice with 10 ml of storage buffer (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 20% glycerol, 5.7 mM MgCl2, 1 mM diithiothreitol) and kept at a 50% slurry at 4 °C. The coupling efficiency, which was typically higher than 95%, was measured by comparing the absorbance at 260 nm of the input periodate-treated RNA to 10% of the unbound material. 175 μl of HeLa nuclear extract containing 5.7 mM MgCl2, 0.90 mM ATP, 36 mM phosphocreatine, 3.58 mM diithiothreitol, and 1.25 unit/ml RNAGuard was incubated with 50 μl of packed beads for 10 min at 30 °C under agitation. The mixture was spun, and the supernatant was transferred to a second tube containing 50 μl of the same packed beads. The beads were washed four times with 1 ml of 70% buffer B (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 20% glycerol, 1 mM diithiothreitol) containing 5 mM MgCl2. The bound proteins were eluted from the column with 100 μl of loading dye (62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.7 mM mercaptoethanol, 0.003% bromophenol blue).

Western Analysis—Equivalent ratios of nuclear extract and proteins eluted from the CE1a RNA column were separated on SDS-10% polyacrylamide gel slabs. These samples were transferred to nitrocellulose filters and probed for the presence of hnRNP A1/A1B/A2/B1 using a rabbit serum containing antibodies against a peptide sequence shared by these proteins (kindly provided by Telogene Inc., Sherbrooke, Québec, Canada). This serum was used at a dilution of 1:1,000, and decorated proteins were revealed using the ECL detection kit (Amersham Biosciences) according to the manufacturer’s instructions.

RESULTS

Additional Sequences within CE1 Modulate 5′ Splice Site Selection—The 150-nt-long CE1 element is located in the intron downstream of common exon 7 in the hnRNP A1 pre-mRNA. CE1 is required for efficient skipping of alternative exon 7B in vitro and stimulates distant 5′ splice site use when inserted between the 5′ splice sites of exon 7 and exon 7B in a model pre-mRNA in vitro (34). We have reported previously that a small 17-nt segment (CE1a) is responsible for a large portion of the effect of CE1 on 5′ splice site selection (34). However, because CE1a does not fully restore the activity of the larger CE1 element, additional sequences within CE1 may also be promoting distant 5′ splice site usage. To identify these sequences, we inserted the 3′ end portion of CE1 (CE1z, 58 nt; Fig. 1A) between the two 5′ splice sites in our model pre-mRNA. Typically, the level of proximal and distant 5′ splice site use can be assessed by monitoring the production of lariat molecules following incubation of a labeled pre-mRNA in a nuclear extract and separating the spliced products on a denaturing gel. In comparison with the control S1 pre-mRNA, incubation of SCE1z RNA in a HeLa extract indicated that CE1z...
stimulated the use of the distal 5′ splice site as observed by an increase in lariat products generated by the use of this site (Fig. 1B, compare lane 4 with lane 1). The amplitude of the stimulation was comparable with the effect of CE1a (Fig. 1B, compare lane 4 with lane 3). As observed previously, the full 150-nt CE1 element promoted a nearly complete loss of proximal 5′ splice site use (Fig. 1B, lane 2). To further define the sequence within CE1z that was responsible for the shift, we fragmented CE1z into two smaller slightly overlapping units (CE1z35 and CE1z31). Whereas CE1z31 had a small effect on 5′ splice site selection, CE1z35 was much more active in switching splicing to the distal 5′ splice site (Fig. 1B, compare lanes 5 and 6 with lane 4). A further dissection of CE1z35 into CE1e (16 nt) and CE1d (19 nt) showed that CE1d, but not CE1e, could shift splicing toward the distal 5′ splice site (Fig. 1B, lanes 7 and 8, respectively). These results indicate that the 19-nt-long CE1d element contains sequences that can promote distal 5′ splice site selection in vitro as efficiently as the previously described CE1a element. When we tested a portion of CE1 that contained both CE1a and CE1d (CE1ad), we observed that the combination of the two elements shifted 5′ splice site selection to a level that was comparable with the level obtained with the complete CE1 element (Fig. 1C, compare lane 3 with lane 2). Thus, the activity of CE1 in HeLa extracts apparently results from the combined effects of CE1a and CE1d. 

hnRNP A1 Binds to CE1d—Because hnRNP A1 has been implicated in the activity of CE1a, we tested whether hnRNP A1 was also involved in the activity of CE1d. First, we assessed

![Diagram](image-url)
the ability of hnRNP A1 to interact with CE1d by performing a gel mobility shift assay using increasing amounts of a recombinant GST-A1 protein (rA1). The RNA used for this assay is 83 nt long and contains plasmid sequences followed by the CE1d element. Since CE1e had no effect on 5' \( /H11032 \) splice site selection, it was used as a control in place of the CE1d element. Slower migrating complexes were observed with CE1d but not with CE1e (Fig. 2C, top panel), indicating a specific interaction of rA1 with CE1d.

The observation that recombinant A1 protein can specifically interact with CE1d suggests that this interaction may be required for the CE1d-dependent switch in 5' \( /H11032 \) splice site selection observed in vitro. To test this possibility, we produced seven mutations spanning the CE1d element and assessed their effects on 5' \( /H11032 \) splice site selection in a HeLa nuclear extract. The mutations are listed in Fig. 2A, and they were tested in a slightly shorter version of the S1 pre-mRNA (pre-mRNA 45) for cloning reasons. Inserting CE1d in pre-mRNA 45 (WCE1d) promotes distal 5' \( /H11032 \) splice site utilization in a manner similar to what was observed in the S1 backbone (Fig. 2B, compare lane 8 with lane 1). We then tested the small 83-nt-long RNA containing the M6 sequence (M6*) instead of CE1d for binding by hnRNP A1. In comparison with a similar transcript containing the M7 sequence (M7*), which was bound by rA1 (Fig. 2C, bottom panel, lanes 5–8), M6* RNA was not bound significantly by rA1 (lanes 1–4). The correlation between A1 binding and the efficiency of distal 5' \( /H11032 \) splice site activation is consistent with the notion that A1 is involved in the activity of CE1d.

The Activity of CE1d Is Mediated by hnRNP A1—To demonstrate that hnRNP A1 mediates the activity of CE1d, we carried out the depletion of hnRNP A1 from a HeLa nuclear extract by affinity chromatography using RNA molecules carrying a high affinity binding site for hnRNP A1. The CE1a RNA was covalently linked to agarose-adipic acid beads and incubated in the presence of a HeLa nuclear extract. The flow-through fraction was recovered and used as the depleted nuclear extract. Western analysis using a polyclonal antibody that recognizes the core hnRNP A1, A2, B1, and A1B proteins (Fig. 3A, lane 1) indicates that the majority of these hnRNP proteins had been removed from the nuclear extract (lane 5). hnRNP A1, A2, B1, and A1B proteins were found in the fractions bound to successive CE1a columns (Fig. 3A, lanes 6 and 7). A mock-depleted nuclear extract was also prepared by load-
ing a HeLa nuclear extract on a column lacking RNA. The mock-depleted extract contains the core hnRNP A/B proteins (Fig. 3A, lane 2), and no signal was detected in the bound fractions (lanes 3 and 4). The mock- and A/B-depleted extracts were then tested for activity using a CE1a-containing pre-mRNA (SCE1a). We have shown previously that 5' splice site usage on this pre-mRNA can be displaced from the distal to the proximal donor site by the addition of an excess of DNA oligonucleotide carrying A1 binding sites and that supplementation with rA1 restores efficient distal 5' splice site use (35). Whereas distal 5' splice site use was predominately observed in the mock-depleted extract (Fig. 3B, lane 1), only the proximal 5' splice site was used in the A/B-depleted extract (lane 2), consistent with a role for hnRNP A/B proteins in the activity of CE1a. SCE1d pre-mRNA was similarly spliced to the proximal 5' splice site in the A/B-depleted extract, suggesting that these proteins may also be required for the activity of CE1d (Fig. 3B, lane 4). The addition of increasing amounts of rA1 to the A/B-depleted extract shifted splicing toward the distal donor site in a CE1d-dependent manner (Fig. 3C). At the highest concentration tested, the addition of rA1 to the SCE1d pre-mRNA nearly completely abrogated proximal 5' splice site use and activated the distal 5' splice site (Fig. 3C, lane 10). In contrast, at the same concentration of rA1, proximal 5' splice site use remained the predominant choice for the control S1 pre-mRNA (Fig. 3C, lane 6). Thus, the greater sensitivity of a pre-mRNA containing CE1d to the supplementation with rA1 indicates that hnRNP A1 can mediate the activity of CE1d.

hnRNP A2 Can Also Mediate the Activity of CE1a and CE1d—To determine whether hnRNP A1 is absolutely necessary for the activity of CE1a and CE1d, we monitored splicing in an extract prepared from a mouse erythroleukemic cell line previously shown to be severely deficient in hnRNP A1 protein expression. The CB3C7 cell line expresses at least 250-fold lower levels of A1 and A1B mRNAs, because one Hnrnpa1 allele has been deleted while the other allele has suffered a retroviral insertion event (41, 42). Similar to what was observed in a HeLa extract (Fig. 1B, lanes 1–3), both CE1 and CE1a improved distal 5' splice site utilization in a nuclear extract prepared from CB3C7 cells (Fig. 4A, compare lanes 2 and 3 with lane 1). We also tested splicing in a nuclear extract prepared from a derivative of the CB3C7 cell line, which is stably restored for hnRNP A1 expression (CB3C7–20; Fig. 4B, lane 3) (42). The relative levels of distal 5' splice site utilization of the SCE1a pre-mRNA were comparable in the CB3C7–20 and in the A1-compromised CB3C7 extracts (Fig. 4A, lane 5). These results suggest that CB3C7 cells contain a factor(s) that functionally replace hnRNP A1, allowing CB3C7 extracts to carry out CE1- and CE1a-dependent shifts in 5' splice site selection.

Several observations suggest that the hnRNP A2 protein can functionally replace hnRNP A1 in its ability to promote distal 5' splice site use in vitro (24). A2 and its splice variant hnRNP B1 can also replace A1 when repression of a 3' splice site is associated with nearby high affinity A1 binding sites (28–30). Because CB3C7 cells express hnRNP A2 and B1 (Fig. 4B), these proteins may compensate for the loss of A1 and A1B in CB3C7 cells. We used RNA affinity chromatography to deplete hnRNP A2 and B1 proteins from the CB3C7 nuclear extract (data not shown). The removal of A2/B1 from the CB3C7 nuclear extract was associated with an incapacity to activate the distal 5' splice site upon incubation with the SCE1d pre-mRNA.
nuclear extracts. CB3C7 are mouse erythroleukemic cells that are deficient in A1 expression. A1 expression was stably restored in CB3C7 nuclear extracts.

RNA are indicated.

rA1 and rA2 had little or no effect on distal 5' splice site selection (Fig. 4C, lanes 5 and 13). To confirm that hnRNP A2 could substitute for A1 in the activity mediated by CE1d, we added increasing amounts of recombinant GST-A2 (rA2) or GST-A1 (rA1) to the A2/B1-depleted CB3C7 nuclear extract. At the highest concentrations, the addition of rA1 or rA2 abolished proximal 5' splice site use on the control S1 pre-mRNA (Fig. 4C, lanes 3 and 4 and lanes 11 and 12, respectively). The reason for the repression of S1 pre-mRNA splicing by rA1 and rA2 in the mouse extract is unknown. Nevertheless, the addition of rA1 and rA2 had little or no effect on distal 5' splice site use on the S1 pre-mRNA. In contrast, the highest concentrations of rA1 and rA2 tested on the SCE1d pre-mRNA promoted both a reduction in proximal use and an increase in distal splice site selection (Fig. 4C, lanes 7 and 8, and lanes 15 and 16, respectively). These results indicate that hnRNP A2 can also mediate the CE1d-dependent activity in 5' splice site selection. Thus, hnRNP A2 is most likely responsible for the lack of a strong splicing defect in the A1-deficient CB3C7 cells.

The ability of hnRNP A2 to bind specifically to CE1d was confirmed by performing a gel shift assay (Fig. 4D). Recombinant hnRNP A2 bound efficiently to CE1d but only weakly to CE1e (Fig. 4D, top left panel). Moreover, rA2 bound efficiently to the CE1d derivative M7* RNA, but the binding to the M6 sequence was reduced considerably (Fig. 4D, bottom left panel). A binding assay performed with recombinant A1B also indicated specific binding to CE1d (Fig. 5D, top right panel) and stronger binding to M7* relative to M6* RNA (bottom right panel). Thus, CE1d can be bound by A1, A1B, or A2. The use of recombinant A1B also allowed a recovery of distal 5' splice site use in a CE1a-dependent manner in hnRNP A/B-depleted extracts (data not shown). Although we have not tested hnRNP B1, the splice variant of A2, we would expect this protein to display a similar binding behavior, because it is also depleted from a HeLa extract using a CE1a RNA column. Moreover, several groups have now reported that B1 also displays a function that is similar to hnRNP A1 in splice site selection (24, 28).

CE4 Also Contains Several A1 Binding Sites That Influence 5' Splice Site Selection—We reported previously that the 24-nt-long CE4 element downstream of alternative exon 7B contains a high affinity A1 binding site. CE4 promotes distal 5' splice site selection in vitro and exon 7B skipping in vivo (35). CE4 contains the sequence UAGAGU (Fig. 5A), which is also present in CE1a and which was shown to be important for A1 binding and the activity of CE1a (34). Because CE1 contains adjacent A1-bound elements that can individually carry out distal 5' splice site selection, we wondered whether a similar organization existed in CE4. When we compared the in vitro splicing of pre-mRNAs carrying either CE4 or a shortened version lacking the CE4p portion that contains the UAGAGU sequence (see Fig. 5A), we noted a significant reduction in the efficiency of distal 5' splice site use (Fig. 5B, compare lane 2 with lane 4), consistent with the notion that the UAGAGU sequence contributes to the activity of CE4 on 5' splice site selection. On the other hand, the remaining portion of CE4 (CE4m) remained as active as CE1a at promoting distal 5' splice site selection (Fig. 5B, compare lane 4 with lane 3), indicating that CE4m can also promote distal 5' splice site selection in vitro.

We have noticed earlier that CE4m is not bound efficiently by recombinant hnRNP A1 in a gel shift assay (35). However, when we repeated the binding assay with recombinant proteins that had been prepared using a high salt procedure (40), we observed that rA1 could bind to CE4m (Fig. 5C, lanes 6-10). The fact that A1 does not strongly interact with the control CE1e RNA indicates that binding to CE4m is specific (Fig. 5C, lanes 1-5). To assess the contribution of A1 in the activity of CE4m, we tested extracts that had been depleted of A/B proteins by RNA affinity chromatography. The control pre-mRNA C5'−/− was spliced almost exclusively to the proximal 5' splice site in a mock-depleted extract or in the A/B-depleted extract (Fig. 5D, lanes 1 and 2), and the addition of rA1 had no significant effect on 5' splice site selection at the concentrations used.
In contrast, whereas the C5' /H11032/4m/4m pre-mRNA was spliced efficiently to the distal donor site in the mock extract (Fig. 5D, lane 5), distal splicing was abolished in the A/B-depleted extract (lane 6). Moreover, the addition of rA1 to the A/B-depleted extract improved the relative efficiency of distal 5' splice site use to an extent that was comparable with the level observed in the mock extract (Fig. 5D, compare lane 8 with lane 5). These results indicate that CE4m can also promote distal 5' splice site utilization in an A1-dependent manner. Thus, the strongest activity of CE4 relative to CE1a and CE4m probably reflects the fact that it contains several distinct A1 binding sites.

DISCUSSION

The activity of the previously identified CE1 and CE4 elements can be attributed in each case to adjacent units individually capable of promoting distal 5' splice site selection in vitro. CE1 is composed of CE1a and CE1d, each bound by members of the hnRNP A/B family of proteins. Likewise, the activity of CE4 on 5' splice site selection can be separated into CE4m and
CE4p, each bound by hnRNP A1 or A2. Recombinant hnRNP A1 and A2 can restore the CE1a-, CE1d-, or CE4m-dependent shift in 5′ splice site selection in extracts that had previously been depleted of their endogenous hnRNP A/B proteins.

The A1 binding sites in CE1a and CE4p are identical and correspond to UAGAGU, a close match to the optimal A1 binding site UAGGGU sequence obtained by selection from a pool of randomized RNA sequences (33). In contrast, the sequence responsible for the binding of A1 and A2 to CE1d and CE4m is less clear. Although CE4m contains the sequences UAGAGU and UAGACU, we have shown previously that mutating the CE1a UAGAGU into UAGACU compromises A1 binding and abrogates its activity (34). Thus, a pyrimidine at position +5 may be incompatible with efficient A1 binding. CE1d also contains a variety of sequences that matches or resembles the A1 binding consensus UAGGR(A/U) (where R represents purine). Notably, CE1d and CE4m share the sequence RRGCUAG and UAGACU, we have shown previously that mutating the ing site UAGGGU sequence obtained by selection from a pool of RNA sequences (35). The existence of elements (CE1 and CE4) that are necessary and sufficient for the preparation of nuclear extracts, Faiz Nasim for plasmid p45, and members of the Chabot laboratory for comments. We thank William Rigby for kindly providing the GST-A2 expression plasmid.

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