

Control of 3' splice site choice *in vivo* by ASF/SF2 and hnRNP A1

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ABSTRACT

The constitutive splicing factor ASF/SF2 has been shown to affect the choice between alternative splice sites by favoring the proximal as opposed to the distal choice. hnRNP A1 antagonizes ASF/SF2 by promoting the distal choice for competing 5' splice sites. We have tested the *in vivo* effects of these proteins on alternative 3' splice site choices. Cotransfection of a dihydrofolate reductase–calcitonin chimeric construct together with a plasmid specifying the SR protein ASF/SF2 into cells of several mammalian lines increased use of a proximal 3' splice site, resulting in the inclusion of a terminal calcitonin exon. This stimulation of 3' proximal splicing was antagonized by cotransfection with an hnRNP A1 plasmid. This effect of hnRNP A1 in promoting distal splicing was also seen in an hnRNP A1-deficient MEL cell line. A similar effect of hnRNP A1 was demonstrated with mutant hamster adenine phosphoribosyltransferase (*aprt*) transcripts that are normally constitutively spliced, suggesting that hnRNP A1 may be a general inhibitor of proximal splicing. Intron size also influenced splice site choice in mutant *aprt* transcripts, with larger introns favoring proximal splicing. These results support the idea that the ratios of particular but general splicing factors and hnRNPs play a role in alternative splicing.

INTRODUCTION

Alternative splicing plays an important role in the tissue-specific regulation of gene expression (1,2), producing mRNA isoforms that specify diverse classes of proteins. It is likely that an understanding of the mechanisms governing alternative splicing will be important not only for the study of development and differentiation, but also for illuminating mechanisms at work in constitutive splicing. The regulation of alternative splicing usually rests on choosing among candidate splice sites, either alternative 5' or alternative 3' sites. Previous work has shown several examples in which the constitutive splicing factor ASF/SF2, as well as other SR proteins, promote the use of the proximal of two 5' sites that can splice to a fixed 3' site (3,4). Conversely, the abundant hnRNP A1 can influence the choice between alternative 5' splices in the opposite manner, promoting splicing at the distal site (5–8). In the case of SR proteins, a splice

site switching experiment has shown that the choice depends on the proximity of the 5' sites to the 3' site, not the local sequence context at or around the 5' site (9).

It is not clear how SR proteins and hnRNP A1 are sensing proximity and distance. Eperon *et al.* proposed a model based on the ability of ASF/SF2 to promote U1 snRNP binding to weak 5' splice sites (10). At low levels of ASF/SF2, U1 snRNP would not be bound, and a weak proximal site would be bypassed in favor of a stronger distal site. At higher levels of ASF/SF2, the weak 5' site would be occupied by U1 snRNP and would be preferentially used due to its effectively higher concentration (i.e., its proximity to a fixed 3' site). This model must be broadened to accommodate the findings of Fu *et al.* (11) that, *in vitro*, ASF/SF2 can also promote a proximal choice between two competing 3' splice sites. In this situation, the 5' site is fixed, and ASF/SF2 would be promoting a weak proximal 3' site. Consistent with this idea, SR proteins like ASF/SF2 can interact with U2AF (12), which itself binds to the branch point near the 3' site. A model for proximity/distance sensing must also take into account the effect of hnRNP A1 in promoting distal splicing and antagonizing the proximal splicing favored by ASF/SF2. Although hnRNP A1 can bind directly to 3' splice site sequences, the specificity of this interaction has been questioned (13,14). However, hnRNP A1 may act via protein–protein interactions, as it can bind to U2 snRNP (15,16) and to a subset of SR proteins (17). Thus, both ASF/SF2 and hnRNP A1 have the potential to play roles in the identification of a 3' site as well as a 5' site. It should be noted, however, that in their *in vitro* studies Fu *et al.* found that A1 did not antagonize an ASF/SF2-promoted proximal 3' splice site choice (11).

These considerations led us to test the effects of ASF/SF2 and hnRNP A1 on transcripts that naturally undergo alternative 3' splicing, and to extend the approach of Fu *et al.* (11) to an *in vivo* condition. We started with the calcitonin/CGRP gene and examined splicing in transfected mammalian cell cultures. In this well-studied example of tissue-specific alternative splicing (e.g., 18–22), splicing of intron 3 proceeds via an alternative 3' splice site choice: splicing at exon 4, a proximal 3' splice site, results in calcitonin mRNA with exon 4 as the terminal exon; whereas splicing at exon 5, a distal 3' choice, results in the exclusion of exon 4 and the generation of CGRP mRNA (Fig. 1A). Calcitonin is produced in thyroid C cells and CGRP is produced in neuronal cells; in cells not normally transcribing this gene, the calcitonin splicing pattern predominates as the default pathway (23). We found that ASF/SF2 promoted proximal 3' splicing and that hnRNP A1

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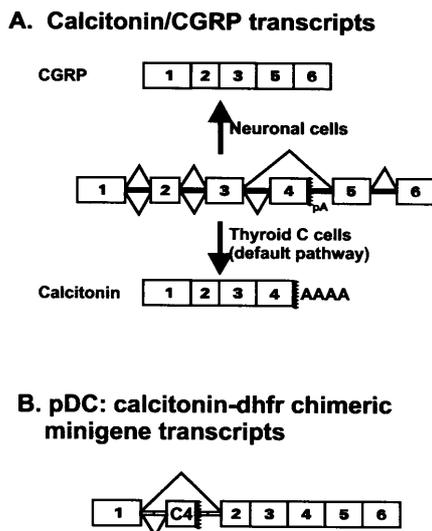


Figure 1. Alternative splicing of calcitonin exon 4. (A) Alternative splicing patterns in the natural calcitonin/CGRP transcript. (B) Alternative splicing patterns in the chimeric construct DC, which carries the rat calcitonin exon 4 within the sole intron of a hamster dhfr minigene.

promoted distal 3' splicing in calcitonin/CGRP transcripts *in vivo*. Moreover, the effect of A1 was also seen in a normally constitutively spliced adenine phosphoribosyltransferase (aprt) transcript that exhibits alternative 3' splicing only by virtue of a splicing mutation. This last result suggests that A1 can act in a general way (e.g., sequence non-specifically) to inhibit proximal splicing. In this mutant aprt transcript, small intron size (154 nt) was seen to promote exon-skipping, suggesting that spatial factors can influence proximal versus distal splice site choice.

MATERIALS AND METHODS

Cell lines and cell culture

DG44 is a CHO cell line with a double deletion at the dihydrofolate reductase (dhfr) locus (24) and U1S is a CHO cell line with a double deletion of aprt (25). XA57 is an APRT-deficient CHO cell mutant with a 5' splice site mutation in intron 4 (26). T98G (ATCC #CRL1690) is a human glioblastoma cell line. These cells as well as human HeLa and 293 cells were grown in monolayer culture in Ham's F-12 medium (Gibco) supplemented with 10% fetal calf serum. PC12 is a rat pheochromocytoma cell line that has acquired a number of properties of sympathetic neurons (27); these cells were grown in the above medium supplemented with 5% horse serum. Two mouse erythroleukemia (MEL) cell lines were used: CB3C7, which is deficient in hnRNP A1, and DP27-17, which has no such deficiency (28). MEL cells were grown in α -modified Eagle's medium (α -MEM; Gibco) supplemented with 5% fetal bovine serum. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Generally the medium was changed every three days.

Plasmid construction

pDC was assembled by inserting a 1.2 kb fragment containing exon 4 of the human calcitonin/CGRP gene, from an intron 3 position 386 bp upstream of exon 4 to a position 361 bp downstream of the polyA site in exon 4, into a *Pst*I site in the sole

intron of the Chinese hamster dhfr minigene construct pDCH1P (29). pF4-neo is a mammalian expression construct carrying the full length rat calcitonin/CGRP gene (22). pCF21 is an ASF/SF2 mammalian expression cDNA construct (30). pCG-A1 is a hnRNP A1 mammalian expression cDNA construct, pCG being the expression vector without the insert (7). A plasmid carrying an aprt splicing mutant gene, pXA57, was constructed by PCR amplification of a region delimited by previously described aprt primers 5' intron 3 and 3' exon 5 (31), cutting with *Not*I and *Xba*I and substituting the mutant DNA for the corresponding 714 nt fragment in pWTaprt (31). pXA57N was created by site-directed mutagenesis in experiments unrelated to this work: TG was substituted for GT at positions 55-56 of exon 4, resulting in a premature translation termination codon at that point. Contrary to expectations (reviewed in 32), the presence of this stop codon in exon 4 did not increase the skipping of this exon, but rather increased inclusion slightly (data not shown).

To expand intron 3 of the aprt gene, we first used site-directed mutagenesis to introduce a *Bgl*II site and a *Bcl*I site at intron 3 positions 18 and 108, respectively; performing a CTG to ATC substitution at positions 20-22 and a G to A substitution at position 110. These changes did not affect the splicing pattern of the aprt transcript (data not shown). The region spanning intron 3 and its flanks was then amplified by PCR; the PCR product contained unique restriction sites in the order *Xho*I-*Bgl*II-*Bcl*I-*Pst*I. This fragment was cut separately with *Bgl*II or *Bcl*I and the resulting fragments were ligated to produce a non-cutting *Bgl*II/*Bcl*I joint between duplicated 90 nt *Bgl*II-*Bcl*I regions. This molecule was once again cut with either *Bgl*II or *Bcl*I and these two fragments were ligated. The resulting ligation product, which contained four copies of the *Bgl*II-*Bcl*I region, was cut with *Xho*I and *Pst*I and cloned into the similarly cut aprt vector pXA57N to create pXA57NT (T for tetramer of the 90 bp region). Transformants were screened by size for the insertion of a tetramer of the 90 nt region. pXA57NH and pXA57NR were constructed by PCR-amplification of regions from positions 10 641 to 11 009 of human hprt gene intron 1, and 165 269 to 165 636 of human Rb gene intron 23, respectively. The primers contained tails with *Bgl*II or *Bcl*I sites and the PCR products were cut with both enzymes and cloned into the large fragment of pXA57NT similarly cut.

Transfection

Adherent cells were transfected according to Wigler *et al.* (33), using 10 μ g of pDC DNA and 10 μ g of effector DNA: pCF21 for ASF/SF2, pCG-A1 for hnRNP A1 and pCG as a control, per 100 mm dish. MEL CB3C7 and DP27-17 cells were transfected by lipofection: $\sim 7.5 \times 10^6$ cells were rinsed successively with PBS and serum-free Opti-MEM (Gibco). A 25 μ l mixture containing 1 μ g of pDC and 10 μ g of pCF21, pCG-A1 and/or pCG in water was added to 0.3 ml of Opti-MEM and mixed with 0.31 ml of Opti-MEM containing 10 μ l of Lipofectin (Life Technologies). This mixture was used to resuspend the cell pellet, and the suspended cells were incubated in a 60 mm petri dish at 37°C for 1 h before adding 5 ml of α -MEM supplemented with 10% fetal bovine serum. RNA was extracted 48 h after the transfection.

RNA analysis

RNA was extracted from cultured cells as follows (34): cells were lysed in a solution of 2% SDS, 200 mM Tris-HCl, pH 7.5 and

1 mM EDTA. DNA and proteins were precipitated with 1.5 M potassium acetate, and centrifuged for 10 min at 4°C in a microcentrifuge. The supernatant was extracted twice with chloroform:isoamyl alcohol and RNA was precipitated with 0.65 vol of isopropanol. RNA extracted from 100 or 60 mm dishes was treated with 30 U of RNase-free DNase I (Boehringer Mannheim), 3 mM MgCl₂ and 100 U RNasin (Promega). We used reverse transcriptase followed by PCR (RT-PCR) to quantify the splicing products (9). For the RT reaction, a 20 µl reaction mixture contained 1 µg RNA (dissolved in water), 0.4 µg of random hexamers, 10 mM dithiothreitol, 40 U of RNasin, 0.5 mM of all four deoxynucleoside triphosphates, 4 µl of 5× RT buffer and 200 U of mouse mammary leukemia virus RT (all above were from Promega). The reaction was carried out at 37°C for 1 h, followed by 5 min heating at 95°C to inactivate the enzyme. The cDNA mixture was used directly as the substrate for PCR. A standard 100 µl PCR reaction contained 4 µCi [³²P]dATP (3000 Ci/mmol; Dupont) and a wax bead (Perkin Elmer-Cetus, AmpliWax™ PCR Gem 100): the lower reagent mix included 2 µl 10× PCR Buffer II (Perkin Elmer), 1.2 µl of a 25 mM MgCl₂ solution, 1 µl of 10 mM dNTPs, 0.4 µg of each primer, in a final volume of 25 µl. One wax bead was added to each tube, and the wax was melted by incubation at 80°C for 6 min. The temperature was lowered to 10°C to resolidify the wax. The upper reagent mix included 8 µl 10× PCR Buffer II, 4.8 µl 25 mM MgCl₂, 1.25 U of *Taq* polymerase (Perkin Elmer) and 5 µl of the cDNA solution in a total volume of 70 µl. The PCR conditions were as follows: denaturation at 95°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min. After 27 cycles, a 7 min extension at 72°C was carried out. A 6 µl sample of PCR reaction solution was electrophoresed in a 5% polyacrylamide gel.

We analyzed 3' alternative splicing with RT-PCR by including, at a 2:1:1 ratio, one common 5' primer and two 3' primers specific for different splicing products. To establish the quantitative response of this method, we first used RT-PCR to generate separate cDNAs from mRNA that had included or excluded exon C4 in a dhfr/calcitonin chimeric construct context. The cDNA was gel purified with the aid of a Qiagen purification system. We prepared mixtures with the ratios of 10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10 (where 1 represents 0.05 pg of each cDNA) and subjected them to PCR using the conditions described above. There was good quantitative agreement between the input ratios and the output of the PCR, as measured by PhosphorImaging (Molecular Dynamics; Fig. 2). Each transfection experiment was carried out at least twice; the average variation for the ratio of exon excluded/exon included species was <20%.

The sequences of oligos used in this study were as follows. Analysis of inclusion or exclusion of exon 4 of calcitonin/CGRP in different constructs—for pDC: Op1 (forward), CGCGCAA-ACTTGGGGG; D3'exon2 (reverse), ACTGAGGAGGTGGTG-GTC; and CD2 (reverse), CCAGCATGCAAGTACTCAGA. For pF4-neo: RC35DF (forward), CCTTAGAAAGCAGCCCAG; RC53DR (reverse), TGAAGGTCCCTGCGGCG; and CD2 (reverse), CCAGCATGCAAGTACTCAGA. For detecting the inclusion of exon 4 of apt: A5'exon2 (forward) and A3'exon5B (reverse); the positions of these primers within the apt gene can be found in Kessler *et al.* (31). Under the conditions used these apt primers are specific for hamster; they do not amplify mouse apt sequences present in MEL cells. For detection of ASF/SF2 expression: ASF401 (forward), TGATGTTTACCGAGATGGCA; and ASF921 (reverse), GGCTTCTGCTACGACTACGG. For

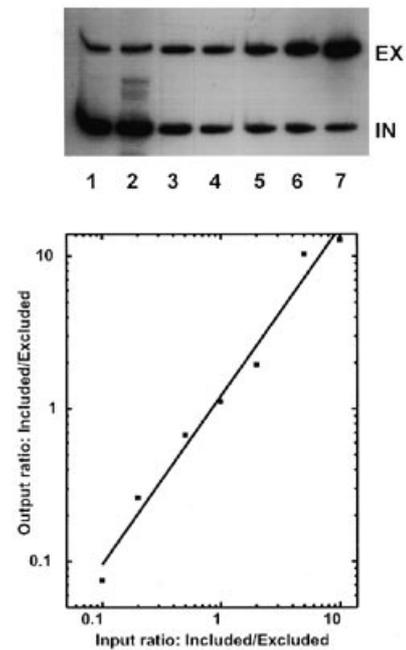


Figure 2. Quantitation of RT-PCR products of alternatively spliced RNA in a reconstruction experiment. Two cDNA molecules, In and Ex, representing inclusion or exclusion, respectively, of calcitonin exon 4 in transcripts of the chimeric calcitonin-dhfr minigene of pDC, were generated by RT-PCR, gel-purified and quantified by their absorbance at 260 nm. Seven mixtures of In and Ex in ratios ranging from 1:10 to 10:1 were prepared; a value of 1 represents 0.05 pg of DNA. These mixtures were used as a template for 27 cycles of PCR in the presence of [³²P]ATP. After polyacrylamide gel electrophoresis, the radioactive bands were quantified by PhosphorImaging. The output ratios represent a faithful rendition of the input ratios over a wide range.

detection of hnRNP A1 expression: A1701 (forward), CAATTT-TGGTTCGAGGAGGGA; and A1893 (reverse), TCCCTTCATC-GGCCCAAAT. For analysis of clathrin light chain B exon EN splicing: SS030 (forward), TGCCTCGAAGGTGAACCGAAC; SS031 (reverse), GGGGTCTCCTCCTTGGATTCT; and SS032 (reverse), TTCCGATGCCACATAGCC.

Digital images acquired by a PhosphorImager were normalized for overall range using the ImageQuant software for the instrument (Molecular Dynamics), converted to TIFF files and printed without further modification using CorelDraw 7.

RESULTS

Effects of ASF/SF2 and hnRNP A1 on the splicing of calcitonin/CGRP transcripts

We constructed a chimeric dhfr/calcitonin gene by inserting the alternatively spliced terminal exon 4 of the rat calcitonin gene (C4) together with its flanking regions into the sole intron of a hamster dhfr minigene (Fig. 1B). This construct was transiently transfected into four different mammalian cell lines and the splicing pattern of the resultant mRNA was determined by RT-PCR. After transfection into human HeLa cells and Chinese hamster ovary (CHO) cells, this construct yielded mainly mRNA that included exon C4 (Fig. 3B and C), whereas human 293 and

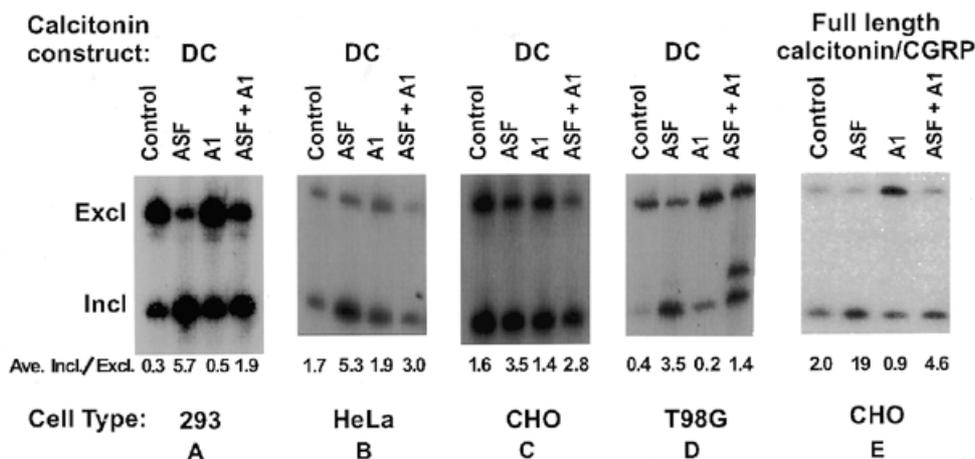


Figure 3. The effect of ASF/SF2 and hnRNP A1 on the inclusion and exclusion of calcitonin exon 4. The indicated cell types were transfected with a plasmid (pDC) carrying either a dhfr/calcitonin chimeric minigene (A–D) or a full length calcitonin/CGRP gene (E). Co-transfections included either an ASF/SF2 gene, an hnRNP A1 gene, or both, as indicated; total DNA was kept constant by addition of the A1 mammalian expression vector lacking the A1 insert. Total RNA was extracted 48 h after transfection and analyzed by RT–PCR. PhosphorImager images are shown: Incl, mRNA that included calcitonin exon 4; Excl, mRNA in which calcitonin exon 4 was skipped. The ratio of the two types of mRNA is indicated below each lane. The ratio does not necessarily reflect the absolute extent of exon 4 inclusion, since it was necessary to use different primers to distinguish the different products. Transfections of each combination of plasmid and recipient were performed at least twice; the average range or SEM was <20% of the mean.

T98G glioblastoma cells tended to exclude exon 4 (Fig. 3A and D). The last are expected to exclude exon 4, as they have been shown to carry out CGRP-type splicing (35). Cotransfection with a plasmid specifying ASF/SF2 increased inclusion in each case; i.e., promoted the use of the proximal 3' splice site (Fig. 3A–D). Cotransfection with an hnRNP A1 plasmid did not change the basal splicing pattern significantly; however, hnRNP A1 did antagonize the proximal splicing promoted by ASF/SF2 when cotransfected together with this gene (Fig. 3A–D). The extent of basal inclusion in these cell lines did not correlate with the level of endogenous ASF/SF2 mRNA, as measured by RT–PCR (data not shown). However, this measurement does not rule out differences in ASF/SF2 protein levels or the degree of protein phosphorylation (36–38). The effects of ASF/SF2 and hnRNP A1 were not due to the specific context of the chimeric dhfr/calcitonin construct, as similar results were obtained using a full length human calcitonin/CGRP gene as a target (Fig. 3E).

For a more sensitive demonstration of the effect of hnRNP A1 on 3' splice site selection, we turned to the hnRNP A1-deficient MEL cell line CB3C7 (6,28) as a recipient. We first confirmed the absence of hnRNP A1 mRNA in this cell line, using RT–PCR (Fig. 4A). As can be seen in Figure 4B, in transfections with the DC construct the proximal 3' site in DC RNA (inclusion of the C4 exon) was used much more in the hnRNP A1-deficient CB3C7 cells than in the hnRNP A1-proficient DP27–17 MEL cell line (6). This effect was partially reversed by inclusion of an hnRNP A1 plasmid in the transfecting DNA (i.e., hnRNP A1 increased exon C4 exclusion; Fig. 4B).

Effects of ASF/SF2 and hnRNP A1 on the splicing of mutant apt transcripts

The above results are consistent with the idea that the ratio of constitutive splicing factors plays a role in determining 3' splice site choices for an exon that is subject to developmentally regulated alternative splicing. Such transcripts may have evolved

sequence elements that make the 3' choice especially sensitive to the balance among certain constitutive splicing factors. Alternatively, when any two 3' sites of similar intrinsic strength are present, a high ratio of ASF/SF2 to hnRNP A1 may in general favor the proximal site. In this latter case, we might expect a constitutively spliced transcript to show a similar response to the ratio of ASF/SF2 to hnRNP A1. To test this idea, we turned to the small 5-exon apt gene. An APRT-deficient mutant of CHO cells, XA57, carries a base substitution (26) that has changed the GT dinucleotide at the 5' splice site of intron 4 to GC, resulting in very little wild-type splicing (Fig. 5B). We noted that in this mutant, exon 3 often spliced to exon 5 (skipping exon 4), as expected from a lack of exon 4 definition (39). However, the inclusion of exon 4 via an exon 3 to exon 4 splice was frequently realized as well, leading to the retention of intron 4 (Fig. 5B). We used PCR to clone the region including apt exon 4 from XA57 cells and constructed a cloned version of this mutant genomic gene, pXA57N. Transient transfection of pXA57N into the CHO apt deletion mutant U1S (25) reproduced the two products of the endogenous gene. However, in this case exon 4 inclusion (with intron 4 retention) predominated (Fig. 5C). Cotransfection of pXA57N with a plasmid specifying ASF/SF2 had little or no effect on the proximal splicing of exon 3 to exon 4 (Fig. 5C, lane 2). hnRNP A1 cotransfection, on the other hand, increased the use of the distal 3' splice site at the expense of the proximal (Fig. 5C, lane 3). Co-transfection with both ASF/SF2 and hnRNP A1 produced a result similar to hnRNP A1 alone (lane 4, note averages of several experiments at bottom of lanes). The mutant apt RNA yielded similar results for the effect of hnRNP A1 in the MEL cell system. In hnRNP A1-proficient cells exon 4 was almost always excluded (distal splicing), whereas in the hnRNP A1-deficient cells the exon along with intron 4 was almost always included (proximal splicing; Fig. 5D, left lanes). Cotransfection of the hnRNP A1-deficient cells with an hnRNP A1 plasmid caused exon 4 exclusion to predominate once again (Fig. 5D).

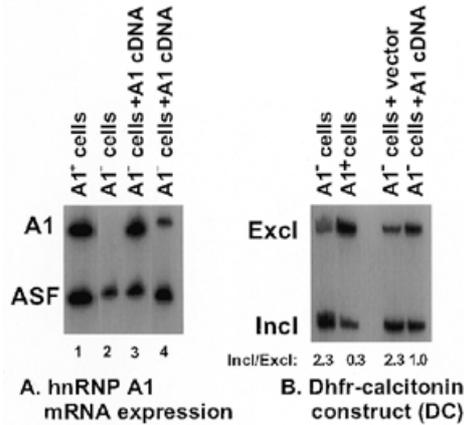


Figure 4. Alternative splicing of calcitonin and apt exons in hnRNP A1-deficient MEL cells. **(A)** Absence of hnRNP A1 mRNA in MEL CB3C7 cells ($A1^{-}$) compared with control MEL DP227-17 cells ($A1^{+}$) and its restoration after transfection with the hnRNP A1 plasmid pCG-A1. Multiplex RT-PCR was used to detect both hnRNP A1 and ASF/SF2 mRNA molecules; a PhosphorImager image of the gel is shown. Note that the relative ratio of hnRNP A1 to ASF/SF2 is underestimated in the transfection experiments, as the transfected cells presumably represent a minority of the population. The RNA in lane 3 was derived from cells transfected additionally with plasmid DC and was the same RNA analyzed in **(B)**, lane 4. The RNA in lane 4 was derived from cells transfected additionally with plasmid XA57N and was the same RNA analyzed in Figure 5D, lane 4. **(B)** Promotion of distal splicing in a dhfr/calcitonin chimeric transcript by hnRNP A1 in hnRNP A1-deficient MEL cells. hnRNP A1-proficient (DP227-17) or -deficient (CB3C7) MEL cells were transfected with the dhfr/calcitonin chimeric construct DC with or without the hnRNP A1 gene, RNA was extracted and the splicing pattern analyzed by RT-PCR. Incl, included exon 4; Excl, excluded exon 4. Shown below each lane is the ratio of exon C4-included to C4-excluded mRNA; the averages of at least two transfection experiments are shown. The 8-fold bias in favor of exon inclusion in hnRNP A1-deficient cells is reduced by cotransfection with an hnRNP A1 plasmid. The control vector used in lane 3 was the mammalian expression plasmid vector (pCG) used for cloning the hnRNP A1 cDNA.

Since there is no reason to believe that specific functional hnRNP A1 binding sites had evolved within the constitutively spliced apt gene, we conclude that hnRNP A1 can influence 3' splice choice in some general way, perhaps by promoting distal at the expense of proximal splicing.

Differential splicing of clathrin light chain B transcripts in hnRNP A1-deficient MEL cells

To test the idea that MEL $A1^{-}$ cells exhibit a general propensity for skipping weak or facultatively spliced exons, we compared the splicing of transcripts of the endogenous clathrin light chain B gene in these MEL cells. Exon 3 of this constitutively produced transcript is included only in neuronal cells (40). As can be seen in Figure 6, the neuronal rat PC12 cells exhibited mostly the non-neuronal pattern of exon 3 exclusion, but also produced easily detectable amounts of mRNA of the size predicted for exon 3 inclusion as well. The non-neuronal MEL $A1^{+}$ cell line contained no detectable molecules that include exon 3, but the MEL $A1^{-}$ derivative produced the same proportion of exon inclusion as the PC12 cells (Fig. 6). Thus, the hnRNP A1 deficiency of this MEL cell line affects at least one endogenous transcript as well as transgene transcripts.

An increase in intron size promotes proximal splicing in mutant apt transcripts

One way of explaining a general promotion of distal splicing by hnRNP A1 is to invoke a loss of flexibility of the RNA upon binding hnRNP A1. The resultant more rigid pre-mRNA would limit the apposition of 5' and 3' splice sites for small introns. An attractive feature of this model is that it explains the promotion of distal splicing irrespective of whether a choice is between alternative 5' or alternative 3' sites. In order to effect such loss of flexibility, one would imagine that hnRNP A1, along with other abundant hnRNPs, would bind along the length of an RNA. Although such ribonucleosomal structures can be produced in purified cell-free reconstitution experiments (41), it is not clear that they exist in continuous arrays in bulk nuclear RNA (42,43). However, it is possible that RNA is first and transiently organized in this manner as it is being transcribed, during which time splice site commitment is taking place. The effect of ASF/SF2 here would be to restore flexibility by replacing hnRNP A1 at a particular binding site, producing a hinge. A prediction of this model is that the effect of A1 may dissipate as the size of the intron increases, allowing the intron to double back on itself.

We tested the idea that increased intron size should diminish the A1-mediated exon-skipping by enlarging the size of apt intron 3 in the mutant XA57N gene. The size of this intron is 154 nt, considerably larger than the minimum size of ~80 nt required for efficient splicing (44). To reduce the chance that new sequence information would affect splice site choice, we expanded the intron by replicating a sequence already present. Three extra copies of a 90 nt sequence from this intron were inserted in tandem; as a result, the intron increased in size from 154 to 424 nt (Fig. 7A). When transfected into CHO cells, transcripts from this construct (pXA57NT) exhibited little exon skipping: exon 3 was spliced to the proximal exon 4 splice site 50 times more efficiently than when the smaller intron was present (compare Fig. 7B, lanes NT and N). This result is consistent with the idea that relatively small introns are required for hnRNP A1 to inhibit the proximal 3' splice site choice.

It is possible that the sequence change inherent in the 90 nt replication was responsible for the increase in proximal splicing, rather than the increase in intron size *per se*. Therefore, we expanded intron 4 to about the same size using two other sequences. Regions of ~370 nt from the introns of the human hprt and Rb genes were chosen that were devoid of sequences with a strong resemblance to a 5' or 3' splice site. As can be seen in Fig. 7B, distal splicing was greatly reduced when intron 4 was expanded with these sequences as well. The Rb sequence was less effective in promoting proximal splicing than either the replicated endogenous apt sequence or the hprt sequence. Perhaps secondary and higher order structures are acting to interfere with the proper juxtaposition of the intron 4 ends, with greater length not guaranteeing adequate flexibility.

DISCUSSION

ASF/SF2 and hnRNP A1 promote proximal and distal 3' splice site choices respectively

The effect of ASF/SF2 on promoting proximal and hnRNP A1 on promoting distal 5' splice site choices has been well-documented in *in vitro* experiments (3-5,8,9,11,45,46) and in some cases has been confirmed by *in vivo* experiments (7,30,47). Effects similar

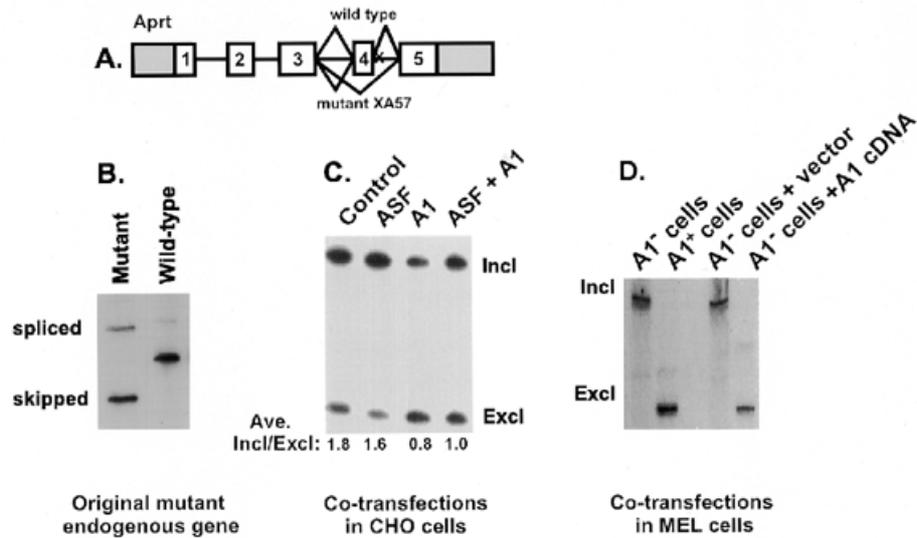


Figure 5. The effect of ASF/SF2 and hnRNP A1 on the inclusion of a mutant aprt exon 4. (A) Map of the mutant aprt gene in CHO XA57, in which the universally conserved GT dinucleotide at the 5' splice site of intron 4 (37) has been altered, and a diagram of the resulting splicing pattern. PhosphorImages of RT-PCR products are shown in (B) and (C). (B) Splicing of the endogenous aprt transcript in CHO XA57 cells yielded both inclusion of exon 4 (Incl, spliced with intron-retention) and exclusion (Excl, exon-skipping). (C) Splicing of aprt gene transcripts in CHO U1S cells (an aprt deletion mutant) transfected with the mutant aprt XA57N gene plus ASF/SF2 and/or hnRNP A1 genes. (D) Promotion of distal splicing in mutant aprt transcripts by hnRNP A1 in hnRNP A1-deficient MEL cells. HnRNP A1-proficient or -deficient MEL cells were transfected with the aprt mutant XA57N gene with or without the hnRNP A1 gene, and RNA was analyzed by RT-PCR. In this experiment, the hnRNP A1 state of the cells resulted in a nearly all-or-none difference in aprt exon 4 inclusion, and this state was reversed by co-transfection with an hnRNP A1 plasmid. Incl, included aprt exon 4 (and intron 4); Excl, excluded exon 4. The control vector used in lane 3 was the mammalian expression plasmid vector (pCG) used for cloning the hnRNP A1 cDNA. The average ratio of the two types of mRNA is indicated below each lane. Transfections of each combination of plasmid and recipient were performed at least twice; the average range or SEM was <20% of the mean.

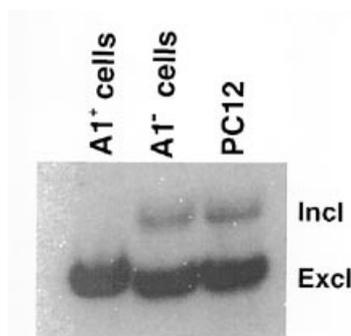


Figure 6. Alternative splicing of clathrin light chain B transcripts in MEL hnRNP A1-deficient cells. A PhosphorImage of the RT-PCR analysis is shown. A1⁺ (MEL DP227-17) cells; A1⁻ (MEL CB3C7) cells; PC12, rat pheochromocarcinoma cells. Incl, splicing in of the brain-specific exon EN; Excl, exclusion of brain-specific exon EN.

to those of ASF/SF2 have also been reported for SC35 (11,48), but these two SR proteins do not always behave identically (3,4,8,45,48). Here we have shown that ASF/SF2 and hnRNP A1 can influence 3' splice site selection *in vivo* in a manner similar to that described for 5' splice site selection. We avoided ambiguity between 5' and 3' targets by studying the alternative splicing of a terminal exon. The ASF/SF2 results are in agreement with the cell-free experiments of Fu *et al.* (11), in which SR proteins were found to promote 3' proximal splicing in β -globin gene constructs.

However, in that study hnRNP A1 did not counteract ASF/SF2-stimulated 3' proximal splicing, although it did so when alternative 5' sites were examined. In contrast, we found such counteractive effects of hnRNP A1 with both calcitonin/CGRP and aprt transcripts. The data of Mayeda *et al.* (49) were also suggestive of an effect of hnRNP A1 at a 3' splice site: hnRNP A1 promoted exon skipping in a 3-exon globin transcript, but this effect was negated by improvement of the 3' splice site upstream of the central exon. In a perhaps related case, ASF/SF2 stimulated the removal of the last intron of the growth hormone gene, and this stimulation was counteracted by hnRNP A1 (8). This effect probably involved the 3' splice site, as the *cis*-acting sequences targeted by ASF/SF2 in this system are located downstream of the 3' splice site in the last exon. The different results with the β -globin transcripts cited above may reflect substrate specificity for hnRNP A1, differences in the assay method used (cell-free splicing versus transient transfection) or relatively high endogenous levels of hnRNP A1 in the cell-free extracts.

HnRNP A1 is very abundant within the nucleus, and is rather difficult to overexpress for this reason (7; data not shown). The MEL hnRNP A1⁻ deficient cell line CB3C7 gave us the opportunity to test the effects of hnRNP A1 activity in the absence of this background. The fact that CB3C7 cells can survive without hnRNP A1 (28) suggests that there are redundant hnRNP A1-like activities in the cell. Although these redundant activities allow cell viability, they may be distinct from hnRNP A1 in their influence on calcitonin/CGRP and aprt transcript splicing.

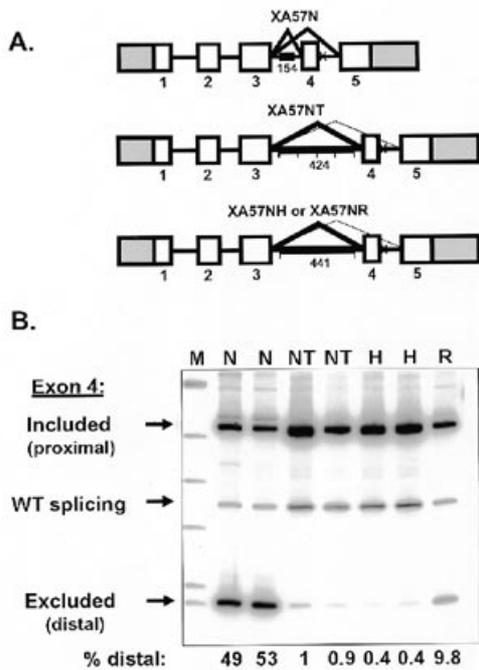


Figure 7. The effect of intron 3 expansion on aprt exon 4 inclusion. Cells of the CHO aprt deletion mutant U1S were transfected with plasmids bearing either the XA57N mutant aprt gene (intron 3 = 154 nt) or variants with insertions in intron 3. RNA was extracted 48 h post-transfection and analyzed for aprt mRNA by RT-PCR. (A) Structures and principal splicing patterns of the aprt genes used. The Xs indicate point mutations that debilitate the intron 4 5' splice site. The size of intron 3 is indicated. Other introns are not drawn to scale. (B) A PhosphorImage of the alternatively spliced aprt mRNA. N, no insertion, original 154 nt intron 3; NT, expansion to 4 tandem copies of an endogenous 90 nt intron 3 sequence, intron 3 is 424 nt; H, insertion of a 369 nt sequence from hprt intron 1, intron 3 is 440 nt; R, insertion of a 368 nt sequence from Rb intron 23, intron 3 is 441 nt; M, markers, ϕ HinI fragments. Each lane represents an independent transfection experiment. The size of the minor central band is consistent with some residual wild-type splicing (with intron 4 removal) taking place. The numbers below each lane indicate the relative amount of distal splice site choice (the small amount of wild-type spliced mRNA was not included in this calculation).

Sensing proximity and distance for splice site choice

The general conclusion that emerges from these and previous studies is that SR proteins promote proximal splicing and hnRNP A1 promotes distal splicing regardless of whether the proximal versus distal choice involves two 5' or two 3' splice sites. That it is the relative position of the alternative splice sites that is determinant, and not their sequence context, has been shown by experiments in which proximal and distal sites were swapped or when the same site was made proximal or distal relative to the other (3–5,9,50). How these proteins determine whether a site is proximal or distal is a basic question that remains unanswered. One possible explanation is that they recognize specific sequence elements but the proximal/distal dichotomy is coincidental, as the number of different substrates studied have been few as yet. Both ASF/SF2 and hnRNP A1 can bind to specific RNA sequences. ASF/SF2 contains RNA recognition motifs (RRMs), and has been shown to bind specific naturally occurring RNA sequences (8,45,46), where this interaction can activate a nearby splice site

(7,30,51). ASF/SF2 binding sequences have also been defined by molecular selection (52,53). SR proteins can also interact directly with other splicing components to influence splice site choice (12,16,54). The SR protein SRp20 has been implicated in activation of the polyadenylation site downstream of calcitonin exon 4 (55); however, earlier evidence argued against a regulatory role for this poly(A) site in the alternative splicing of calcitonin/CGRP transcripts (56). hnRNP A1 has been found to bind to the polypyrimidine tract region located upstream of 3' splice sites (15,16) and hnRNP A1 tight-binding RNA sequences have been isolated from synthetic libraries using molecular selection techniques (57,58). hnRNP A1 has RNA-annealing activity, and can bind to U2 snRNP (59,60). Indeed, Chabot *et al.* have shown an example in which binding of hnRNP A1 to a specific sequence influenced alternative 5' splice site choice (47). Thus, each protein could be binding close to a particular splice site, be it 5' or 3', proximal or distal, and could act to promote splicing at that site via the recruitment of additional general splicing factors. The effect of such binding may be most apparent through the enhancement of intrinsically weak splice sites, typically found at alternatively splicing sites. In support of this idea is the finding that hnRNP A1 promotes proximal rather than distal splicing of Ich-1 transcripts, a reverse of the usual situation (61). In this exceptional case, hnRNP A1 binding may be activating a specific proximal splice site. Two arguments against the idea that hnRNP A1 generally acts through specific binding sites are: (i) the demonstration of promiscuous binding by hnRNP A1 that depends more on base composition than sequence (13,14); and (ii) the fact that the normally constitutively spliced transcripts of the aprt and β -globin genes are similarly affected by these two proteins when their splice sites are mutated or placed in different contexts. It is difficult to see why high-affinity *cis*-targets of ASF/SF2 and hnRNP A1 should have evolved in these two genes, the normal transcripts of which have apparently strong splice sites. Furthermore, neither of the hexameric sequences implicated in strong specific hnRNP A1 binding (47,57) are present in the aprt transcript studied here.

Alternatively, as proposed by Eperon *et al.* (10), ASF/SF2 could produce a bias in favor of proximal splicing by acting as a general splicing enhancer and hnRNP A1 could promote distal splicing if it acted as a general splicing inhibitor. These authors found that ASF/SF2 could stimulate U1 snRNP binding to 5' splice sites *in vitro*; in the presence of a strong distal and a weak proximal 5' site, the addition of an ASF/SF2 extract resulted in U1 snRNP binding to both sites, with the proximal site favored for splicing, presumably due to its greater effective concentration relative to the 3' site. The effect of hnRNP A1 on promoting distal splicing would fit this explanation if a splicing inhibitory role could be ascribed to this factor, with weak proximal sites being more easily inhibited. Whereas hnRNP A1 has been sometimes described as an essential splicing factor (62,63), under some circumstances hnRNP A1 can inhibit splicing, e.g., when it is preincubated with pre-mRNA (5); and it can compete with snRNPs for binding to RNA (16). A weakness of this model is its requirement that the proximal site never be stronger than the distal site.

We offer a third model for the inhibition of proximal splicing by hnRNP A1. This model postulates a topological constraint imposed by the non-specific binding of hnRNP A1, perhaps as part of an hnRNP core particle. The resulting higher order structure may reduce the flexibility needed to juxtapose the 5' and

3' splice sites, at least for smaller introns. The role of ASF/SF2 may be to displace some hnRNP A1 molecules, thus acting as a hinge. The results of the intron 4 expansion in the *aprt* gene are in accord with this idea: proximal splicing of the 154 nt intron 4 was inhibited by hnRNP A1 and promoted by expansion of the intron to ~400 nt. That hnRNP A1 is acting via non-specific binding is also consistent with the data of Williams and colleagues, who found that hnRNP A1 bound with similar affinities to many disparate sequences, the SELEX winner being the sole exception (13,14). However, it is difficult to see how this model would apply to large introns.

Splice site choices and exon definition

The exon definition model of splice site recognition proposes that the splice sites at each end of an internal exon cooperate to establish the splicing of the adjacent introns (39). By stimulating or inhibiting splicing at a proximal 5' or 3' site, ASF/SF2 and hnRNP A1 would be expected to promote exon inclusion or exon skipping, respectively, in transcripts containing two or more introns. In cell-free splicing experiments, Mayeda *et al.* (49) showed this to be the case for a naturally weak alternatively spliced internal exon (β -tropomyosin exon 6) and for the central exon of the human β -globin gene when it was shortened by internal deletions. Here we have extended such experiments to *in vivo* splicing and to two additional genes, the alternatively spliced calcitonin exon 4 and the normally constitutively spliced exon 4 of the *aprt* gene. In both cases, overproduction of ASF/SF2 promoted exon inclusion whereas overproduction of hnRNP A1 promoted exon skipping. The case of *aprt* exon 4 is especially interesting, as the 3' splice site upstream of this exon was weakened indirectly by interference with exon definition, i.e., by mutating the splice site at the 5' site downstream of the exon. Compelling evidence for the exon definition model lies in the observations that a mutation in either the 3' or the 5' splice site that borders an internal exon usually leads to exon skipping; i.e., splicing to the remaining unaffected site or to a cryptic site is exceptional (64,65). Mutation at the 5' splice site downstream of exon 4 in the *aprt* gene represents one such exception, in that splicing at the upstream 3' site was reduced but not eliminated. As a result of this partial failure of exon definition, molecules retaining intron 4 were produced about half the time in CHO cells. Removal of hnRNP A1 from this system (by transfection into hnRNP A1-deficient cells) increased exon 4 inclusion, allowing much more use of a 3' site that was no longer well-defined by a downstream exon. Restoration of hnRNP A1 by co-transfection reproduced exon 4 skipping, confirming that it was the deficiency in this protein and not some other unrecognized factor that was responsible for these splicing phenotypes. This result implies that hnRNP A1 can play a role in exon definition within a normally constitutively spliced transcript, by inhibiting splicing at a 3' site when a downstream 5' site is no longer present. The *in vivo* overproduction of an analogue of hnRNP A1 in *Drosophila* caused the skipping of the two internal exons of the dopa decarboxylase gene (66), in support of a role for this similar protein in exon definition. As noted above, hnRNP A1 promoted the skipping of an abbreviated central exon in a β -globin gene construct; expansion of this exon eliminated the influence of hnRNP A1 (49). In that case, according to the topological constraint model described above, hnRNP A1 may have been acting to prevent the juxtaposition of

the two ends of the short exon, a bridging that may be necessary for exon definition (67).

Our results also relate to the interplay between exon definition and intron definition in the commitment of splice sites. Sterner *et al.* (68) have proposed that exon definition comes into play only when introns are large; when adjacent introns are sufficiently small, intron definition occurs and exon definition is not required. It might be expected from this idea that the expansion of intron 3 in the *aprt* gene would result in a greater reliance on exon definition, which in the case of the XA57 mutant would be manifested by the skipping of exon 4 (with its mutated downstream 5' splice site). We obtained the opposite result: the expansion of intron 3 from 154 to >400 nt greatly reduced exon 4 skipping; i.e., with the larger intron, intron definition prevailed over exon definition. The sensitivity of splicing choices to higher order structures produced by the inevitable sequence changes accompanying insertions or deletions may confound conclusions about the effects of size.

HnRNP A1 and alternative splicing

The fact that the splicing of calcitonin exon 4 could be influenced by the ratio of ASF/SF2 to hnRNP A1 lends support to the idea that the ratio of constitutive splicing factors could be determining tissue-specific splicing patterns. In an effort to find examples of differential exon inclusion among the endogenous mRNA molecules in the hnRNP A1-deficient cells, we examined *src*, α -tropomyosin and clathrin light chain B mRNA by RT-PCR. Each of these transcripts contains an exon that is included only in neuronal cells (40). No differences between the hnRNP A1⁺ and hnRNP A1-MEL cell lines were found for the first two species (data not shown). However, the hnRNP A1⁻ MEL cells, like neuronally-derived rat PC12 cells, did produce as a minor species mRNA of the size predicted for the inclusion of clathrin light chain B exon 3. The hnRNP A1⁺ cells failed to demonstrate this exon inclusion. Thus, endogenous substrates can also be subject to increased exon inclusion in this hnRNP A1⁻ cell line. Obviously, the amount of missplicing among endogenous transcripts must be small, as these cells remain viable. Indeed, a survey of different tissues has revealed that hnRNP A1 levels are quite variable (69), and the ratio of ASF/SF2 to hnRNP A1 is more variable yet (70). Perhaps exon definition is mediated by a family of redundant splicing factors whose collective activities ensure accurate splicing; only a minority of exons would be affected by a deficiency in any one particular factor. In this view, tissue-specific alternative splicing could be effected by quite different ratios between specific combinations of constitutive splicing factors without there being widespread splicing differences.

The mechanism of hnRNP A1 action in exon definition is likely related to its function in repressing proximal splice site choice. Exon definition may be a major factor in the distinction between true and false splice sites in large transcripts. Factors like hnRNP A1 may act by preventing the inclusion of false exons (causing them to be appropriately skipped) rather than by directly pinpointing the true splice sites.

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