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Antisense RNA inhibits splicing of pre-mRNA *in vitro*

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Antisense RNAs complementary to human β-globin pre-mRNA or to a chimeric globin/adenovirus E2a pre-mRNA specifically and efficiently inhibit pre-mRNA splicing *in vitro*. The level of inhibition depends on the length, position and concentration of the antisense RNA relative to the pre-mRNA substrate. Antisense RNAs complementary to sequences >80 nucleotides downstream of the globin 3′ splice site inhibit at least as efficiently as those extending across the splice sites. Thus splicing is sensitive to perturbations involving exon sequences some distance from the splice sites. Inhibition is mediated by factors which affect the annealing of antisense and substrate RNAs. Direct analysis of RNA duplex formation demonstrates the presence of an activity in HeLa cell nuclear extract which promotes the rapid annealing of complementary RNAs in an ATP-independent manner. Both annealing and inhibition are greatly reduced when antisense RNA is added to the splicing reaction ≥5 min after substrate. This result may reflect a transition between an open structure, in which annealing of antisense RNA with pre-mRNA is facilitated, and a closed complex in which pre-mRNA is sequestered at an early stage of spliceosome assembly.

Key words: mRNA splicing/antisense RNA/globin pre-mRNA/adenovirus pre-mRNA

Introduction

Short conserved sequences present at 5′ and 3′ splice sites in nuclear pre-mRNA and at the branch point site a short distance upstream of the 3′ splice site are essential but not sufficient to determine unambiguously the site of splicing (for reviews see Green, 1986; Padgett *et al.*, 1986; Krainer and Maniatis, 1988). Since sequences which resemble splice site consensus sequences occur at random throughout most transcription units at much higher frequencies than active splice sites (Munroe, 1983) the context in which splice sites occur is critical in determining their activity. Accumulating evidence suggests that sequences distant from the splice site are often crucial for splicing. Small deletions within the early (Khoury *et al.*, 1979) or late (Somasekhar and Mertz, 1985) regions of SV40 alter the efficiency with which splicing occurs at downstream splice sites. Substitution of sequences within the second exon of human β-globin with homologous sequences from corresponding mouse or rabbit genes alters the splicing *in vitro* of constructs containing duplicated 3′ splice sites (Reed and Maniatis, 1986). Similarly, substitution of sequences within an exon from the rat fibronectin gene affects the use of alternative splice sites within the fibronectin gene *in vivo* (Mardon *et al.*, 1987). Relatively small deletions in introns can also block splicing of an alternatively spliced transcript (Hwang *et al.*, 1984). In contrast to these results, however, other studies show that splicing *in vitro* or *in vivo* is often insensitive to deletions within intron or exon sequences which do not affect the splice site consensus sequences (Wieringa *et al.*, 1984; Furdon and Kole, 1986; Parent *et al.*, 1987). Truncation of most of the 3′ exon sequences in human or rabbit β-globin pre-mRNA transcripts, for example, has little apparent effect on splicing *in vitro* (Furdon and Kole, 1986; Parent *et al.*, 1987).

The manner in which sequences remote from the splice site affect splicing is at present unclear. A number of possible mechanisms may account for the role that non-essential exon sequences play in splice site selection. One possibility is that such exon sequences are involved in stabilizing a conformation which is essential for splice site activity. A second possibility is that these exon sequences play a critical role in establishing a functional ribonucleoprotein (RNP) organization along the length of the transcript. These and other possible models are not mutually exclusive. While studies using deletion and point mutations have provided important evidence establishing the requirements for conserved, splice site consensus sequences (Aebi *et al.*, 1986; Eperon *et al.*, 1986; Krainer *et al.*, 1984; Reed and Maniatis, 1986) and their role as binding sites for snRNPs (Chabot and Steitz, 1987), mutational analysis has been less effective in analyzing the role of sequences in the pre-mRNA which are more distant and less clearly defined. Antisense RNA molecules complementary to the pre-mRNA provide an alternative approach to probing for required sequences within a particular gene or pre-mRNA transcript. Naturally occurring antisense RNAs have been shown to block gene expression at the level of translation or transcription (Green *et al.*, 1986). Artificial antisense RNA molecules block gene expression in eukaryotes in the nucleus (Izant and Weintraub, 1984) and the cytoplasm (Melton, 1985).

In this study I describe the use of antisense RNA to probe requirements for mRNA splicing *in vitro*. Antisense RNAs complementary to different regions of pre-mRNA transcripts inhibit splicing *in vitro* in a specific and efficient manner. While initial experiments were directed at exploring the possible role of specific, splice site proximal RNA secondary structure in the adenovirus 2 E2a (ad2-E2a) region, further, more detailed studies using a human β-globin pre-mRNA suggest a general role for exon sequences in modulating splicing. Results presented here also demonstrate the presence of several distinct activities within the nuclear extract which mediate interactions between complementary RNA molecules during splicing.

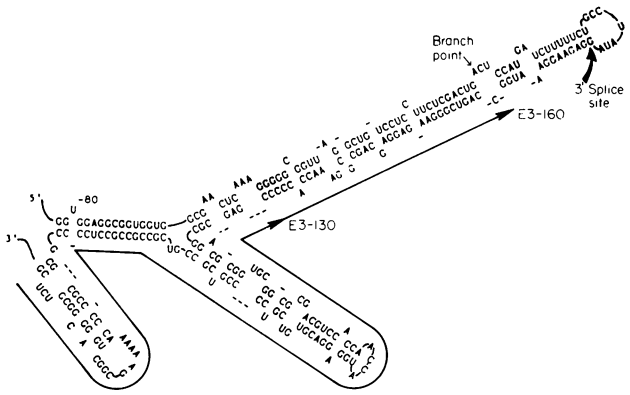

Fig. 1. Secondary structure of sequence near the 3′ splice site at the third exon of adenovirus 2 E2a. The solid line indicates overlap with two 5′ co-terminal antisense RNAs, designated E3-160 and E3-130. Arrowheads on this line represent 3′ ends of these RNAs.

Results

A chimeric globin/adenovirus transcript is efficiently spliced in vitro

Previous work from this laboratory (Munroe, 1984) demonstrated that the 3′ splice site and branch point sequences adjacent to the third exon of the ad2-E2a major mRNA are base paired with downstream exon sequences to form an exceptionally stable hairpin structure in solution (Figure 1). To investigate the possible role of this local secondary structure in mRNA splicing a series of short transcripts including exon 2, intron 2 and part of exon 3 of the ad2-E2a region were prepared and tested for their ability to undergo splicing *in vitro*. Although it was reported that ad2-E2a transcripts are inefficiently spliced *in vitro* (Goldenberg, 1984), the shorter transcripts tested here are spliced inefficiently, if at all. An ad2-E2a substrate consisting of exon 2, intron 2 and 160 nucleotides (nt) of exon 3 gives rise to very faint bands corresponding to the size expected for spliced product (S) and free 5′ exon (E1, Figure 2A, lane 9). Under these conditions globin pre-mRNA is efficiently spliced as shown in Figure 2A, lanes 1−3. The products obtained from this ad2-E2a precursor were not analyzed further. To obtain efficient splicing *in vitro* at the 3′ splice site of exon 3 of ad2-E2a a chimeric transcript was prepared in which the 5′ half of the ad2-E2a transcript is replaced with exon 1 and adjacent intron sequences of human β-globin, as shown in Figure 2B. This pre-mRNA undergoes splicing with much higher efficiency than other ad2-E2a transcripts. Two bands, S and E1, appear after a 2-h incubation with ATP. These bands correspond to RNAs 320 and 160 nt in length, the sizes expected for spliced product and free 5′ exon, respectively (Figure 2A, lanes 4−6). Band E1 co-migrates exactly with exon 1 of globin and appears before band S as expected for an intermediate of the splicing reaction.

The identity of the putative splice product was confirmed by RNase protection assays in which the eluted band S was hybridized to a series of plasmids containing globin and ad2-E2a sequences. For comparison, bands representing exon 1 of globin and exon 3 of ad2-E2a were generated by hybridizing labeled, unspliced chimeric transcript to globin and ad2-E2a cDNA clones as outlined schematically in Figure 3B. As expected the products of these protection

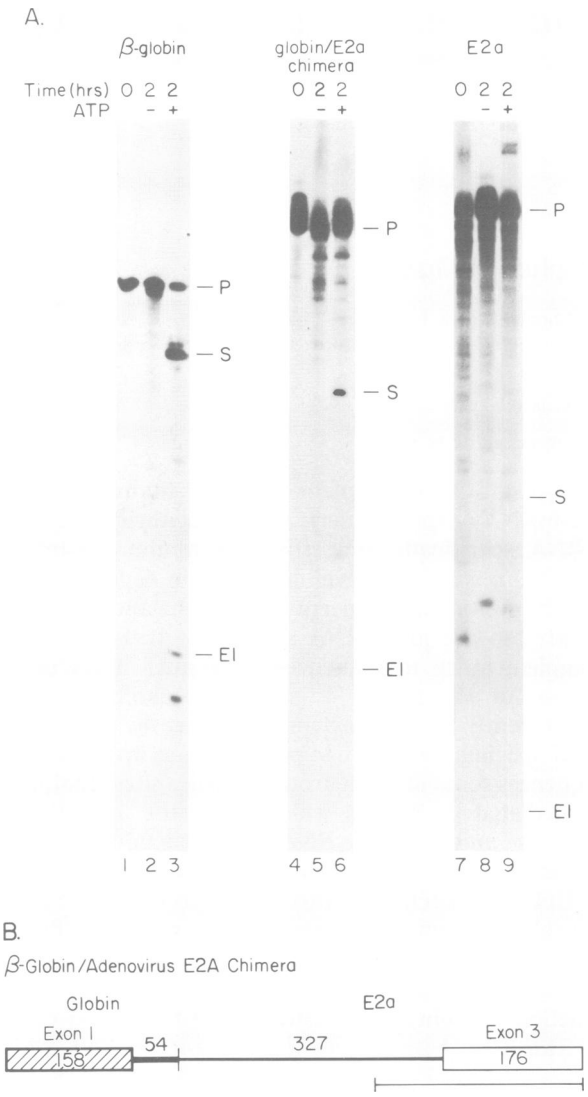

Fig. 2. Splicing of globin and adenovirus transcripts *in vitro*. (**A**) Splicing of globin (**lanes 1−3**), globin/E2a chimera (**lanes 4−6**) and ad2-E2a (**lanes 7−9**) pre-mRNAs. Each reaction contained 5 nM substrate RNA. P, S and E1 in this and subsequent figures indicate pre-mRNA, spliced product and cleaved exon 1 respectively. The excised intron lariat and intron−exon lariat for globin migrate immediately ahead of band E1 and between P and S, respectively, in lane 3. (**B**) Structure of globin/E2a substrate. Boxes represent exons, single connecting line the intron. Vertical slash in intron represents junction of globin (heavy line) and ad2-E2a sequences. Lengths of intron and exon segments are indicated. Line underneath transcript at right represents the region of ad2-E2a sequence shown in Figure 1.

assays are two bands, each ~160 nt in length, corresponding to ad2-E2a exon 3 (Figure 3A, lane 1) and globin exon 1 sequences (lane 2). Unspliced globin transcript hybridized to the chimeric plasmid DNA gives a band (E1 + I) corresponding to the globin exon plus intron sequences in the chimera (lane 3). Globin sequences hybridize more efficiently than ad2-E2a sequences under these conditions. When labeled RNA from band S was hybridized to genomic globin and ad2-E2a sequences, the most prominent bands (lanes 6 and 7) were identical in size and intensity to the corresponding exon bands. A larger RNA species seen in lane 7 (E1 + I) represents protection of a small amount of degraded, unspliced RNA which contaminates the eluted

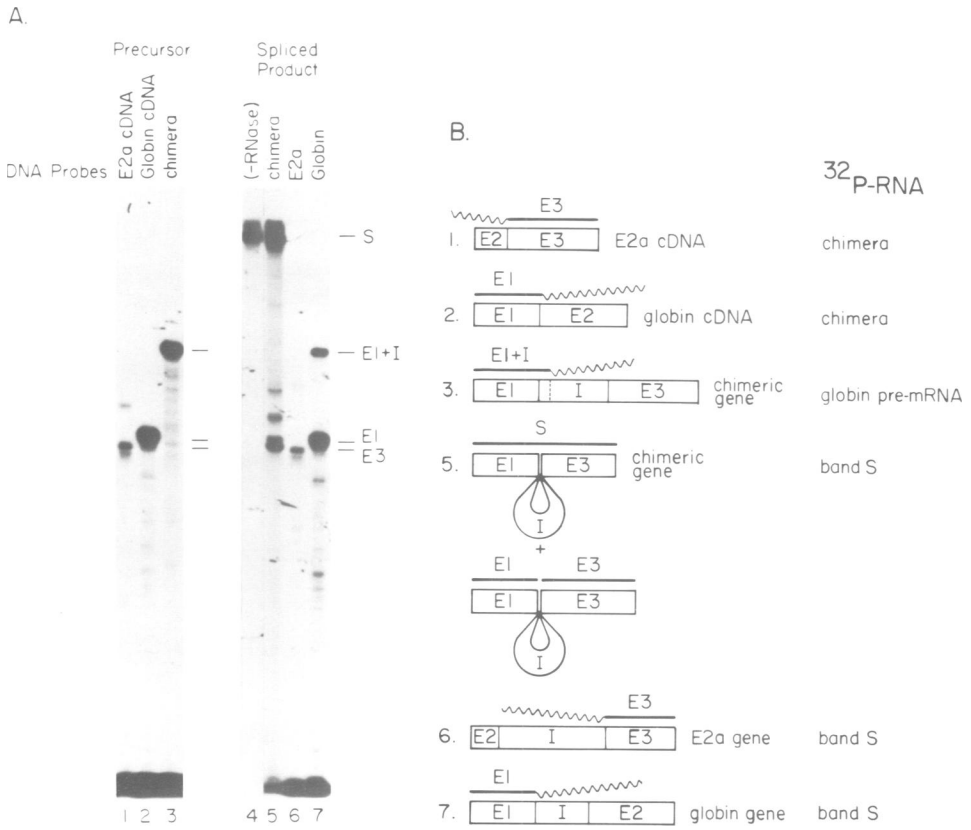

Fig. 3. Identification of globin/E2a spliced product. (**A**) Labeled RNA representing unspliced chimeric transcript (**lanes 1,2**), unspliced globin transcript (**lane 3**) or eluted spliced product (band S, **lanes 4–7**) were hybridized to a 50-fold excess of the plasmid DNA indicated above each lane. In **lanes 1–3** and **5–7** hybrids were digested with RNase to remove single-stranded RNA. **Lane 4** shows one-fifth of the hybridization reaction in **lane 5** prior to RNase digestion. (**B**) DNA/RNA hybridizations are shown schematically. Numbers at left correspond to lanes in (A).

band S RNA (compare with lane 3). Finally, hybridization of band S with the chimeric plasmid protected the full length of the eluted band S as well as individual exon (lane 5) since nicking at the point of DNA loopout is inefficient. In this reaction the intensities of globin and ad2-E2a exon bands were similar, consistent with the hybridization of product RNA to both complementary segments of the DNA simultaneously prior to cutting between the two spliced exons (lane 5 in Figure 3A,B). Taken together, these results demonstrate that the chimeric globin/adenovirus transcript is accurately spliced *in vitro*.

Antisense RNA inhibits splicing of the chimeric pre-mRNA

RNA complementary to the 3′ exon of the globin/E2a chimeric transcript was used to probe requirements for RNA secondary structure in splicing *in vitro*. An antisense RNA molecule (designated E3-160) complementary to 160 nt of the third exon (E3) of the ad2-E2a pre-mRNA was hybridized to the chimeric substrate prior to splicing. Since this antisense RNA is complementary to all of the 3′ exon except 13 nt adjacent to the splice site, its annealing should disrupt the intramolecular structure around both splice site and branch point consensus sequences without directly interacting with either of these sites. If the hairpin stem blocks accessibility of factors to the splice site, disrupting it with antisense RNA might stimulate splicing at this site. Conversely, if the stable intramolecular secondary structure present *in vitro* represents a conformation favorable to splicing, such as disruption might inhibit splicing. Results clearly demonstrate that the antisense RNA blocks the appearance of spliced product (Figure 4, compare lanes 3 and 4 with lanes 6–8). A 20-fold excess of the same antisense RNA has no effect on splicing of globin pre-mRNA (Figure 4, lanes 9–14). Increasing the ratio of antisense to substrate RNA over the range of 1–10 leads to a continuous decrease in spliced product (results not shown). These results suggest a specific interaction between the complementary substrate and antisense RNAs. Further experiments show that antisense RNA also inhibits when it is added with substrate directly to the splicing reaction. A shorter antisense RNA, E3-130 also complementary to the 3′ exon but hybridizing 46 nt downstream of the splice site (Figure 1), also blocks splicing (results not shown). This shorter RNA does not overlap the large base-paired stem which sequesters the branch point and splice site consensus sequences as shown in Figure 1 (Munroe, 1983, 1984). Although the shorter antisense RNAs may indirectly perturb the secondary structure of the splice site it is also possible that this inhibition by antisense RNAs reflects a more general property of the splicing reaction. Further experiments were carried out with RNAs complementary to human β-globin pre-mRNA transcripts to examine this possibility.

Antisense RNA inhibits splicing of globin pre-mRNA

Antisense RNAs complementary to the 3′ exon of a transcript consisting of exon 1, intron 1 and exon 2 of human β-globin pre-mRNA inhibit splicing of this transcript in a manner

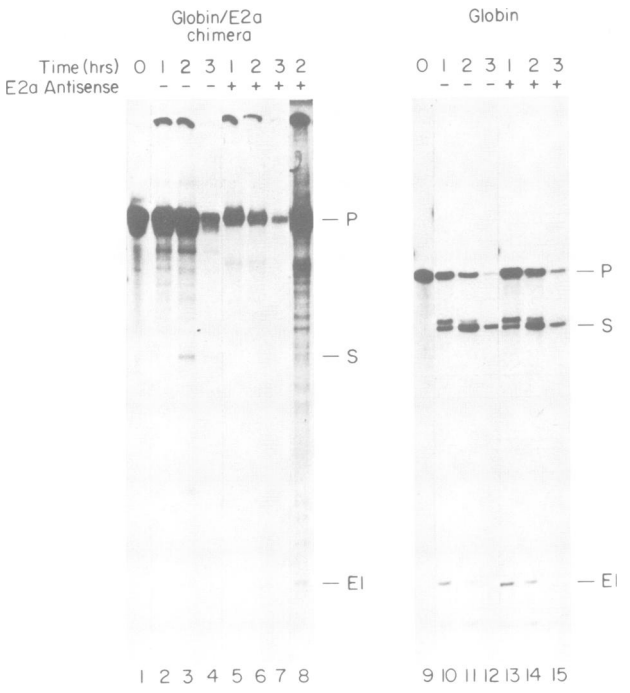

Fig. 4. Antisense RNA inhibition of the splicing globin/E2a transcript. Globin/E2a (**lanes 5–8**) and globin pre-mRNAs (**lanes 13–15**) were incubated with a 20-fold molar excess of antisense RNA under hybridization conditions prior to their addition to splicing reactions at a final concentration of 2–3 nM. Reactions were incubated for times indicated. **Lane 8** is an overexposure of **lane 6** showing absence of spliced products. **Lanes 1** and **9** are input RNA. **Lanes 2–4** and **10–12** show reactions without ad2-E2a antisense RNA.

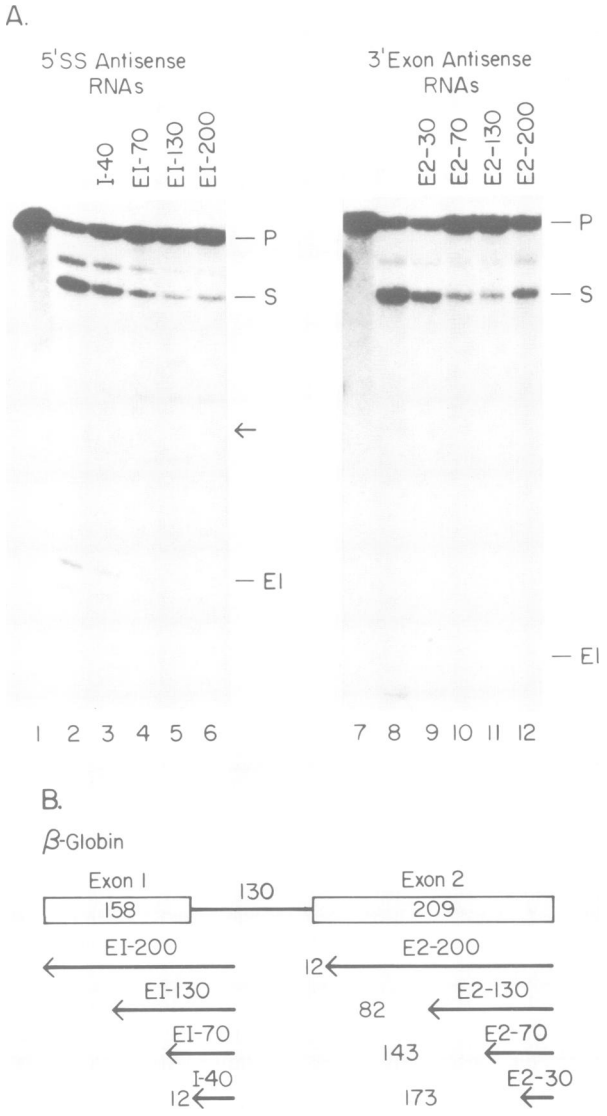

Fig. 5. Antisense RNA inhibits splicing of human β-globin pre-mRNA. (**A**) Antisense RNAs complementary to the 5′ splice site (**lanes 3–6**) or 3′ exon (**lanes 9–12**) were included in splicing reactions. Arrow indicates position of band seen in all splicing reactions inhibited with 5′ antisense RNAs. **Lanes 2** and **8** show reactions incubated without antisense RNA. **Lanes 1** and **7** show reactions stopped at time zero. All other samples were incubated for 2 h. (**B**) Structure of globin pre-mRNA and antisense RNAs. Lengths of intron and exons are indicated. Distance from 3′ end of antisense RNA to nearest splice site is shown to left of each arrow representing antisense RNA.

similar to that observed for the chimeric globin/adenovirus transcript (Figure 5A, lanes 8–12). Antisense RNA E2-200, E2-130 and E2-70 complementary to the 3′ exon all inhibit splicing strongly, even though they are complementary to regions of the exon well removed from the splice site as shown in Figure 5B. Antisense RNAs complementary to the 5′ splice site were also tested (Figure 5A, lanes 2–6). These RNAs inhibit splicing to a roughly similar extent as those of comparable size complementary to the 3′ exon. Weak or negligible inhibition is observed with the two shortest antisense RNAs tested, E2-30 and I-40 (Figure 5A, lane 9). This may reflect either weaker interactions of the short antisense RNAs with substrate or the position of the antisense RNAs relative to the substrate. Further experiments using globin substrates with an extended 3′ exon and antisense RNAs with a complementary 5′ extension show that even a large antisense RNA hybridizing 170 nt downstream of the 3′ splice site inhibits weakly (results not shown).

The specificity of inhibition by antisense RNA was examined by several additional experiments. Titration of splicing demonstrates that inhibition is observed at moderate concentrations (3- to 10-fold molar excess) of antisense RNA relative to substrate (Figure 6A). No inhibition is observed with non-complementary transcripts. Sense transcripts representing either the 5′ half (Figure 6B, lane 5) or 3′ half (not shown) of the globin transcript do not inhibit splicing at concentrations at which antisense RNAs inhibit efficiently. In fact, an excess of the 5′ half-sense transcript relieves inhibition by a complementary antisense RNA (Figure 6B, lanes 2–4). Annealing of the 5′ sense transcript in excess to antisense RNA probably reduces the amount of antisense RNA available to anneal to substrate. Finally, RNAs which block the splicing of a complementary pre-mRNA do not inhibit the splicing of a truncated transcript where the complementary sequences are deleted and only weakly inhibit splicing of transcripts where the level of overlap is reduced to 40–50 nt (Figure 8A, lanes 8–10 and results not shown).

The level of inhibition observed with E2-200 is slightly, but consistently, reduced from that observed with shorter antisense RNAs, E2-130 and E2-70, which are also complementary to regions more distant from the 3′ splice site (Figure 5A, lanes 10–12). Parallel titrations of splicing with E2-130 and E2-200 (not shown) show that a 3-fold higher concentration of E2-200 is needed to achieve a comparable level of inhibition. The stability of these, and other, antisense

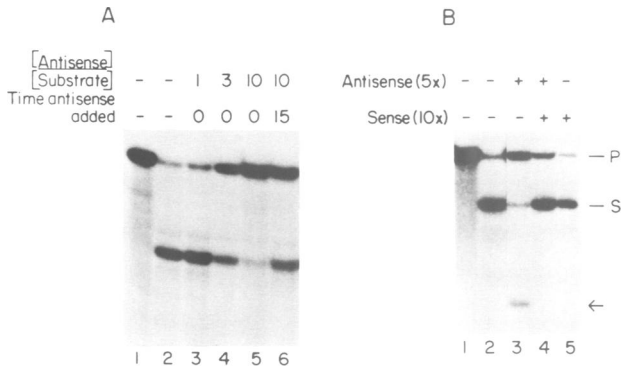

Fig. 6. Effects of complementary RNA molecules on splicing of globin pre-mRNA. **(A)** Titration of globin substrate with increasing concentrations of RNA complementary to 3′ exon (**lanes 3–6**). Ratios of antisense to substrate are indicated at top. Reaction in **lane 1** was stopped at time zero, others were incubated for 3 h. Reaction in **lane 2** shows splicing without antisense RNA, that in **lane 6** splicing in presence of antisense RNA added 15 min after start of reaction. **(B)** Inhibition of splicing by antisense RNA is blocked by addition of excess of sense transcripts complementary to antisense RNA. A 10-fold excess of sense transcript representing 5′ exon and 55 nt of adjacent intron was included in splicing reactions with (**lane 4**) or without (**lane 5**) a 5-fold excess of antisense RNA EI-200.

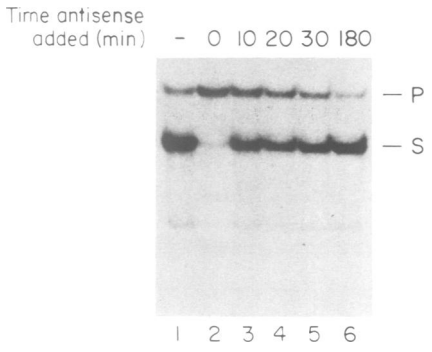

Fig. 7. Inhibition of splicing by antisense RNA decreases when antisense RNA is added after start of splicing reaction. A 10-fold excess of antisense RNA EI-200 was added to splicing reactions at times indicated. Reactions were stopped at 3 h.

RNAs does not differ significantly during the incubation (results not shown). The level of inhibition by E2-200 and E2-130 may reflect differences in the folding of these RNAs which affect their interactions with the pre-mRNA substrate, variations in the sensitivity of splicing to duplex structure at different downstream positions or the presence of factors which either block or unwind complementary RNAs annealing near the 3′ splice site. Consistent with the latter possibility is the observation that an antisense RNA which spans the 3′ splice site inhibits splicing poorly in comparison with antisense RNAs of the same length which are complementary to the 3′ exon or 5′ splice site (result not shown).

Differences in the stability of substrate or antisense RNAs during splicing might affect interpretation of the results. While a variable level of degradation is seen during splicing *in vitro*, in many reactions the amount of degradation is small. In Figure 6A, for example, the amount of degradation is only 5–20% while 80% of the input RNA is spliced in the uninhibited reaction. Thus selective degradation of spliced or unspliced transcripts cannot account for the level of inhibition observed. Interactions with antisense RNA also affect the stability of the pre-mRNA substrate. Addition of antisense RNA complementary to the 5′ splice site induces cleavage in the intron between the branch point and 3′ splice site, 22 nt upstream of the 3′ splice site. The arrows in Figures 5A and 6B indicate a 230-nt 3′ fragment released by cutting at this site in the presence of antisense RNA, as determined by primer extension.

Antisense RNA inhibits efficiently only when added at the start of splicing

When antisense RNAs are added to splicing reactions shortly after the substrate, the amount of inhibition is greatly reduced. This effect is seen with both 3′ antisense RNAs (Figure 6, cf. lanes 5 and 6) and 5′ antisense RNA (Figure 7). The lack of inhibition observed upon late addition of antisense RNA suggests either that a factor essential for inhibition is rapidly inactivated during the first few minutes of incubation or that the pre-mRNA substrate is rapidly sequestered in some way making it inaccessible to antisense RNA. To test the first possibility, antisense RNA and labeled substrate were added to the reaction 15 min after initiating splicing of an unlabeled substrate. Since efficient inhibition of splicing is observed when both antisense RNA and substrate were added late, it is likely that a reduction in the accessibility of the substrate to annealing with antisense RNA, and not the stability of some factor, interferes with inhibition by late-added antisense RNA.

RNA complementary to the 3′ exon inhibits only at the adjacent splice site

The splicing of two pre-mRNAs containing duplicated 3′ globin exons was examined to characterize further the effect of antisense RNAs on splicing at distance sites (Figure 8). In each of these transcripts, originally constructed and characterized by Reed and Maniatis (1986), sequences directly adjacent to the duplicated 3′ splice site site are identical. In the first case the transcript contains a 55-nt-long, truncated copy of exon 2 at the internal 3′ splice site, and a 209-nt-long copy of exon 2 at the external site downstream (Figure 8B, top). In this construct the internal site is inactive, although a transcript with a single copy of exon 2 truncated at 53 nt is spliced *in vitro* (Figure 8A, lane 8). In the presence of E2-130, which is complementary only to the external 3′ exon (Figure 8B), no product corresponding to splicing at the internal splice site is seen, although splicing at the external splice site is efficiently blocked (Figure 8A, lanes 4–7). This result indicates either that inhibition of splicing at the downstream site is not sufficient to activate the internal 3′ splice site or that the downstream antisense RNA inhibits splicing at both 3′ splice sites.

To test whether an RNA complementary to the 3′ exon inactivates all splicing within a given pre-mRNA transcript, splicing of a second transcript with duplicated 3′ splice sites was studied. In this transcript (Figure 8B, bottom) substitution of the globin sequences in the internal 3′ exon with rat insulin sequences leads to splicing at the internal site (S1) and inactivation of the outer site (S2). As shown in lanes 11–13, neither E2-130 nor E2-200 inhibits splicing at the internal site. The distance between the site of E2-200 annealing and the internal splice site is the same as that between E2-30 and the 3′ splice site in the normal globin transcript (Figures 5B and 8B). It appears that inhibition by antisense RNA complementary to the 3′ exon of globin

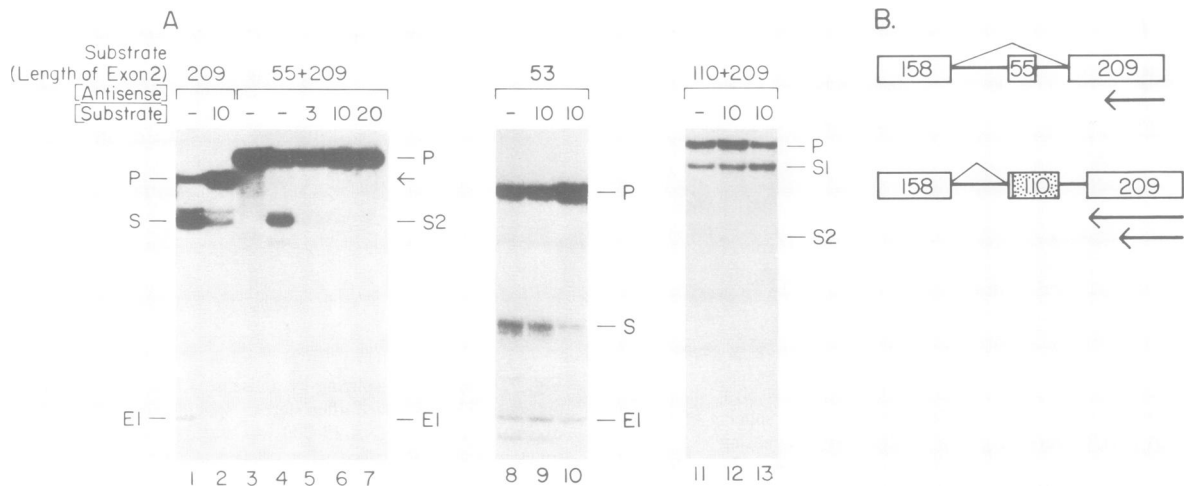

Fig. 8. Antisense RNA inhibition of splicing with globin pre-mRNAs containing duplicated or truncated 3′ exons. (**A**) A 3- to 20-fold excess of antisense RNA complementary to the full-length (209 nt) 3′ globin exon was added to splicing reactions containing normal globin substrate (**lanes 1** and **2**) or a substrate containing an internal duplication which includes the 3′ exon truncated 55 nt downstream of the splice site (**lanes 3–7**). **Lanes 8–10** show the effect of adding a 10-fold excess of E2-130 (**lane 9**) and E2-200 (**lane 10**) to substrate containing a 3′ exon truncated 53 nt downstream of the 3′ splice site. **Lanes 11–13** show splicing of globin transcript with duplicated exons 110 and 209 nt long. The internal 3′ exon contains primarily non-globin sequences, the external 3′ exon is identical to exon 2 of the standard globin substrate. A 10-fold excess of antisense RNAs E2-130 or E2-200 was added to reactions shown in **lanes 12** and **13** respectively. Reactions in **lanes 1** and **2** and **4–7** were incubated for 4 h, those in **lanes 9–13** for 3 h and that in **lane 3** for 0 h. (**B**) Structure of substrates with duplicated 3′ exons used in splicing reactions shown in **lanes 3–7** (top) and **lanes 11–13** (bottom). Stippled region represents non-globin sequence. Overlap with antisense RNA E2-130 and E2-200 is indicated.

pre-mRNA is either localized to the adjacent 3′ splice site, or restricted to splice sites closer than 180 nt.

HeLa cell nuclear extract facilitates RNA/RNA annealing

The specific inhibitory effect of complementary RNA molecules on splicing *in vitro* strongly suggests that these RNAs anneal to the pre-mRNA substrate. To examine this annealing directly, splicing reactions containing ^{32}P-labeled substrate were treated at different times with high concentrations of RNase T1 to obtain resistant double-stranded molecules. After only 2 min incubation of substrate with a 10-fold excess of EI-200 antisense RNA an intense band corresponding to the length of the expected duplex appears (Figure 9, lane 5). The intensity of this band increases slightly over the next 18 min (lanes 6–8) until it represents ~40% of the input RNA. No RNA is present at this position in the gel in the absence of antisense RNA (lane 1), demonstrating that the T1 resistant band represents an intermolecular duplex. In contrast to the result obtained in the presence of nuclear extract, very little annealing of substrate to antisense RNA was seen in the course of a 30-min incubation in buffer without nuclear extract (Figure 9, lanes 2 and 3). Pretreatment of the nuclear extract with Proteinase K also destroys the annealing activity (not shown). The annealing activity does not required added ATP since duplex formation proceeds efficiently in the absence of both ATP and creatine phosphate (Figure 10, lanes 7 and 8).

The annealing of the complementary RNAs was investigated as a function of time of addition of the antisense RNA. As shown in Figure 10 the addition of E2-200 at 0, 5 and 15 min leads to a progressive decrease in the rate of annealing. The decrease in duplex formation seen after late addition of the antisense RNA is paralleled by an increase in the level of splicing seen in other aliquots of these reactions collected at 2 h without T1 digestion (not shown). This result demonstrates that the block in antisense inhibition observed following late addition of antisense RNA reflects a failure of the antisense RNA to anneal efficiently to the substrate, rather than a decrease in the sensitivity of the committed substrate to inhibition by the base-paired RNA.

Interactions between substrate and antisense RNAs were studied further by examining the effect on the level of splicing when the time of addition of ATP as well as antisense RNA was varied. Preincubation of substrate and antisense RNA in the absence of added ATP slightly enhances the level of inhibition observed when antisense RNA and substrate are incubated together in the nuclear extract for the same time (not shown). Since annealing appears slightly faster when ATP is omitted (Figure 10, lanes 7 and 8), ATP may antagonize the formation of the antisense duplex required for inhibition.

Discussion

The results of this study demonstrate that antisense RNAs complementary to different regions of β-globin and the adenovirus E2a pre-mRNAs efficiently block splicing *in vitro*. In particular, RNAs complementary to sequences >80 nt downstream of the nearest splice site block splicing as efficiently as those complementary to splice site sequences. Inhibition is specific in that inhibition is only observed in the presence of complementary RNAs. It is likely that antisense RNA interferes with some step in the assembly of the spliceosomes which precedes formation of the covalent intermediates (Frendewey and Keller, 1985; Grabowski *et al.*, 1985). Inhibition at an early step in splicing is indicated by the requirement for simultaneous addition of antisense RNA and substrate to the splicing reaction (Figures 6A and 7) and the failure to observe accumulation of intermediates over the course of extended incubations. The appearance of exon 1 and the lariat intermediate (Figures 4 and 5A)

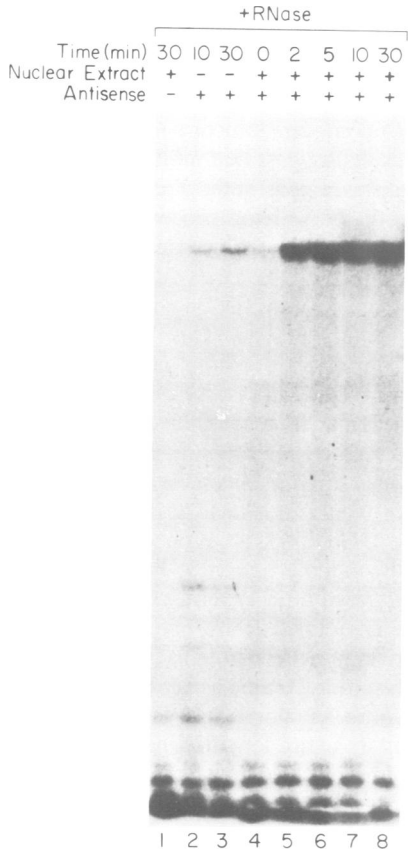

Fig. 9. Time course of RNA/RNA annealing. **Lanes 2–8** show splicing reactions containing 1 nM globin pre-mRNA and 10 nM EI-200 antisense RNA incubated for 0–30 min as indicated, then stopped with the addition of 500 U/ml T1 RNase. **Lane 1** shows splicing reaction without antisense RNA.

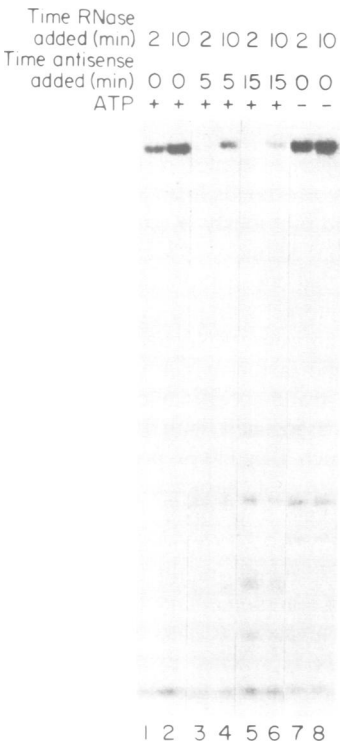

Fig. 10. RNA/RNA duplex formation is blocked upon late addition of antisense RNA. RNA/RNA duplex formed in splicing reactions containing a 10-fold excess of antisense RNA E2-200. Antisense RNA was added at 0 min (**lanes 1, 2, 7** and **8**), 5 min (**lanes 3,4**) or 15 min (**lanes 5,6**) after starting the reaction. ATP and creatine phosphate were omitted from reactions in **lanes 7** and **8**. Aliquots were collected and treated with T1 RNase 2 or 10 min after addition of antisense RNA.

probably corresponds to a low level of splicing observed in the presence of inhibitor. Although the mechanism of this inhibition remains to be established, these experiments demonstrate that exon sequences distant from a splice site affect splice site recognition or activity.

Antisense RNAs appear to inhibit splicing by annealing to complementary sequences on the pre-mRNA substrate. First, antisense RNAs only inhibit the splicing of complementary substrates. Similarly, inhibition of splicing is greatly diminished when the complementarity is reduced to 40 nt by truncating either the substrate or antisense RNA. Direct observation of duplex formation by RNase protection (results not shown) and the effects of intramolecular base-pairing on splicing (Solnick and Lee, 1987) indicate that RNA base-pairing shorter than 40 nt is unstable under conditions of splicing *in vitro*. Second, a fragment of the substrate which by itself has no effect on splicing, relieves inhibition when present in excess over antisense RNA, suggesting that it competes with substrate for binding to antisense RNA. This result also indicates that the presence of double-stranded RNA *per se* does not inhibit splicing. Third, direct evidence for antisense RNA annealing is provided by the rapid formation of T1 RNase-resistant duplexes. Conditions for duplex formation parallel those required for inhibition by antisense RNA: late addition of antisense RNA leads to a marked decrease in both annealing and inhibition.

The level of inhibition of splicing observed with different combinations of antisense and substrate RNAs probably reflects both the accessibility of the substrate to annealing with antisense RNA and interactions of the different RNAs with factors which facilitate or antagonize their annealing. Previous studies have suggested that rapid binding of proteins and snRNPs at the RNA branch point or 3′ splice site blocks hybridization of 3′ splice site sequences with oligodeoxyribonucleotides (Ruskin and Green, 1985). Binding of such factors may also interfere with annealing of longer antisense RNAs, such as E2-200, to the 3′ splice site region. It is not clear whether stable or transient annealing of complementary RNAs is required for inhibition. Only 25–50% of the input substrate is recovered as duplex under conditions where inhibition is nearly complete. While this probably reflects the stringency of the assay, in that partially annealed molecules are degraded and only full-length duplexes are scored, it may also indicate that splicing is inhibited by antisense RNAs when they are only transiently or partially annealed to substrate.

At least three distinct activities affect interactions between substrate and antisense RNAs at an early stage in splicing. One of these promotes the annealing of complementary RNA molecules in an ATP-independent manner. A similar activity was noted by Konarska *et al.* (1985). The presence of this activity is demonstrated by the rapid formation of duplex in the presence of nuclear extract (Figures 9 and 10). This rate is greatly accelerated over that observed in the presence of buffer or proteinase K treated extract. Thus annealing is

facilitated by one or more factors in the nuclear extract which include an essential protein component. Another activity, acting slightly later, blocks further annealing of complementary RNAs and may reflect the sequestering of the pre-mRNA within an RNP complex. A third activity which appears to antagonize duplex formation in the presence of ATP probably corresponds to an ATP-dependent unwinding activity reported previously (Konarska *et al.*, 1985; Ruskin and Green, 1985; Solnick, 1985; Wollenzien *et al.*, 1987).

It is possible that one or more of these activities represents factors which interact with pre-mRNA molecules *in vivo* in the absence of antisense RNA and are involved in splicing. For example, the factors which mediate annealing activity may be single-strand-specific nucleic acid binding proteins such as those associated with pre-mRNA *in vivo*. Such proteins, of which T4 gene 32 protein and *Escherichia coli* ssb protein are the best characterized examples, can promote nucleic acid annealing under physiological conditions (Alberts and Frey, 1970; Christiansen and Baldwin, 1977). *In vivo* these proteins facilitate both dissociation and reassociation of complementary DNA strands during replication and recombination (Chase and Williams, 1986). Many abundant hnRNP proteins exhibit single-strand-specific nucleic acid binding properties (Thomas *et al.*, 1981; Kumar *et al.*, 1986, 1987; Swanson *et al.*, 1987). At least one of these proteins, a hnRNP C protein, appears to be essential for pre-mRNA splicing (Choi *et al.*, 1986; Sierakowska *et al.*, 1986).

Inhibition of splicing by RNA complementary to 3′ exon sequences might be explained in a number of ways. Possible mechanisms include: (i) blocking of specific binding sites on the exon; (ii) interference with the scanning of exon sequences by an essential splicing factor; (iii) blocking of a factor that binds specifically to the 3′ end of the splicing substrate; (iv) disruption of pre-mRNA secondary structure important for splicing; (v) disruption of RNP structure required for assembly of a functional RNA-splicing complex. Several points are relevant to a consideration of these models. First, inhibition by blocking binding sites for specific splicing factors on the exon is inconsistent with the ability of truncated pre-mRNAs lacking most of the 3′ exon to undergo efficient splicing. Second, no substantive evidence for any kind of scanning mechanism in mRNA splicing has been reported, despite several efforts to test this type of mechanism (Green, 1986; Padgett *et al.*, 1986; Krainer and Maniatis, 1988). A specific requirement for exon scanning would imply either the presence of a specific entry site for binding of the factor, or a free 3′ end. A free 3′ end is clearly not essential, since RNAs complementary to the 3′ end of some transcripts do not inhibit (Figure 8 and results not shown). Data presented here are most consistent with some type of general perturbation of the splicing process involving disruption of either relatively long-range RNA secondary structure or RNA–protein interactions along the length of the 3′ exon.

Solution studies of secondary structure of these sequences indicate that the 3′ exons and splice site regions have considerably different intramolecular secondary structures. While strong base-pairing interactions between the 3′ exon and upstream splice site sequences were found in ad2-E2a transcripts, the structure of the globin 3′ exon was essentially identical in both spliced messenger and unspliced pre-mRNA, indicating little if any interactions between exon and intron sequences (Munroe, 1984; Munroe and Duthie, 1986; and unpublished results). Thus the similar level of inhibition observed with antisense RNA complementary to globin and ad2-E2a sequences suggests that local secondary structure is not crucial to the inhibitory effects. While a specific role for local RNA secondary structure in splicing cannot be ruled out, these results suggest that inhibition by antisense RNA annealed to distal regions of the 3′ exon interferes with some other aspect of the splicing process.

The rapid annealing of antisense RNA to the pre-mRNA substrate reported here indicates that the substrate is initially present in an open conformation which facilitates annealing of complementary strands. This 'open' complex is then rapidly converted to a closed complex in which exon sequences are sequestered and unavailable for annealing to complementary strands. If the formation of the closed complex is essential for splicing, RNA complementary to the 3′ exon may inhibit splicing by blocking this step. Such a mechanism would be plausible if the transition from the open to closed complex is a concerted one, in which the entire length of the 3′ exon is involved. This transition might be triggered by a general conformational change in the partially assembled RNA–protein complex or by the co-operative binding of proteins along the length of the exon. The resulting complex may be a highly co-operative structure, whose functional state is sensitive to interactions at sites along its entire length. One prediction of this two-state model for RNP assembly is that an antisense molecule which blocks splicing by annealing to the distal end of the 3′ exon should lock in the accessible, open conformation, thereby facilitating late annealing of a second RNA complementary to a separate site on the exon. Further experiments examining the effect of antisense RNAs on RNP complex formation and spliceosome assembly will serve to test this model and characterize these hypothetical early intermediates in the assembly of an active splicing complex.

Materials and methods

Plasmids

A series of plasmids containing sequences from the E2a region of adenovirus 2 was constructed as follows. Plasmid pE2E3 contains cDNA sequences from the spliced early E2a mRNA for the adenovirus DNA-binding protein which were subcloned from a cDNA plasmid (Gattoni *et al.*, 1985) generously provided by Drs J.Stevenin and R.Gattoni (Strasbourg). An E2a cDNA fragment, cut at the *Pvu*II site 6 nt upstream of the 5′ end of exon 2 and at a *Pst*I site inserted next to the *Msp*I site 380 nt downstream of exon 3, was inserted between the *Hin*dIII and *Pst*I sites in pGEM 3 (Promega Biotec) after ligating the *Pvu*II-cut end with *Hin*dIII linker. pEIE was constructed by cutting pE2E3 in exon 2 and replacing E2a cDNA sequences downstream of this site with a genomic fragment containing the remaining portion of exon 2, intron 2 and part of exon 3. The resulting plasmid contained all of exon 2, intron 2 and 1200 nt of exon 3 inserted between the *Hin*dIII and *Sph*I site of pGEM 3. pXPR was prepared by inserting a *Pst*I/*Xho*I fragment with 320 nt of intron 2 and 160 nt of exon 3 between the *Pst*I and *Sal*I sites of pSP65. This plasmid was modified to eliminate the *Ava*I site in the polylinker region by inserting a *Xba*I linker between the adjacent *Sma*I and *Xba*I sites to produce pXPRX.

A further series of plasmids containing sequences from the 5′ half of the human β-globin gene was constructed by subcloning sequences from the 5′ end of the globin insert in pSP64-HβΔ6 (Krainer *et al.*, 1984). pHBS was prepared by excising a fragment containing all of exon 1 and 50 nt of adjacent intron 1 with *Hin*dIII and *Nsp*(7524)I (Amersham) and inserting this fragment between the *Hin*dIII and *Sph*I sites of pGEM 3. pHB500 was constructed by excising the *Hin*dIII/*Bam*HI fragment encompassing the first two exons and intron 1 in pSP64-HβΔ 6 and inserting it between the *Hin*dIII and *Bam*HI sites of pGEM 4 (Promega Biotec). pHBNB was constructed by inserting the *Nsp*(7524)I/*Bam*HI fragment, including 80 nt of intron 1 and 210 nt of exon 2 of human β-globin, between the *Sph*I and *Hin*dIII

sites of pGEM 3 after ligating the *Bam*HI end of the fragment, to a *Bam*HI/*Hin*dIII non-palindromic linker (New England Biolabs). pSP64-H$\beta\Delta$6-IVS1,2 (Krainer *et al.*, 1984) was obtained from Dr A.Krainer (Cold Spring Harbor). Plasmids p3'D55 and p3'S96A containing duplicated 3' splice sites and portions of exon 2 of human β-globin have been described previously (Reed and Maniatis, 1986). These plasmids were the generous gift of Dr R.Reed (Cambridge, MA). Finally an adenovirus E2a/β-globin chimera, pHBE2a, was constructed by inserting the same *Pst*I/*Xho*I fragment of ad2-E2a region present in pXPR between the *Pst*I and *Sal*I sites of pHBS. This fragment contains 330 nt of intron 2 and 160 nt of exon 3 from the E2a region (Figure 2B).

In vitro transcription

RNA transcripts were synthesized *in vitro* from the plasmids described above using either SP6 or T7 polymerase. All transcription reactions contained either an unmethylated (Pharmacia/PL-Biochemical) or 7-*N*-methylated (New England Biolabs) diguanosine triphosphate cap nucleotide, and a single ^{3}H- or ^{32}P-labeled triphosphate ribonucleoside. Cap nucleotide was present in a 10- or 20-fold molar excess over GTP. Transcriptions were carried out as previously described (Melton *et al.*, 1984; Munroe, 1984) for 1–2 h and incubated for 10 min with DNase I to remove template DNA. Following addition of EDTA, RNA was extracted with phenol/chloroform/isoamyl alcohol (50:49:1) and purified from unincorporated nucleotides and digested DNA by either two precipitations with ethanol in the presence of 1 M ammonium acetate, or by G-100 Sephadex column chromatography. After a final ethanol precipitation, RNA was rinsed once with 80% ethanol, dried and dissolved in water pretreated with diethylpyrocarbonate. The integrity of RNA transcripts was checked by autoradiography or fluorography. The concentration of the RNA was calculated from the incorporation of labeled nucleotide.

Specific transcripts were prepared by run-off transcription of plasmids cleaved with restriction enzymes as follows. The chimeric Ad2-E2a/globin transcript was synthesized with SP6 RNA polymerase from pHBE2a cut with *Bam*HI immediately downstream of the insert. Antisense RNAs complementary to the third exon of the E2a mRNA were transcribed with SP6 RNA polymerase from pXPR cut with *Hae*III (E3-160) and from pXPRX cut with *Ava*I (E3-130). Globin antisense molecules EI-200, EI-130, EI-70 and I-40 which are co-terminal at their 5' ends in intron 2 were transcribed with T7 RNA polymerase from pHBS cut with *Hin*dIII, *Mst*II, *Fok*I and *Mae*III respectively. Globin antisense RNAs E2-200, E2-130, E2-70 and E2-30 complementary to the second exon were transcribed with T7 RNA polymerase from pHB500 cut with *Acc*I, *Mst*II, *Bst*NI and *Dde*I. The 500-nt globin pre-mRNA substrate containing 209 nt of exon 2 was transcribed either with SP6 polymerase from pSP64-H$\beta\Delta$6 cut with *Bam*HI or with T7 RNA polymerase from pHB500 cut with *Bam*HI. These two substrates differed only by 5–9 nt at the capped 5' end and were identical in their stability and splicing efficiency *in vitro*. The truncated globin substrate containing 53 nt of exon 2 was synthesized from pHB500 cut with *Xho*II using T7 RNA polymerase. Transcripts containing duplicated 3' splice sites were transcribed from p3'D55 and p3'S96A cut with *Bam*HI. Sense RNAs representing the 5' or 3' halves of the 500-nt globin substrate were transcribed with SP6 RNA polymerase from pHBS cut with *Eco*RI or with T7 RNA polymerase from pHBNB cut with *Xmn*I respectively.

In vitro splicing

Nuclear extracts were prepared according to the procdure of Dignam *et al.* (1983). The final extract was dialyzed against either 20% or 5% glycerol. Several different extracts were used in the course of this work, differing slightly in their splicing activity, nuclease activities and annealing activities. Dr Krainer generously provided one sample of nuclear extract. Splicing reactions were carried out exactly as described by Krainer *et al.* (1984) except that 12 μl of nuclear extract was used in each 25-μl reaction. Substrate concentration was 1 nM unless indicated otherwise. Splicing reactions were incubated at 30°C for 1–4 h as indicated. Following incubation, RNA was isolated as previously described (Krainer *et al.*, 1984) and analyzed on urea–polyacrylamide gels containing 4–6% polyacrylamide and 7 or 8.3 M urea. RNA samples were heated at 85–95°C in either 5 M urea or 98% formamide to dissociate RNA/RNA duplexes prior to electrophoresis.

Nucleic acid hybridizations

The band corresponding to chimeric spliced product was excised and electroeluted from urea–polyacrylamide gels. RNA/DNA hybridization was carried out in 80% formamide, 0.04 M Pipes (pH 6.5), 0.4 M NaCl, 1 mM EDTA for 2 h at 55°C with ^{32}P-labeled RNA and a 50-fold molar excess of plasmid DNA prior to digestion with RNase A and RNase T1 as previously described (Zinn *et al.*, 1982). RNA/RNA annealing prior to splicing was carried out at 45°C under similar conditions. Assays of RNA/RNA annealing in nuclear extract were carried out by adding 500 or 2500 U/ml T1 RNase to the splicing reactions followed by incubation for 10 min at 30°C. RNA was extracted as usual following treatment with proteinase K (0.25 mg/ml) for 30 min at 30°C.

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