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Pathways for selection of 5′ splice sites by U1 snRNPs and SF2/ASF

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We have used protection against ribonuclease H to investigate the mechanisms by which U1 small nuclear ribonucleoprotein particles (snRNPs) determine the use of two alternative 5′ splice sites. The initial binding of U1 snRNPs to alternative consensus splice sites was indiscriminate, and on a high proportion of pre-mRNA molecules both sites were occupied simultaneously. When the sites were close, this inhibited splicing. We propose that double occupancy leads to the use of the downstream site for splicing and that this is the cause of the proximity effect seen with strong alternative splice sites. This model predicts that splicing to an upstream site of any strength requires a low affinity of U1 snRNPs for the downstream site. This prediction was tested both by cleaving the 5′ end of U1 snRNA and by altering the sequence of the downstream site of an adenovirus E1A gene. The enhancement of downstream 5′ splice site use by splicing factor SF2/ASF appears to be mediated by an increase in the strength of U1 snRNP binding to all sites indiscriminately.

Key words: ASF/ribonuclease H protection/SF2/splice site selection/U1 snRNPs

Introduction

The sequences used as 5′ splice sites are so diverse (Mount, 1982; Ohshima and Gotoh, 1987; Shapiro and Senapathy, 1987; Jacob and Gallinaro, 1989) that candidates would be found at ~0.5–1% of all positions in a random RNA sequence. Given that the modal mean length for mammalian introns is ~1000 nucleotides (nt), non-terminal exons being ~150 nt (Hawkins, 1988; Traut, 1988), the splicing of most introns would involve a choice between multiple candidate sites. Although this choice may be restricted in many cases by process of exon definition (Robberson *et al.*, 1990), in which the closest 3′ and 5′ splice sites would be paired, the existence of both natural and artificial examples of alternative 5′ splice sites and of cryptic 5′ splice sites (reviewed by Rogers, 1985) argues that the splicing mechanism is flexible and must, therefore, discriminate between candidate sites.

The use of alternative 5′ splice sites is dependent on the sequences of the sites (Eperon *et al.*, 1986; Zhuang and Weiner, 1986; Fu and Manley, 1987; Zhuang *et al.*, 1987; Mayeda and Ohshima, 1988). Positions close to the 5′ splice site appear to be recognized by base pairing with the 5′ end of U1 snRNA (Zhuang and Weiner, 1986; Séraphin *et al.*, 1988; Siliciano and Guthrie, 1988; Nandabalan *et al.*, 1993; Séraphin and Kandels-Lewis, 1993). However, the mechanisms by which U1 small nuclear ribonucleoprotein particles (snRNPs) affect selection are unknown. The obvious possibility is that the affinity of U1 snRNPs for 5′ splice sites determines the outcome, either because binding to the best site prevents binding to alternative sites or because either site can be bound, but dissociation is so rapid that there is on average only one site bound at any time and the sites are used according to the relative probabilities that they are occupied (Eperon *et al.*, 1986).

Neither of these schemes is supported by the evidence so far. U1 snRNPs bind specifically to 5′ splice sites (Mount *et al.*, 1983), but the affinities for different sequences do not correlate with splicing preferences (Chabot and Steitz, 1987a; Tatei *et al.*, 1987; Mayeda and Ohshima, 1988; Nelson and Green, 1988). It has not been possible to show whether the binding of U1 snRNPs discriminates between authentic and cryptic sites, because initial binding to the latter sites is undetectable even when they are used for splicing (Chabot and Steitz, 1987a; Nelson and Green, 1990). With alternative strong 5′ splice site sequences (from rabbit β-globin IVS-2 and a consensus sequence), ribonuclease (RNase) T1-immunoprecipitation studies found no discrimination in binding to match that of splicing (Nelson and Green, 1988). When these sites were only nine nucleotides apart, they were protected simultaneously, which could arise either from steric interference with RNase T1 cleavage or from binding to both sites. The latter interpretation implied that multiple candidate sites on a pre-mRNA might be occupied simultaneously (Nelson and Green, 1988), although this could not be tested with RNase T1-immunoprecipitation assays, and it raised further questions about the stage of the splicing reaction at which the 5′ splice site was selected and the role of U1 base pairing.

We report here that U1 snRNPs do bind to both of two alternative consensus 5′ splice site sequences on a single pre-mRNA molecule, but that with rabbit β-globin IVS-2 sites only one site develops the interactions with 3′ components that confer protection against RNase H. We propose and test a model for the role of U1 snRNPs in splice site selection which explains the proximity effect for splice site selection *in vitro* (Reed and Maniatis, 1986), predicts the situations in which it will be seen and suggests one route by which factors such as splicing factor SF2/ASF affect the selection of alternative 5′ splice sites (Krainer *et al.*, 1990a; Ge and Manley, 1990; Ge *et al.*, 1991; Mayeda and Krainer, 1992).

Results

Our principal objective was to determine whether both of two alternative 5′ splice sites on a single pre-mRNA

molecule are occupied by U1 snRNPs before spliceosomes assemble. Simultaneous occupancy would eliminate models in which U1 attachment *per se* determined the site to be incorporated into the active spliceosome. To determine the occupancy of the two sites, we tested whether they were protected against oligonucleotide-direct cleavage by RNase H. The fragments produced by this assay depend on the sites protected, as shown in Figure 1. The pre-mRNA substrates used were derived from rabbit β-globin IVS-2 by inserting an extra 5′ splice site sequence (consensus or β-globin IVS-2) into the *Bam*HI site of the upstream exon (Eperon *et al.*, 1986); spacer sequences were then inserted between the two alternative 5′ splice sites which, when the sites were >40 nt apart, caused splicing to occur almost exclusively via the downstream natural site (described in Cunningham *et al.*, 1991). The substrates are named according to the sequence of the respective 5′ splice sites and the distance in nucleotides between them, i.e. **G25G**, **C175G**, etc. In some cases, the natural splice site was mutated to the consensus sequence too, as in **C24C** and **C174C**.

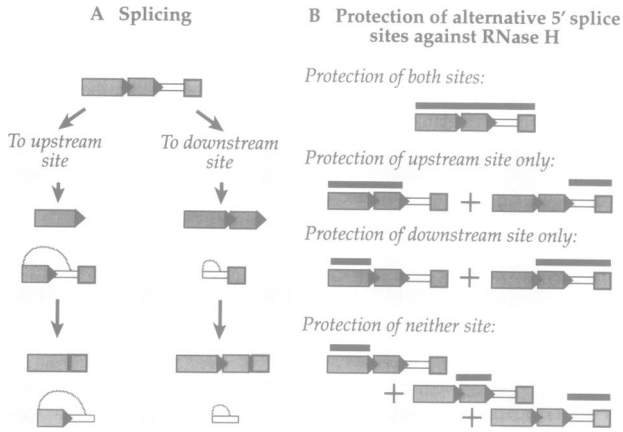

Fig. 1. Products of splicing (A) and RNase H protection assays (B) from pre-mRNA substrates with two alternative 5′ splice sites. (**A**) The β-globin and adenovirus E1a substrates contained two alternative 5′ splice sites, shown as black arrowheads. In the β-globin substrates, these sites were consensus or globin 5′ splice sites and the distance between them varied from 24 to 176 nt; in the E1a case, the sites were the natural 13S and 12S sequences, swapped around in some cases, separated by 138 nt. The arrows show the two successive steps of splicing when either 5′ splice site is used. The stippled line represents the 2′−5′ bond formed in the lariats. (**B**) After incubation of the splicing reactions for various times, deoxyoligonucleotides complementary to both 5′ splice sites were added simultaneously. In the presence of RNase H, unprotected 5′ splice sites were cleaved. The diagrams show the RNA fragments (heavy lines above the diagrams of the pre-mRNA) produced when a pre-mRNA is protected at both, one or neither of the 5′ splice sites. Where there was protection, each possible combination produced one fragment that was unique.

Simultaneous binding of U1 snRNPs to alternative consensus 5′ splice sites before spliceosome assembly

Pre-mRNA substrates were incubated with nuclear extracts at 30°C for various times before the oligonucleotides directing RNase H cleavage were added. Early experiments showed that consensus 5′ splice sites (the upstream site in **C175G** and both sites of **C24C**) began to be protected before globin 5′ splice sites (Figure 2 and Table I). This suggested that the assay was able to detect early interactions at consensus 5′ splice sites. In the case of **C175G**, the site bound was an upstream site not used for splicing under normal conditions, and with **C24C** both sites were bound simultaneously. We excluded the possibility that the apparent protection was an artefact of inefficient cleavage in the presence of the extract by showing that the protection was

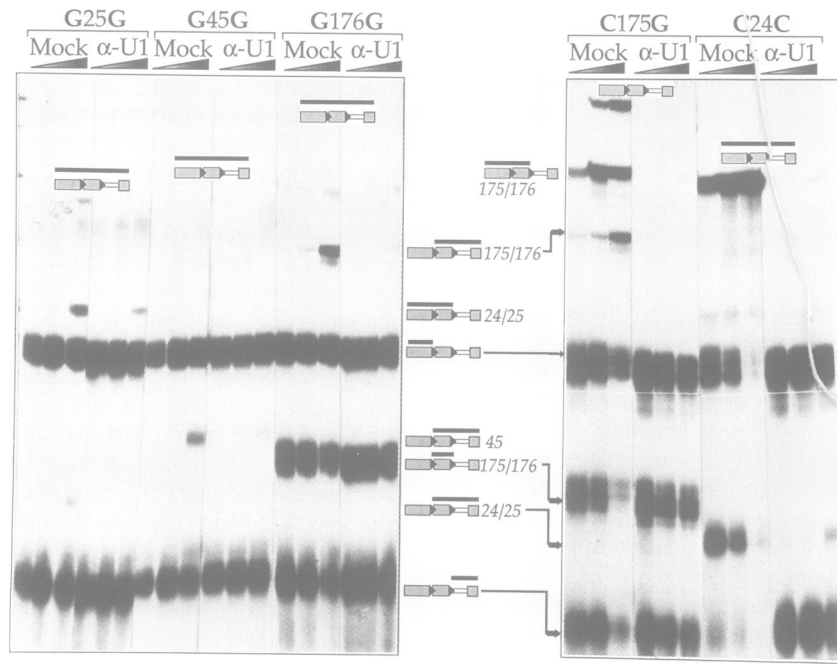

Fig. 2. Time courses of U1-dependent protection of alternative globin and consensus 5′ splice sites. Extracts were pre-treated with RNase H in the absence (Mock) or presence (α-U1) of oligonucleotide α-U1 5′. Pre-mRNA was incubated for 0, 3 or 20 min before challenge with oligonucleotides that direct RNase H cleavage to the 5′ splice sites. The times are represented by the bar of graduated thickness above successive lanes of the 5% polyacrylamide gel. The products of RNase H cleavage are shown by the bars in the diagrams alongside the autoradiograph; the diagrams of the fully protected molecules are just above the actual or expected positions of the corresponding bonds. In parallel reactions (not shown), branch site protection was also assayed at the same times and at 135 min; splicing efficiencies were measured from the latter reactions (Table II).

Table I. Relative levels of protection at alternative 5′ splice sites

Substrate	5′ splice sites protected	Time (min)			
		0	3	20	
G25G	Both	–	0.3	1.8	
	Upstream only	–	1.9	11	
	Downstream only	–	0.7	4.9	
G45G	Both	–	–	–	
	Upstream only	–	–	–	
	Downstream only	3.8	3.7	17	
G176G	Both	–	–	–	
	Upstream	–	0.2	2.1	
	Downstream only	–	2.3	34	
C24C	Both	50	80	95	
	Upstream only	3.2	2.2	1.5	
	Downstream only	31	9.4	1.7	
C175G	Both	0.6	4.9	17	
	Upstream only	7.1	28	27	
	Downstream only	2.7	5.0	18	
		0	5	10	15
C174C	Both	4.8	36	48	53
	Upstream only	3.7	6.5	6.3	6.5
	Downstream only	35	39	31	28

The figures show the proportion (in %) of molecules protected at one or both 5′ splice sites after various times of incubation. The levels of radioisotope in all of the bands visible in the mock lanes of Figures 2 and 5 (for **C174C**) were measured by a PhosphorImager and scintillation counting, respectively, and corrected values used for calculations of the relative numbers of molecules in each band within each track (see Materials and methods). For the comparison of downstream site protection at 20 min (values above) with the efficiency of splicing to that site (Table II), the level of fully protected precursor was included for substrate **C175G** because protection depended on spliceosome assembly at the downstream site and only the downstream site was used for splicing.

eliminated when the extract had been pre-incubated with an oligonucleotide complementary to the first 14 nt of U1 snRNA (Figure 2, lanes labelled α-U1). This treatment appeared to produce some degradation of all U1 snRNA molecules in the extract (see below) and it was included as a control in many of the subsequent experiments.

In view of the seemingly indiscriminate but U1-dependent protection of consensus 5′ splice sites, we sought to determine whether it arose from direct binding by U1 snRNPs (and any accessory factors), even in the absence of interactions with components recognizing 3′ splicing signals. Direct protection by U1 snRNPs was shown to be possible in principle by experiments in which we found that the incubation of purified U1 snRNPs (gift of Dr R.Lürhmann) with pre-mRNA in splicing buffer did provide protection (data not shown). If this happened in extracts, then protection would be seen in nuclear extracts in circumstances where U1 snRNPs would bind, but either commitment complexes or spliceosomes would not have formed. We tested this: (i) in the absence of splicing signals to the 3′ side of the 5′ splice sites, when the only factors known to associate specifically are the U1 snRNP and a 115 kDa protein (Bennett *et al.*, 1992); (ii) after removal of the 5′ end of U2 snRNA; (iii) at early time points before the formation of complexes at the branch site; and (iv) in the absence of exogenous Mg^{2+} and ATP (Mount *et al.*, 1983; Chabot and Steitz, 1987b; Michaud and Reed, 1991; Bennett *et al.*, 1992; Fu and Maniatis, 1992; Wassarman and Steitz, 1992; Wyatt *et al.*, 1992).

When the first condition was tested using truncated substrates lacking 3′ splicing signals, only the consensus 5′ splice sites were protected against RNase H cleavage (Figure 3); with substrates bearing two alternative consensus sites (**C24C** and **C174C**), most molecules were protected at both sites. Globin 5′ splice sites were not protected. Likewise, when U2 snRNA was cleaved, the protection after 20 min of consensus 5′ splice sites was unaffected, whereas the protection of globin 5′ splice sites was reduced on average by a factor of 4 (Figure 4). To test whether protection of 5′ splice sites preceded that at or near the branch site, the proportions of pre-mRNA protected at early times (Figure 5 shows **C174C**; other data not shown) were calculated relative to the values at 20 min. Consensus 5′ splice site protection preceded that at branch sites, which in turn preceded globin 5′ splice site protection. Branch site protection was largely dependent on intact U1 snRNA. A similar kinetic effect can be seen in Figure 6 where, in the presence of a nuclear extract that gave unusually low levels of protection in general (see below), molecules protected at both alternative consensus 5′ splice sites appeared in the mock-treated extracts before significant branch point protection began to increase. Finally, consensus 5′ splice sites were protected in the absence of Mg^{2+} and ATP (Figure 6). Analogous experiments using psoralen cross-linking showed that U1 snRNA binding to pre-mRNA containing either consensus or globin 5′ splice sites was almost eliminated by pre-treatment of the extract with oligonucleotide α-U1 5′, but it did not require either 3′ splicing signals or intact U2 snRNA and it developed more rapidly than the U2:pre-mRNA cross-links (data not shown). We conclude that the initial protection of consensus 5′ splice sites against RNase H cleavage in extracts is produced by the binding of U1 snRNPs, possibly in association with accessory factors, whereas the protection of globin 5′ splice sites requires interactions with 3′ components.

Where the substrate contained two consensus 5′ splice sites (**C24C** and **C174C**), the high levels of intact precursor under these conditions indicate that both sites in one pre-mRNA were bound by U1 snRNPs. The time courses showed that significant levels of RNase H-resistant precursor formed either without incubation at 30°C or before protection close to the branch site developed. Thus, on at least a proportion of substrates, both of two alternative consensus 5′ splice sites are occupied by U1 snRNPs before spliceosomes assemble.

Selective interactions with 3′ components

The protection of globin 5′ splice sites was shown above to be dependent on interactions with 3′ components, possibly formed when commitment or splicing complexes assemble. The inability of RNase H to detect earlier interactions could indicate either that there were none or that the probe oligonucleotides displace U1 snRNPs from sites with less potential than consensus sites for base pairing. The results from psoralen cross-linking (described above) supported the second interpretation.

With pre-mRNA substrates in which only the downstream globin 5′ splice site was used for splicing, this was the only

site protected; when two close sites were used (**G25G**), the substrates were protected at either site (Table I and II). The efficiency of splicing to the natural (downstream) β-globin splice site in **G25G**, **G45G**, **G176G** and **C175G** (Table II) showed a significant correlation with the level of protection of that site at 20 min (Table I), with a coefficient = 0.95

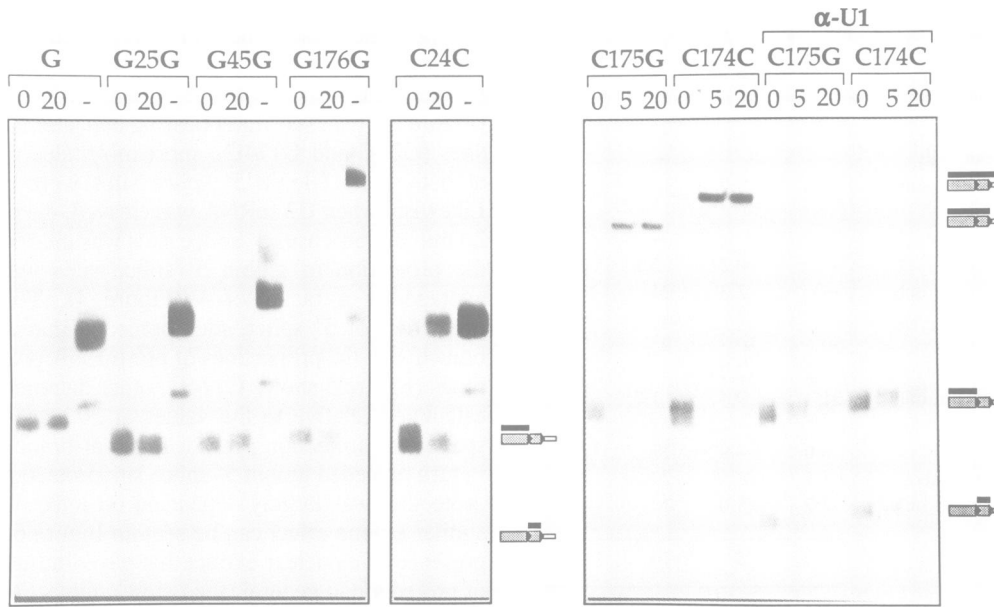

Fig. 3. Protection of alternative 5′ splice sites in the absence of 3′ splicing signals. Truncated versions of the β-globin substrates lacking the 3′ portion of the RNA were incubated in splicing reaction mixtures for 0, 5 or 20 min, as shown, before challenge with oligonucleotides that direct RNase H cleavage to the 5′ splice sites. The expected position of fully protected pre-mRNA can be deduced from the position of untreated precursor RNA in the adjacent lanes marked with a dash (left-hand panel) or from the diagrams beside the autoradiograph (right-hand panel). Pre-mRNA remained only in the samples of **C24C** and **C174C**, where it is the major product. The other products of RNase H cleavage are shown by the bars in the diagrams alongside the autoradiograph. The reactions labelled 'α-U1' were incubated in an extract which had been pre-incubated with an oligonucleotide directing cleavage of the 5′ end of U1 snRNA. Electrophoresis was on a 5% polyacrylamide gel.

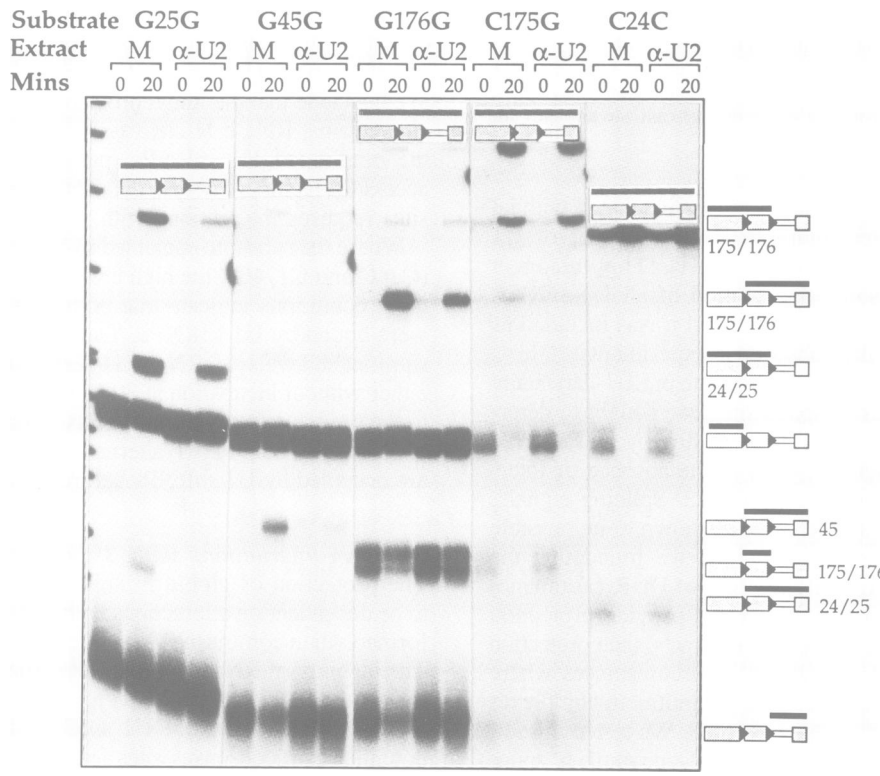

Fig. 4. Requirement of the 5′ end of U2 snRNA for 5′ splice site protection. Extracts were pre-treated with RNase H in the presence (α-U2) or absence (M, mock) of an oligonucleotide complementary to the 5′ end of U2 snRNA, and incubated with pre-mRNA for the times shown before oligonucleotide challenge. The 5′ splice site cleavage reactions, electrophoresis and labelling of the autoradiograph were as described for Figure 2. The incubation times are shown in minutes. The fully protected precursor bands are just below the diagrams in each set of lanes.

and $p(\varrho = 0) = 2.5\%$. We conclude that the 3′ interactions that stabilize binding to a globin 5′ splice site are likely to be or follow those that specify use of the site for splicing.

Inhibition of splicing by simultaneous U1 snRNP binding to close sites

Splicing is inhibited when two alternative 5′ splice sites are in close proximity, especially when both are consensus sites (Nelson and Green, 1988; Cunningham *et al.*, 1991; Tables I and II). We have interpreted this previously as suggestive evidence for simultaneous U1 snRNP binding and mutual interference (Cunningham *et al.*, 1991), and this interpretation is consistent with the very high level of protection of **C24C** described above (Table I). Cleavage of U1 snRNA in the extracts by RNase H reduced the efficiency of splicing of most substrates, but activated splicing of **C24C** and **G25G** (Table II). We attribute this activation to a reduction in the strength of binding of U1 to the 5′ splice sites, which reduces the interference. We have determined the extent of cleavage of U1 snRNA in these extracts by primer extension, and the results are highly reproducible: no full-length RNA is detectable and the 5′ termini of the RNA are distributed within the normal U1 snRNA sequence as follows: 10% at position 6, 3% at position 7, 6% at position 8, 8% at position 9 and 73% at position 10. Thus, only 13% of the U1 RNA is complementary to the 5′ exon and the conserved GU at the 5′ splice site, and none is complementary to the last three nucleotides of the 5′ splice site. To confirm the role of U1 snRNPs, pure intact U1 snRNPs were added back to a treated extract; the reproducible effect was to suppress again the splicing of **C24C** (Figure 7), whereas for **C174C** splicing was unaffected or stimulated slightly (data not shown). This phenomenon is most reasonably explained as the consequence of initial binding of U1 snRNPs to both 5′ splice sites on every molecule of pre-mRNA, the strong binding being sufficient for sites in close proximity to prevent the development of extended interactions with the substrate (Chabot and Steitz, 1987b). The less marked activation of **G25G** after U1 snRNA cleavage suggests that both sites are occupied initially, as with **C24C**, but that the weaker binding results in reduced inhibition.

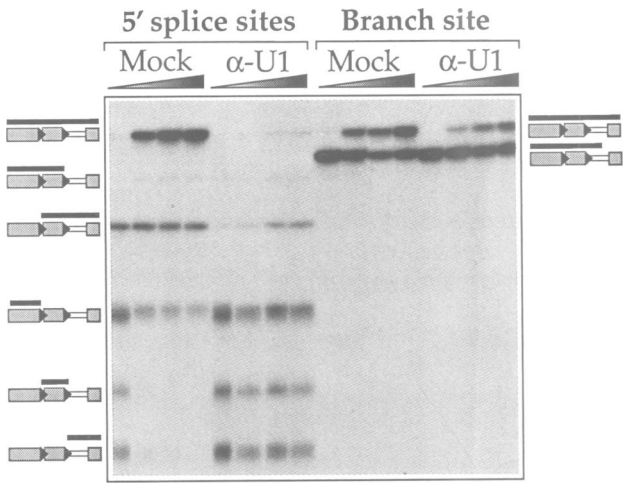

Fig. 5. Time courses for U1-dependent protection of alternative consensus 5′ splice sites and the branch site of **C174C**. Extracts were pre-treated with RNase H in the presence of a control oligonucleotide (Mock) or oligonucleotide α-U1 5′ (α-U1). **C174C** pre-mRNA was incubated for 0, 5, 10 or 15 min before challenge with the oligonucleotides directing RNase H cleavage to either the 5′ splice sites or a region upstream of the branch site. The time points are in successive lanes of the 6% polyacrylamide gel, underneath the bar of graduated thickness. The products of RNase H cleavage are shown by the bars in the diagrams alongside the autoradiograph.

A model for alternative 5′ splice site selection in vitro

Except for those substrates in which the alternative splice sites are within 45 nt of each other, and cause mutual interference, splicing uses predominantly the downstream site in our normal extracts and, with **GXG** substrates, *in vivo* (Cunningham *et al.*, 1991; Table II). However, the upstream site was also clearly shown to be occupied initially in **C174C** and **C175G**. Why, then, were the upstream sites not used for splicing? Our results with consensus sites suggest that, whenever the upstream site is bound, the downstream site is bound as well. If so, then a strong preference for the closest occupied 5′ splice site by the components associated with the 3′ splice site would lead to exclusive use of the downstream site. If this explanation is correct, then the observed patterns of splicing suggest that, for consensus and globin 5′ splice sites, both sites should be occupied on almost

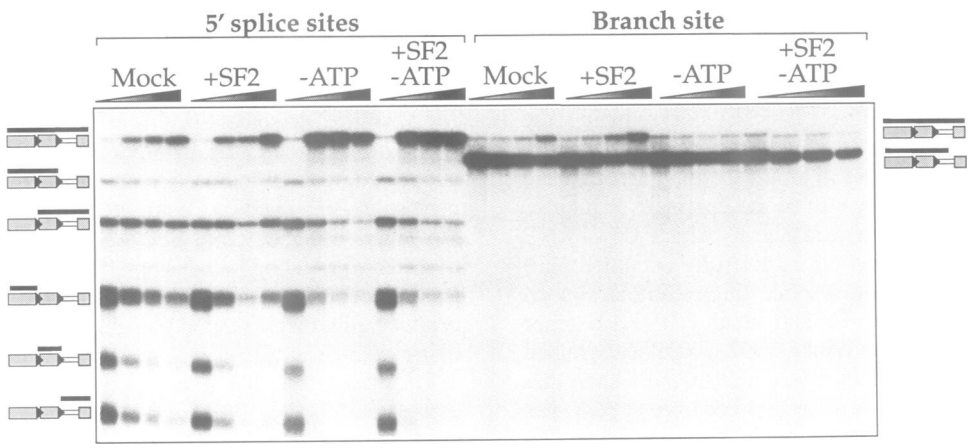

Fig. 6. Effects of SF2/ASF and absence of Mg^{2+} and ATP on protection of alternative consensus 5′ splice sites and a region upstream of the branch site. The substrate was **C174C** and the extract contained potassium glutamate instead of potassium chloride. Water replaced the $MgCl_2$, ATP and phosphocreatine in the appropriate reaction mixtures ('−ATP'). All the reactions were pre-incubated at 30°C before SF2/ASF or buffer D (Mock reactions) and the pre-mRNA were added. The incubation times, protection assays and electrophoresis were as described in Figure 5.

Table II. Efficiencies of splicing

Extract	G25G	G45G	G175G	C24C	C175G
Mock	7.9:28	0:53	0:62	0:0	0:67
α-U1	31:26	5.0:38*	0:43	7.2:48	6.7:22

The figures correspond to the proportions (%) of pre-mRNA spliced to the upstream and downstream 5′ splice sites. The splicing intermediates and products in 135 min reactions parallel to the 5′ splice site protection experiments in Figure 2 were analysed by gel electrophoresis and the yields of the various bands quantified. Branch site protection was analysed in the same reactions, but it did not prevent calculations of the levels of the various forms of RNA. The 5′ exon splicing intermediates were included with the mRNA products for calculations of the proportion of molecules in each lane which remained unspliced or had spliced to either the upstream or downstream sites. The proportions of molecules which had spliced to the two sites are shown separated by a colon.
*An additional 11% spliced after α-U1 treatment to a cryptic site in between the normal sites.

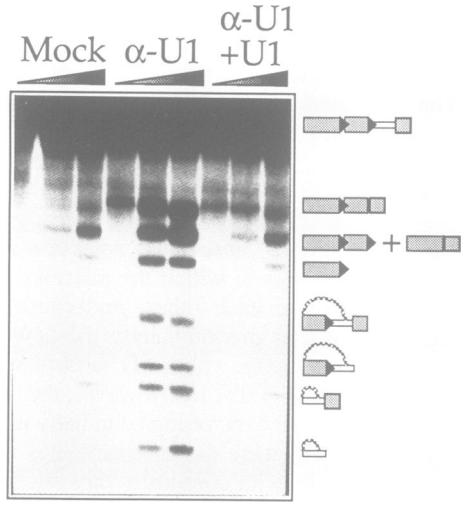

Fig. 7. Inhibition of splicing from close alternative 5′ splice sites by U1 snRNPs. Pre-mRNA C24C was incubated for increasing times (0, 1 and 2.5 h, shown by the bar above each set of lanes) in reaction mixtures pre-treated with a control oligonucleotide ('Mock') or oligonucleotide α-U1 5′ (α-U1), followed by addition of buffer D or purified U1 snRNPs (see Materials and methods). Electrophoresis of the reactions was on a 6% polyacrylamide gel.

every pre-mRNA molecule in the normal splicing extracts. The scheme is shown in Figure 8 and is discussed below.

Three predictions can be made: (i) either weakening the interaction of U1 snRNPs with the 5′ splice site, by removing the 5′-most nucleotides of U1 snRNA, or reducing the concentration of U1 snRNPs, should result in activation of the upstream site; (ii) the use of an upstream alternative site will depend, not on the strength of the upstream sequence, but on providing a downstream site to which U1 snRNP binding is so weak that the site is not fully occupied; (iii) the effects of factors that alter splice site preferences (such as SF2/ASF or hnRNP A1: Ge and Manley, 1990; Krainer *et al.*, 1990a; Mayeda and Krainer, 1992) could be explained by effects on the overall affinity of U1 snRNPs such that the proportion of pre-mRNA molecules occupied at both sites is altered. This last suggestion avoids the need to invoke selective or polar properties of the factors themselves.

The effect of reducing the potential base pairing between U1 snRNA and the splice site (prediction i) can be seen in Table II, where there is a clear increase in the relative proportion of splicing to the upstream site. This effect has been reproduced in a number of experiments with substrates of the form CXG. The remaining predictions were examined as described below.

Selection of adenovirus E1a alternative 5′ splice sites

The model described above was based on results with β-globin-based transcripts. In order to test the predictions about the use of an upstream alternative site in a different system, we used a modified adenovirus 5 E1a gene. A pre-mRNA was produced which was very similar in general organization to the globin series shown in Figure 1. An exon of 201 nt preceded the 12S 5′ splice site (the sequence of which we designate as sequence 1), which was 138 nt upstream of the alternative 13S 5′ splice site (sequence 2); a 3′ exon of 44 nt included a 3′ hairpin structure. As expected (Schmitt *et al.*, 1987), splicing used only the 13S site (Figure 9, substrate E1a 1,2). Although both 5′ splice site sequences could form base pairs of equal strength with U1 snRNA, we had established previously that, *in vitro*, splice site sequence 2 had an intrinsic strength equal to that of the globin sequence, whereas sequence 1 was extremely weak (Eperon *et al.*, 1986; Lear *et al.*, 1990). The nine nucleotide consensus regions of these sites were exchanged and the product pre-mRNA named E1a 2,1. This substrate spliced to both sites (Figure 9); laser densitometry showed that only 23% of the 5′ exon intermediates had used the downstream 5′ splice site. Addition of purified splicing factor SF2/ASF stimulated preferentially the downstream site, use of which rose to 51% (Figure 9). In contrast, a mutant with identical strong sequences at both sites (E1a 2,2) used only the downstream site. These results agree exactly with the first prediction made above.

RNase H protection assays showed that the patterns of protection of the alternative E1a 5′ splice sites were consistent with the inferred strength of U1 snRNP binding to the two sequences: sequence 1 was never protected, even when it was used for splicing (E1a 2,1), whereas sequence 2 was protected indiscriminately, and protection did not require intact U2 snRNA or 3′ splicing signals (data not shown). With substrate E1a 2,2, a significant proportion of pre-mRNA molecules were protected at both 5′ splice sites.

Modulation of alternative splicing by SF2/ASF

SF2/ASF is an essential splicing factor that, at high concentrations, causes alternative 5′ splice site selection to shift in favour of downstream sites (Krainer *et al.*, 1990a,b; Ge and Manley, 1990; Ge *et al.*, 1991). According to our model for 5′ splice site selection, this effect could be the result of a general increase in the affinity of U1 snRNPs for 5′ splice sites, leading to an increase in the proportion of pre-mRNA molecules occupied at both alternative 5′ splice sites. However, if the action of SF2/ASF was intrinsically selective then, depending on the step affected, the shift in splicing preferences caused by SF2/ASF would be expected to produce either a corresponding shift in the binding detected or no change at all.

The effect of SF2/ASF addition on binding to alternative consensus 5′ splice sites can be seen in Figure 6 where, as predicted, the level of C174C precursor protected at both sites rose (Table III). The level of protection near the branch site also rose. SF2/ASF alone in the absence of extract did not cause protection at these sites (not shown). The extract in this case was different from the others used in this work

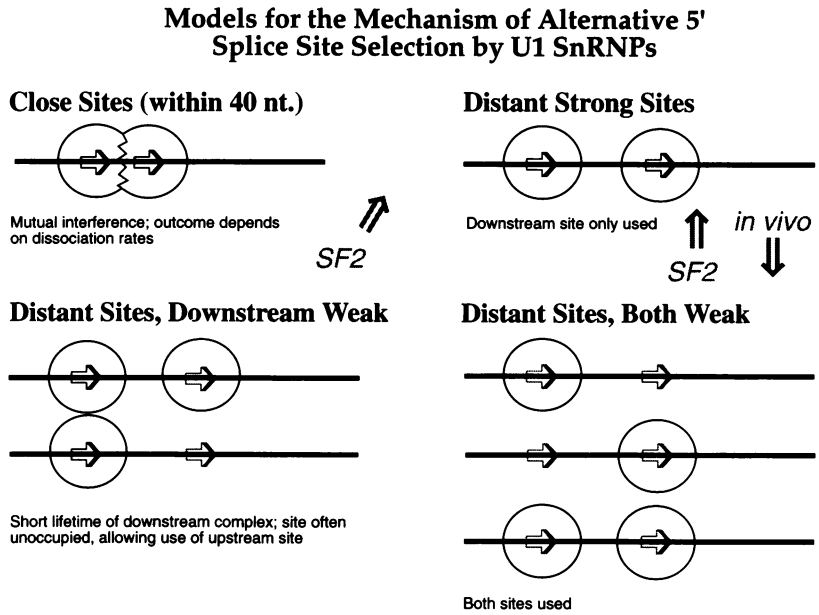

Fig. 8. Models for alternative 5′ splice site selection mediated by U1 snRNP binding. The pre-mRNA is shown as a solid line (5′ on the left), candidate 5′ splice sites as closed arrows and U1 snRNPs with accessory factors as circles. Grey arrows represent weak 5′ splice sites, e.g. sequences which do not perform well in a *cis*-competition assay (Eperon *et al.*, 1986; Lear *et al.*, 1990). A pre-mRNA with two close strong 5′ splice sites will be impeded in splicing by simultaneous occupation and mutual interference (top left). With two strong sites >40 nt apart (top right), we propose that both sites are occupied on each molecule of pre-mRNA and that the downstream site will be used for splicing. The upstream site will not be used, whatever its intrinsic strength. The upstream site will be used only if the downstream site is so weak that it is unoccupied on some molecules (bottom left). When the sites are weaker (bottom right), some molecules of pre-mRNA are bound at only one site and in these cases whichever site is occupied will be spliced; selection will depend on the affinity of U1 snRNP for each site. The open grey arrows show that the effect of an increase in the concentration of SF2/ASF on splice site selection could be explained by an increase in the affinity of U1 binding, which would cause weak sites to behave as strong sites. Exactly opposite effects are predicted if U1 binding is weakened by, for example, reducing the length of U1 snRNA able to base pair with the 5′ splice sites. The different patterns of splice site selection seen *in vivo* might arise from weaker interactions with the 5′ splice sites, such as might be caused by a lower effective concentration of U1 snRNPs.

in that it contained 80 mM potassium glutamate instead of potassium chloride (Black, 1992), and it unexpectedly showed substantial use of the upstream 5′ splice site, consistent with the rather low level of protection at both sites simultaneously. A partially purified preparation of SF2/ASF, which contains other SR proteins that affect 5′ splice site selection similarly (Mayeda *et al.*, 1992), also increased the level of protection at both sites in this extract and caused the use of the upstream site to fall from 45% to 18% (not shown). Other experiments in our normal extract mixture with substrate **C48G** showed that the level of protection of the unused upstream site was increased in the presence of partially purified SF2/ASF with both intact substrates and substrates lacking 3′ splicing signals (not shown). Although it is probable that occupancy of such a site is close to 100% under our normal conditions, an increase in U1 snRNP binding affinity would be expected to produce an increased level of resistance in displacement by oligonucleotides. We conclude that SF2/ASF acts non-specifically at 5′ splice sites to stabilize the initial interactions of U1 snRNPs.

Discussion

Previous studies have shown that, with both consensus and globin 5′ splice sites, U1 snRNPs bind either of two alternative sites even when only one is used for splicing (Nelson and Green, 1988). The significance of this was unclear without a method for determining (i) the proportion of sites bound, (ii) whether the sites were occupied simultaneously and (iii) whether spliceosome assembly was selective or also indiscriminate, resulting in the latter case in an accumulation of abortive complexes on the 'wrong' site. Our experiments on very similar substrates and with the same sites have shown that alternative consensus 5′ splice sites are protected indiscriminately and that both of two alternative consensus 5′ splice sites can be protected before, or in the absence of, interactions with 3′ components. In contrast, the results with globin 5′ splice sites showed that sites that were not used for splicing were not protected and, because protection required interactions with 3′ components, we suggested that these interactions form only with the site to be used for splicing.

We interpreted the protection of consensus 5′ splice sites as a measure of high-affinity binding by U1 snRNPs because: (i) it was almost eliminated by oligonucleotide treatment to remove the 5′ end of U1 snRNA; (ii) it could be produced by pure U1 snRNPs; (iii) it was seen in conditions which prevent the binding of most other components and in which psoralen cross-linking confirmed that U1 snRNA was base paired to the pre-mRNA; and (iv) it was consistent with the earlier immunoprecipitation data. As with the latter experiments, it is not possible to exclude the possibility that other proteins are associated with the bound U1 snRNP and, indeed, such factors clearly affect the affinity of the interaction with the 5′ splice site (see below), but the predicted effect on splice site selection of cleaving the U1 snRNA is a clear indication that U1 snRNPs are a major determinant of binding affinity.

The simultaneous occupation of two close 5′ splice sites by U1 snRNPs on almost all molecules of **C24C** pre-mRNA was associated with a substantial inhibition of splicing. A similar proportion of **C174C** pre-mRNA was protected at

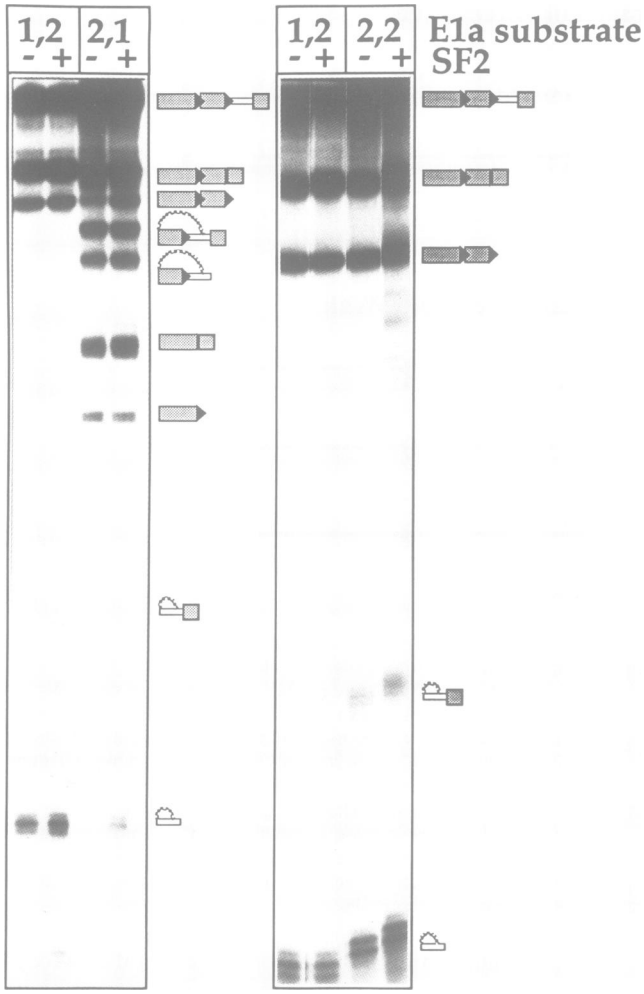

Fig. 9. Splicing of adenovirus E1a substrates. The sequences of the two splice sites responsible for production of the 12S and 13S mRNA isoforms (sites 1 and 2, respectively) were swapped around as described by the nomenclature of the substrates (E1a, 1,2 has the wild-type arrangement) and in the main text. The positions of the intermediates and products for the two isoforms are shown alongside the panels.

both sites in the absence of Mg^{2+} and ATP or with 3′ truncated molecules, although the proportion was lower in normal splicing reactions with an intact substrate. The differences could reflect either reduced levels of binding by U1 snRNPs in the normal reaction or reduced resistance to displacement by the RNase H oligonucleotides. Based on our observations, we proposed a scheme in which the occupation of pre-mRNA molecules at both of the two strong 5′ splice sites would lead to splicing to the downstream 5′ splice site. This would reconcile the discriminate use of splice sites with the indiscriminate binding of U1 snRNPs. For strong 5′ splice sites, the scheme asserts that selection takes place when interactions develop with 3′ components on the basis of proximity, whereas the use of competing sites of low affinity depends on the probability that they are occupied, i.e. on the basis of U1 snRNP dissociation rates.

According to the model proposed, the absolute preference for the more downstream of two globin 5′ splice sites indicates that these too should behave as strong sites. Although the RNase H assay was unable to detect the initial binding of U1 snRNPs to globin 5′ splice sites, this interaction is known to be non-selective (Nelson and Green, 1988), which agrees with our own preliminary data from psoralen cross-linking (not shown), and the level of binding in nitrocellulose filter assays to these sites is ~60% of that to consensus 5′ splice sites (Tatei *et al.*, 1987; Mayeda and Ohshima, 1988). Thus, it seems probable that the initial binding of U1 snRNPs to globin sites is similar to that seen with consensus 5′ splice sites.

Table III. Effects of SF2/ASF and absence of Mg^{2+} and ATP on the proportion of C174C pre-mRNA protected at both alternative 5′ splice sites

Extract	Incubation time (min)			
	0	5	10	15
Mock	0.35	5.1	7.4	12
Mock + SF2/ASF	3.3	8.6	25	37
$-Mg^{2+}$/ATP	4.4	53	71	79
$-Mg^{2+}$/ATP + SF2/ASF	3.9	55	73	73

The figures represent the proportions of pre-mRNA protected at both alternative 5′ splice sites. The levels were calculated from the experiment shown in Figure 6, according to the formula described in Materials and methods and used in Table I.

The scheme based on this reasoning made several predictions, which were tested, about the effect of weakening the base pairing of U1 snRNA with the downstream site either by making the sequence of the site less favourable or by cleavage of the U1 snRNA. Although the shift of splicing to the upstream site in the former case could be explained by several mechanisms, the effect of U1 cleavage would not have been expected and it is, therefore, a powerful argument in support of the model. There have been other reports of continued activity after cleavage of U1 snRNA in this manner (Black and Steitz, 1986; Pan *et al.*, 1989), and it is likely that it depends upon the maintenance of some residual ability to form base pairs with the exon portion of the splice site. Further support for our model came from the unexpected effect of an extract preparation in potassium glutamate buffer, which allowed the upstream site to be used even in the presence of a consensus downstream 5′ splice site; in this case, the proportion of the precursor protected at both sites was lower and splicing to the upstream site too would have been expected (Figure 6). A strong preference for a downstream alternative 5′ splice site has been observed in some other studies (Reed and Maniatis, 1986), but not all (Mayeda and Ohshima, 1988; Nelson and Green, 1988) and this may depend on whether the nuclear extract used favoured the simultaneous occupation of both sites.

The choice of the E1a site 1 sequence for the experiment in Figure 9 was based on its weakness in competition experiments *in vivo*, wherein synthetic splice sites were placed in turn 25 nt upstream of the globin site (Eperon *et al.*, 1986; Lear *et al.*, 1990). Our results now suggest that this system measured the relative lifetimes of bound U1 snRNPs at two interfering sites. The relative resistances to RNase H challenge of bound complexes at the four splice site sequences tested in this report (data not shown) reflect the proportionate use of these four sequences *in vivo*: consensus, 100%; E1A sequence 2, 64%; globin, 63%; E1A sequence 1, 0% (Eperon *et al.*, 1986). It is remarkable that the two E1a sites behave so differently because the predicted values for the free energy of base pairing to U1 snRNA for the two sites are the same (Lear *et al.*, 1990), and it is likely

that other factors have a sequence-specific effect on the stability of the bound complex.

We have discussed previously several mechanisms that might be responsible for the effect of SF2/ASF on alternative 5′ splice site selection (Mayeda and Krainer, 1992). An obvious possibility was that SF2/ASF caused selective positive effects at downstream sites or negative effects at upstream sites. An alternative was that high concentrations of SF2/ASF might equalize the strengths of alternative 5′ splice sites, resulting in a preference for the proximal site by an unknown mechanism (Mayeda and Krainer, 1992). The scheme proposed in Figure 8 provides a basis for explaining the proximity effect and led us to predict that SF2/ASF could equalize sites by increasing the affinity of U1 snRNPs for all sites, which would considerably increase the occupancy of weak sites and thus increase the proportion of pre-mRNA molecules with multiple occupancy. Our results, showing that SF2/ASF caused an increase in protection of both consensus splice sites (Figure 6), are inconsistent with the expectations of models based on discrimination by SF2/ASF and are consistent with our prediction.

SF2/ASF is an essential splicing factor (Krainer *et al.*, 1990b). Our results do not show that its effects are restricted to interactions at 5′ splice sites, but they argue strongly that the effect of SF2/ASF on increasing the affinity of binding to a 5′ splice site is part of its mechanism of action. The increase in affinity might be related to the activity of SF2/ASF in promoting the annealing of complementary RNA (Krainer *et al.*, 1990b). However, SF2/ASF was necessary for the development of RNase H protection of the consensus 5′ splice site in an S100 preparation (data not shown), even though U1 snRNPs alone confer protection and would be found in the S100 fraction. We have found also that SF2/ASF does not enhance the protection by pure U1 snRNPs (I.C.Eperon, unpublished results). It is possible that the main action of SF2/ASF is to inhibit a factor in extracts that weakens U1 snRNP binding.

There are several factors or activities in nuclear or S100 extracts that might weaken the binding of U1 snRNPs. One candidate is hnRNP A1, which has effects antagonistic to those of SF2/ASF on splice site selection (Mayeda and Krainer, 1992; Mayeda *et al.*, 1993). This possibility is supported by preliminary results suggesting that hnRNP A1 in excess can reduce the binding of U1 snRNPs to a 5′ splice site (Buvoli *et al.*, 1992). The increased detection of U1 binding in the absence of Mg^{2+} and ATP (Figure 6) suggests two further possibilities. One is that an RNA helicase acts to destabilize U1 snRNP binding, such that with weak sites simultaneous multiple binding becomes improbable and the affinity for U1 snRNPs determines the site most likely to be occupied and spliced. Several splicing proteins from *Saccharomyces cerevisae* have sequence motifs found in RNA helicases (Wassarman and Steitz, 1991). The last possibility is that the first Mg^{2+}-dependent step identified, the base pairing of U5 snRNA near the 5′ splice site, might weaken the base pairing of U1 snRNA (Wassarman and Steitz, 1992). However, the effect of SF2/ASF on 5′ splice site protection is seen even with 5′ half molecules (data not shown), which suggests that other snRNPs are not involved (Bennett *et al.*, 1992). Furthermore, it follows that the effects of SF2/ASF on selection are unlikely to involve the mediation of U1−U2 interactions at the 3′ splice site in the manner of SC35 (Fu and Maniatis, 1992), another SR protein (Zahler *et al.*, 1992) which has similar effects on splice site selection (Fu *et al.*, 1992).

Some comparisons have been reported between 5′ splice site selection *in vivo* and *in vitro* (Kedes and Steitz, 1987, 1988; Lowery and Van Ness, 1987, 1988; Noble *et al.*, 1987; Schmitt *et al.*, 1987; Cunningham *et al.*, 1991). Except for SV40 T/t splicing, where use of the downstream site is limited by intron length and complex branch sites, but enhanced by SF2/ASF (Fu and Manley, 1987; Noble *et al.*, 1987; Ge and Manley, 1990), splicing *in vitro* showed much reduced use of the upstream alternative sites; in the case of the β-globin derivatives examined in this paper, the use of an upstream consensus 5′ splice was eliminated. Our work suggests one explanation: that the effective concentration of U1 snRNPs is lower *in vivo*, and that the affinity of the site is then more important. This would be consistent with observations of U1 snRNP accumulations within foci in mammalian cell nuclei (Carmo-Fonseca *et al.*, 1991), together with some but not all splicing components (Spector *et al.*, 1991; Zamore and Green, 1991), leaving only a fraction of the snRNPs distributed diffusely and in interchromatin granules and perichromatin fibrils, sites at which it is believed splicing may occur (Spector *et al.*, 1991; Huang and Spector, 1992). Similarly, amphibian germinal vesicles contain particles (A snurposomes) which contain only U1 snRNPs (Wu *et al.*, 1991). It is possible that the disruption of such structures during the preparation of nuclear extracts liberates U1 snRNPs, such that they are disproportionately represented *in vitro* compared with other splicing factors.

Materials and methods

Preparation of transcription templates

β-Globin templates were prepared by the polymerase chain reaction (PCR; Saiki *et al.*, 1988), such that a phage T7 promoter sequence was appended to the first nucleotide of exon 2 of rabbit β-globin (nt 272; van Ooyen *et al.*, 1979). This defined the 5′ end of the pre-mRNA. The oligonucleotide defining the 3′ end of the pre-mRNA was designed such that the transcript ran to nt 570 of β-globin, then incorporated nt 1032−1086 (the branch site, 3′ splice site and the first 19 nucleotides of exon 3), followed by AATTC-CAGCACG, which produced an inverted repeat in the 3′-most 20 nt. The substrates for PCR have mostly been described before (Cunningham *et al.*, 1991); **G** came from an unmodified β-globin, and for **C** this gene was mutated such that CAGG/GTGAGT was altered to CAG/GTAAGT. 3′-Truncated pre-mRNA was prepared either from PCR reactions in which the 3′ primer was complementary to nt 542−558 of β-globin IVS-2, or by RNase H cleavage of intact pre-mRNA in the presence of the same oligonucleotide. The adenovirus 5 E1a substrates were prepared likewise and extended from 200 nt 5′ of the 12S splice site at position 973/974 to 20 nt into the 3′ exon, followed by an arbitrary self-complementary sequence of 24 nt (TGCAATCCGATGCATCGGATTGCA). The splice site sequences in the non-mutant substrate E1a 1,2 were AGG/GTGAGG and ACA/GTAAGT at the 12S and 13S sites, respectively. In the mutant E1a 2,1, these sequences were swapped, and in E1a 2,2 the 12S site was mutated to fit the 13S sequence.

Splicing in vitro

Nuclear extracts were prepared from HeLa cells grown in 1.5 l spinner cultures, with triethanolamine in buffer D (Dignam *et al.*, 1983; Tazi *et al.*, 1986). In one case, the extract preparation contained 80 mM potassium glutamate instead of 100 mM potassium chloride (Black, 1992). The splicing reaction mixtures were as described by Krainer *et al.* (1984). Splicing reactions with the adenovirus substrate used 10 U Inhibit-Ace (5 prime-3 prime Inc.), which brought the concentration of KCl to 65 mM and added 10 mM NaCl. For simple splicing reactions, 10 μl of extract were added to 0.5−1.0 μl RNA, and reactions were incubated in inverted, sealed microtitre plates. Reactions were initiated such that the specified incubation

times elapsed at the same moment. Processing in microtitre plates before gel electrophoresis was as described previously (Cunningham *et al.*, 1991).

For RNase H cleavage of snRNAs, 8 μl (6.4 U) of RNase H (Pharmacia) were added per 295 μl reaction mixture. Aliquots were incubated in the presence of oligonucleotides at 30°C for 45 min, and were then kept on ice until 10 μl portions were mixed with an RNA substrate. For cleavage of U1 snRNA, 600–670 pmol (in 0.6–14 μl) of the oligonucleotide α-U1 5′ (TGCCAGGTAAGTAT) were used. In mock reactions, the oligonucleotide was replaced by an equal volume of water or an equimolar concentration of an unrelated oligonucleotide of equal length.

For experiments with SF2/ASF, buffer D in the reaction mixture was replaced by SF2/ASF in buffer D. For the experiment in Figure 6, highly purified SF2/ASF (Krainer *et al.*, 1990b) was added to 23 ng/μl after incubation of a mixture containing all the other components at 30°C for 45 min. Because the extract in this case contained potassium glutamate, an equal volume of normal buffer D was added in the same way to the control reactions. Similar results were found with partially purified SF2/ASF (Mayeda *et al.*, 1992), which contains other SR proteins with activities similar to SF/ASF. In other experiments, SF2/ASF, an S100 preparation and buffer D were mixed to replace the extract in a reaction mixture.

Highly purified U1 snRNPs were a gift from Dr R.Lürhmann (Marburg). SnRNPs in buffer D were added to U1-cleaved extracts to a concentration of ~5.4 ng/μl.

RNase H protection

The oligonucleotides appropriate to each pre-mRNA were added to splicing reactions of 5–10 μl after the specified times of incubation at 30°C (for details, see Eperon and Krainer, 1993); 100 pmol of each oligonucleotide were added to each reaction in a total of 2 μl buffer D. The 5′ splice site cleavage oligonucleotides were CGACTCACCCTGGG (complementary to the upstream globin 5′ splice site), AAACTCACCTGAA (downstream globin site), CGACTTACCTGGGG (upstream consensus site) and AAACTTACCTGAAG (downstream consensus site). The oligonucleotides are fully complementary to the pre-mRNA target; the underlined sections are complementary to the nine nucleotides of the 5′ splice site. To determine branch site protection, the oligonucleotides used were TGAACATGGTTAGCAgag (the last three nucleotides are not complementary to the substrate), which anneals across the branch site, or CTCCATATAACATGAAT which anneals 16 nucleotides to the 5′ side of the site but gives similar results. The oligonucleotide used in some of the mock reactions was CATCTTCCCATTCT, which is not complementary to the pre-mRNA.

Cross-linking

Splicing reactions were prepared as above, but with ~40-fold more RNA in a volume of 20 μl, and 4′-aminomethyl-4,5′,8-trimethylpsoralen (AMT) was added as described by Rinke *et al.* (1985). After incubation at 30°C for various times, the open microtitre plate was placed on ice and irradiated from 5 cm with long-wave UV (λmax = 366 nm) emitted at 700 μW/cm^2 for ~1 h. Subsequent processing was as above. For reactions done under normal conditions with full-length and 3′ truncated substrates, RNA was eluted from the bands after gel electrophoresis and digested with RNase H in the presence of specific oligonucleotides directed against the pre-mRNA or U snRNA; the reaction products were analysed by gel electrophoresis. The assignments of the cross-linked products after pre-treatment of the extract to cleave U2 snRNA, or after the short splicing reaction time courses, were based on their mobilities.

Quantitative analysis

The levels of radioisotope in each of the 5′ splice site cleavage bands in Figure 2 were determined by a PhosphorImager (Molecular Dynamics). Laser densitometry was used to determine the relative levels of autoradiograph bands in the parallel splicing reactions. Scintillation counting was used for the bands in Figures 5 and 6. All values were corrected for the uridine content of the RNA molecules. The proportion of fully protected RNA in each lane was calculated as [precursor]/[precursor + 5′ fragment protected at upstream site +5′ fragment cut at upstream site] (see Figure 1). Likewise, the proportion protected at only the upstream site was calculated using the same denominator. The proportion protected at the downstream site only was calculated as [3′ fragment protected at the downstream site]/[precursor + 3′ fragment protected at the downstream site + 3′ fragment cut at downstream site]. As far as possible, each calculation included only fragments that either did or did not include a 5′ cap, because this affected the stability of the molecules. The inclusion of precursor in the last calculation is an exception, but of little significance because the calculation was used primarily for substrates of the form **GXG**, where the levels of fully protected precursor were very low. The splicing efficiency for step 1 was calculated using the measured ratios of each form of RNA within one lane; E2 and E2E3 were treated as products, and both intact RNA and RNA cleaved at the branch site were treated as precursors.

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