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Differential Block of U Small Nuclear Ribonucleoprotein Particle Interactions during In Vitro Splicing of Adenovirus E1A Transcripts Containing Abnormally Short Introns

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We have studied the consequences of decreasing the donor site-branch site distance on splicing factor-splice site interactions by analyzing alternative splicing of adenovirus E1A pre-mRNAs in vitro. We show that the proximal 13S donor site has a *cis*-inhibiting effect on the 9S and 12S mRNA reactions when it is brought too close to the common branch site, suggesting that the factor interactions in the common 3′ part of the intron are impaired by the U1 small nuclear ribonucleoprotein particle (snRNP) binding to the displaced 13S donor site. Further analysis of the interactions was carried out by studying complex assembly and the accessibility to micrococcal nuclease digestion of 5′-truncated E1A substrates containing only splice sites for the 13S mRNA reaction. A deletion which brings the donor site- branch site distance to 49 nucleotides, which is just below the minimal functional distance, results in a complete block of the U4-U5-U6 snRNP binding, whereas a deletion 15 nucleotides larger results in a severe inhibition of the formation of the U2 snRNP-containing complexes. Sequence accessibility analyses performed by using the last mini-intron-containing transcript demonstrate that the interactions of U2 snRNP with the branch site are strongly impaired whereas the initial bindings of U1 snRNP to the donor site and of specific factors to the 3′ splice site are not significantly modified. Our results strongly suggest that the interaction of U1 snRNP with the donor site of a mini-intron is stable enough in vitro to affect the succession of events leading to U2 snRNP binding with the branch site.

Splicing of pre-mRNAs in eukaryotes is performed within a multicomponent ribonucleoprotein complex, known as the spliceosome (6; for reviews, see also references 33 and 44). The assembly of the spliceosome requires a very intricate succession of events, since at least four small nuclear ribonucleoprotein particles (the U1, U2, U4-U6, and U5 snRNPs) and a set of protein factors are involved. Analyses of this process have been performed by biochemical approaches including native gel electrophoresis (21, 26, 54), RNase protection experiments (7, 23, 39), directed cleavage of U snRNP (1, 3, 12, 25), and affinity chromatography (2, 5, 17, 37). Moreover, genetic (34, 42, 50, 52, 53) and immunological (3, 7, 10) approaches have completed these studies.

These approaches have yielded a considerable amount of information about the different steps of spliceosome formation. U1 snRNP binds to the donor site by means of base pairing between sequences at the 5′ end of the U1 snRNA and the 5′ exon-intron junction (42, 52). U2 snRNP binding to the pre-mRNA, which is facilitated by base pairing between the branch site sequence and an internal region of the U2 snRNA (30, 34, 35, 50, 53), also requires the prior interaction of one or several factors with the 3′ splice site. Involvement of a factor, designated U2AF, has been clearly demonstrated (40, 51), but other factors, either related to U5 snRNP (16, 45) or not (14), could also be required. Binding of U2 snRNP results in the formation of a stable presplicing complex, which can be detected after nondenaturing gel analysis (21, 22). The next step involves the binding of U4-U6 and U5 snRNPs, probably as a preformed complex (4, 11, 22) and leads to the formation of the active spliceosome (2, 22). However, there remain uncertainties concerning the earliest events preceding the stable U2 snRNP binding to the pre-mRNA. For example, although a pivotal role for U1 snRNP in yeasts has been demonstrated, in which the donor site and the branch site are clearly involved (27, 37), a similar role for U1 snRNP in higher eukaryotes seems less evident. Zillmann et al. (54, 55) have shown that site-directed degradation of U1 snRNA leads to the absence of presplicing complexes, suggesting that U1 snRNP could have a primary role in the early events linked to the 3′ splice site and/or branch site recognition. In apparent contradiction to this observation, it has been shown that a purified fraction containing protein factors and U2 snRNP is sufficient to obtain a stable binding of U2 snRNP to a minimal substrate containing a branch site and 3′ splice site (24, 40). Moreover, pre-mRNAs which are mutated in the donor site are still able to form U2 snRNP-containing complexes (2, 21, 26, 36), thus suggesting that early events occurring at the levels of the 5′ and 3′ splice sites are not necessarily related. To further complicate this situation, U1 snRNP-donor site interactions are not stable in the majority of experimental conditions, unlike the U2 snRNP-branch site interactions (2, 21, 54, 55), and several groups have proposed that the first crucial event in the constitution of the spliceosome is the binding of the U2 snRNP to the branch site (23, 26). Therefore, for higher eukaryotes, the predominance of the role of U1 snRNP in the early events of the spliceosome assembly is not established definitively, and it was necessary to find new approaches to analyze this problem.

The analysis of transcripts containing introns too short to

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be efficiently excised (mini-introns) could be interesting in this respect. Previous analyses of intron selection mutants first showed that an intron size above 60 to 80 nucleotides (nt) is required to obtain efficient splicing (38, 46, 49). It was proposed (13, 38) and then demonstrated (20, 43) that the minimal intron length requirement for splicing is based on the distance between the donor site and branch site. This minimal distance is presumed to reflect spatial requirements for the simultaneous interactions of large splicing factors with each other and with the intron. With mini-intron-containing transcripts, it can be anticipated that the succession of events continues until the mechanism jams as a result of the abnormal proximity of donor and branch sites. Detailed analyses of the interactions still occurring with these substrates should provide useful information about the earliest splicing events because, except for the small size of the intron, all other parameters of the splicing assay are kept normal.

By using adenovirus E1A pre-mRNAs which are progressively deleted in the 13S intron sequence (46, 47) as substrates in an in vitro splicing system, we made an unexpected observation suggesting that the binding of U1 snRNP to the donor site of a mini-intron-containing transcript strongly impairs the U2 snRNP-branch site interaction. This prompted us to use this model to analyze in detail the consequences of the shortening of the intron on the interactions between splicing factors and splice sites and on the assembly of the splicing complex.

MATERIALS AND METHODS

Plasmid construction. The wild-type plasmid Sp4 contains the natural E1A sequences from positions 533 to 1342 (*Xba*I site) of the adenovirus type 2 genome, inserted in the pSP65 vector polylinker (41). The modified Sp4-91, Sp4-101, and Sp4-111 plasmids were constructed by replacing the wild-type *Xma*I-*Xba*I fragment (positions 1006 to 1342) of the Sp4 plasmid by the corresponding fragment isolated from the PJU-91, PJU-101, and PJU-111 clones, respectively (a kind gift from the group of G. Akusjärvi [46]). The Sp4 ΔD plasmid was constructed as follows. First, 12 bp was deleted from the polylinker, which removes the *Acc*I site. Then the 13S donor site was removed by cleavage at the *Acc*I site located 7 bp upstream of the 13S donor site, followed by mild *Bal* 31 digestion, which deleted 19 bp from positions 1101 to 1119. Sp4ΔA-X was constructed from Sp4-111 by removing the polylinker *Acc*I and the E1A sequence between the *Acc*I and *Xho*I sites.

The Sp1 clone family contains the *Xma*I-*Xba*I fragment of the E1A unit inserted in the pSP65 vector (41). Sp1-91, Sp1-101, and Sp1-111 are derived from the corresponding Sp4 clones. The Sp1-92 clone is derived from the PJU-92 construction (47). This last clone contains, in addition to the deletion of 23 bp as in Sp1-91, another deletion of 19 bp from positions −35 to −17 upstream from the intervening sequence (IVS) exon 2 junction, which removes the branch site region. The Sp1-112 clone is derived from Sp1-92 but contains, instead of its 23-bp deletion upstream of the *Xho*I site, the 51-bp deletion of the Sp1-111 construction.

RNA synthesis and in vitro splicing. DNA templates linearized in the pSP65 polylinker with *Hin*dIII were transcribed with SP6 RNA polymerase, using a cap analog (GpppG), and RNAs uniformly labeled with [α-^{32}P]CTP (470 Ci/mmol) were treated with DNase I and deproteinized as previously described (41). Standard splicing reactions were carried out in a 25-μl reaction mixture containing 10 μl of HeLa cell nuclear extract and ca. 50,000 cpm of ^{32}P-labeled transcript (ca. 2 ng), as described previously (41). Incubation was carried out at 30°C for various times, depending on the experiments. For direct analysis of the RNA on acrylamide gels, splicing reactions were stopped by the addition of sodium dodecyl sulfate (SDS) and proteinase K, and phenol-$CHCl_3$ extraction was performed.

Oligonucleotide-directed cleavage of snRNAs in nuclear extract. Cleavage of individual small nuclear RNAs (snRNAs) within the nuclear extract was performed, as described by Black et al. (3), in the presence of RNase H and oligonucleotides. The U1-specific oligonucleotide was complementary to U1 snRNA nucleotides 1 to 15. U2-specific oligonucleotides complementary to nucleotides 1 to 15 (U2 5′) or 28 to 42 (U2 loop) are identical to oligonucleotides E15 and L15 used previously (3). RNase H digestion was carried out for 50 min in a 24-μl reaction mixture containing 10 μl of nuclear extract, 0.8 U of RNase H, and either 0.6 μg of U2 loop, 0.6 μg of U1, or 0.2 μg of U2 5′ oligonucleotides, under splicing conditions described above, before the addition of labeled transcripts. The efficiency of cleavage of U1 and U2 snRNAs was greater than 95%, and the residual splicing efficiency was lower than 10%.

Nuclease treatments. In standard experiments, nuclease digestion was carried out after incubation of the substrates under splicing conditions for 15 min at 30°C. Micrococcal nuclease (MNase) digestion under mild conditions was performed in the presence of 3 to 12 U of MNase/ml of nuclear extract and 1 mM $CaCl_2$ for 15 min at 26°C. Other conditions used for digestion are indicated in the results. For naked-RNA digestion, the final concentration of MNase was 250- to 500-fold lower. Reactions were stopped by incubation in the presence of 4 mM EDTA, 0.5% SDS, 30 mM Tris-HCl (pH 8.3), and 0.1 mg of proteinase K per ml for 1 h at 30°C, and the cleavage products were purified by phenol-$CHCl_3$ extraction.

For RNase T_1 digestion, the samples were incubated with a final concentration of 20 to 50 U of RNase T_1 per ml of nuclear extract for 15 min at 30°C. Reactions were stopped by phenol-$CHCl_3$ extraction in the presence of 0.5% SDS. After ethanol precipitation, nuclease cleavage products were analyzed either directly on sequencing gel or by primer extension experiments.

Primer extension analysis. Primer extension was performed by using oligodeoxynucleotides complementary to sequences downstream of the intron-exon 2 junction as indicated in the figure legends. A sample of MNase- or RNase T_1-treated RNA (2 to 3 fmol) was coprecipitated and hybridized with an excess of 5′-end-labeled primer (15 to 20 fmol), and the primer was extended by avian myeloblastosis virus reverse transcriptase as previously described (41). The cDNA products were analyzed on 8% acrylamide gels.

Nondenaturing gel electrophoresis of splicing complexes. Complex assembly analysis was performed as previously described (21). After addition of heparin to 0.6 mg/ml, aliquots of the splicing-reaction mixture (5 μl) were loaded directly on 4% polyacrylamide (acrylamide/bisacrylamide ratio, 80:1) gels in 50 mM Tris-glycine buffer. Gels were run at a constant voltage of 10 V/cm for 4 to 5 h at 10 to 15°C and were dried prior to autoradiography.

RESULTS

***cis*-Inhibition of 12S and 9S mRNA splicing reactions by the donor site of the 13S mini-intron.** Previous in vivo studies of the sequence requirements for the occurrence of the 13S

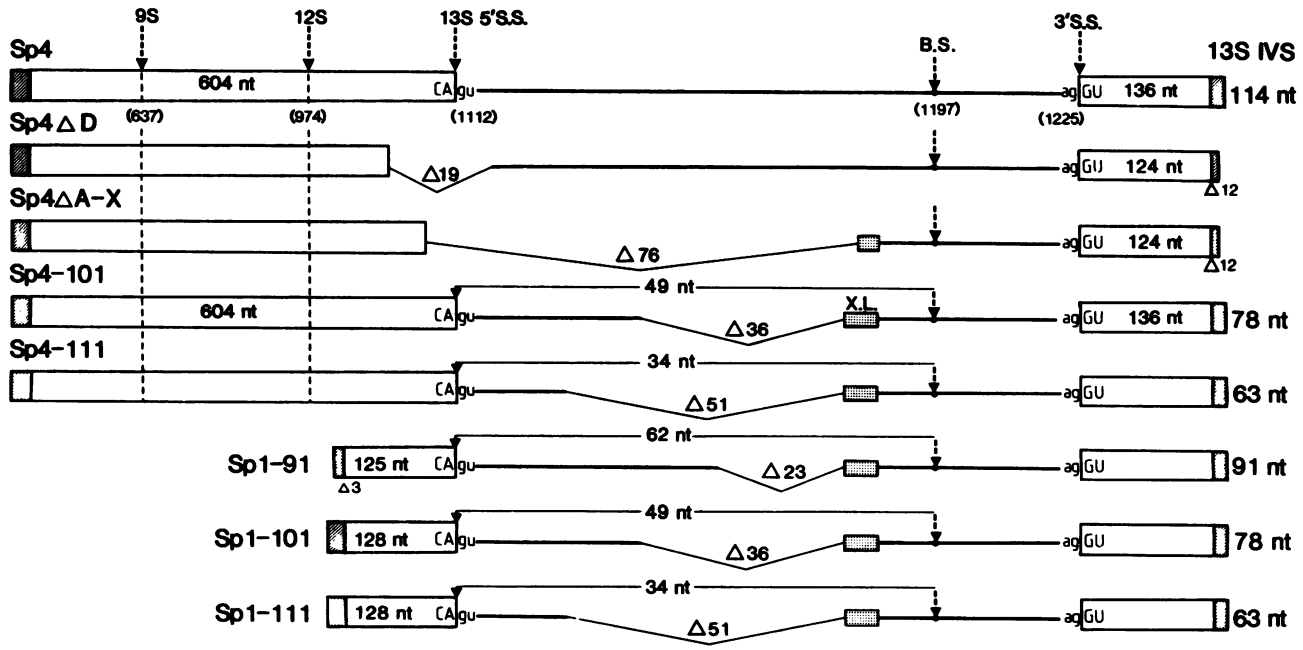

FIG. 1. Schematic representation of mRNA precursors derived from the adenovirus type 2 E1A unit. The schematic structure of Sp4 and Sp1 precursors is given. Symbols: □, exons; ——, introns; ▨, pSP65 sequences present at each end of the E1A transcripts; ▩, *Xho* linker sequence (denoted X.L.). The positions of the splice sites (5'S.S. and 3'S.S.) and of the branch sites (B.S.) are numbered from the 5' end of the genome. The net deletion for each transcript is indicated, and the size of the 13S IVS is given at the right.

mRNA splicing reaction from E1A transcripts containing progressively increasing deletions within the 13S intron had shown that the splicing of a 13S intron of 78 nt or shorter was dramatically or completely inhibited, respectively (46). To verify that the sequence requirements for in vitro and in vivo splicing were similar, we used the same constructions, inserted in the Sp4 clone (Fig. 1), and subjected the resulting transcripts to splicing under saline conditions that allow the alternative splicing reactions for the 13S, 12S, and 9S mRNAs (41). The wild-type E1A substrate (Sp4) is spliced predominantly into 13S mRNA, which accumulates as a 740-nt species after 2 h of incubation (Fig. 2). In contrast, the 9S mRNA reaction, which can be assessed by the accumulation of its corresponding mRNA of 265 nt or, even better, by that of the 9S intron, is only a minor reaction, as during the early period of adenovirus infection (41). As noted previously, the 12S mRNA reaction is relatively underrepresented in vitro with the wild-type substrate (41). The 12S mRNA, which is expected to accumulate only after a 1-h incubation as a 602-nt RNA species, is poorly separated from the 13S exon 1 (604 nt), which is the major species at 30 min of incubation.

Splicing assays with the Sp4-101 transcript, which has a 13S intron of 78 nt, show that the 13S reaction is already strongly inhibited since the 13S exon 1 and the 13S mRNA are practically undetectable after 30 min and 2 h of incubation, respectively. This result is in agreement with results of the in vivo studies in which a 30-fold reduction of the 13S splicing was obtained (46), indicating a similarity between the in vivo and in vitro studies. The expected consequence of a reduction of 13S splicing was that the 12S and 9S mRNA reactions should be stimulated with this deleted transcript as well as with the Sp4-111 transcript, which has a 13S intron of 63 nt (Fig. 1), since all of the pre-mRNA molecules become available for these two splicing reactions. Such a result is obtained with the Sp4ΔD transcript, in which a 19-nt deletion, which precisely removes the 13S donor site (Fig. 1), results in a dramatic stimulation of the 9S and 12S mRNA reactions, compared with the wild-type Sp4 transcript (Fig. 2). However, although the Sp4-101 and Sp4-111 transcripts do not undergo the 13S splicing reaction, the 12S and 9S mRNA reactions were not significantly stimulated and were even partially inhibited with the Sp4-111 transcript (Fig. 2). To determine whether the deletions within the Sp4-111 and Sp4-101 transcripts resulted in the loss of essential sequences for 12S and 9S splicing, we tested another deleted Sp4 substrate (Sp4ΔA-X) in which the deletion of the Sp4-111 transcript was extended up to 3 nt upstream from the 13S donor site (Fig. 1). With this last transcript, the inhibition of the 12S and 9S mRNA splicing observed with the Sp4-111 transcript was relieved (data not shown). Therefore, these results indicated that it is the presence of the 13S donor site which has a *cis*-inhibiting effect on the 12S and 9S mRNA reactions within the mini-intron transcript, rather than the loss of essential sequences.

Previously, it was demonstrated that synthetic donor sites inserted close to the genuine donor site or cryptic donor sites of the β-globin gene, although inactive for the splicing reaction, interact with the U1 snRNP factor (8, 29). Thus, the most plausible explanation for our results was that U1 snRNP may normally interact in vitro with the 13S donor site of the Sp4-111 transcript. However, its binding abnormally close to the 3' part of the intron common to the 13S, 12S, and 9S reactions could result in disturbances of the interactions between the 3' splice site and/or the branch site and their specific factors. Since this could represent one of the mechanisms by which the splicing of transcripts containing a mini-intron is inhibited, we have further analyzed this phenomenon.

Splicing complex assembly with transcripts containing nor-

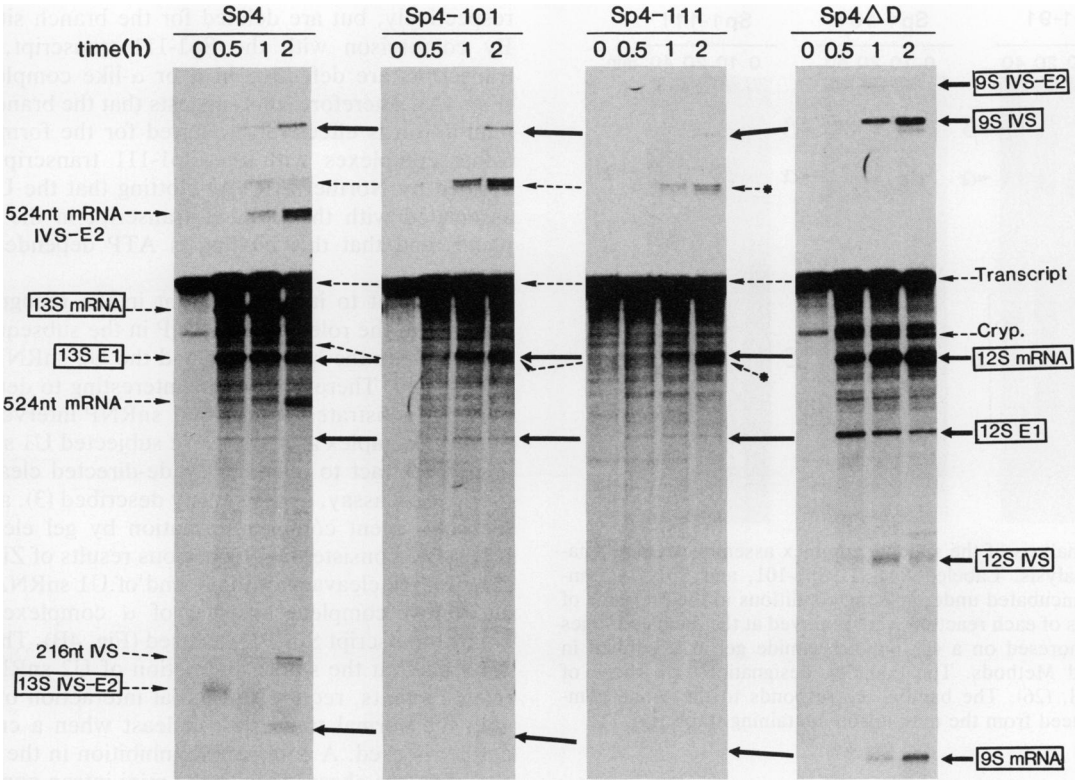

FIG. 2. Analysis of in vitro splicing products of Sp4 and deleted Sp4 transcripts. The different ^{32}P-labeled transcripts were processed for the times indicated, and the splicing products were analyzed on a 5% denaturing polyacrylamide gel as described in Materials and Methods. Intermediate and final products of the 13S splicing reaction, as well as those of the minor 216-nt intron reaction, which produces the 524-nt mRNA from the 13S mRNA, are indicated on the left (9, 15). The 216-nt intron is located between the 9S donor site and a 3′ splice site 216 nt downstream. The splicing products of the 12S and 9S mRNA reactions, except the 9S exon 1, which migrates below the 9S mRNA, are indicated on the right. The triplet below the 9S IVS and the minor band near the 12S mRNA, which are both marked with an asterisk, are the IVS-E2 intermediate and mRNA species produced by the 216-nt intron reaction on the initial transcripts Sp4, Sp4-101, and Sp4-111. The minor band (denoted Cryp.) on the right corresponds to the mRNA species formed from the Sp4ΔD transcript by the use of the cryptic donor site at position 1023 and of the common acceptor site at position 1226 (52).

mal or abnormally short introns. To facilitate studies of interactions between splicing factors and splicing sites from normal and abnormally short introns, we used truncated forms of the Sp4 transcripts, synthesized from the Sp1 plasmid family, which start 109 nt upstream from the 13S donor site (Fig. 1). The Sp1-91 substrate, which has a *Xho* linker (like the other modified substrates) but only a 23-nt deletion in the intron, undergoes efficient splicing and was chosen as a control transcript. We verified that the 13S mRNA reaction had a poor efficiency (lower than 2 to 3%) with the Sp1-101 transcript and was completely inhibited with the mini-intron-containing transcript Sp1-111. With this last transcript, the use of the cryptic donor site located 89 nt upstream of the 13S donor site (52) results in a very minor reaction, with a splicing efficiency lower than 2 to 3%.

To obtain information about the specific step (or steps) at which splicing defaults, we analyzed the ability of the modified E1A transcripts to assemble into presplicing and splicing complexes. Complex formation was monitored by native gel electrophoresis, which, however, results in the release of U1 snRNP from splicing complexes (21, 22). The time course of complex formation with the control transcript (Sp1-91) shows a classical pattern as initial complexes (Σ complexes), formed instantaneously at time zero, are rapidly transformed into U2 snRNP-containing complexes (α complexes) (Fig. 3). Thereafter, β complexes, which also contain U4, U5, and U6 snRNPs, are detected after a lag time of ca. 10 min, and these then accumulate. The Sp1-101 transcript, containing the 78-nt intron, is efficiently assembled into α complexes since a high accumulation of these complexes is observed with time (Fig. 3). However, only a faint band of β complexes is detected, which is compatible with the low efficiency of splicing for this transcript. With the Sp1-111 transcript, which contains the mini-intron, a complex with an electrophoretic behavior similar to that of α complexes was formed, but with an efficiency reduced by a factor of ca. 3 to 5 compared with the previous transcript. Thus, the data in Fig. 3 clearly demonstrate that the splicing block for the 78-nt intron transcript is primarily at the level of the U4-U5-U6 snRNP-binding step. In contrast, the formation of α complexes is already severely impaired with the mini-intron transcript, suggesting that the main splicing defect is at the level of the U2 snRNP binding or at an earlier step. Because we do not know whether the complexes assembled from the mini-intron transcript represent the genuine α complex, since complexes with similar characteristics can also be formed in the absence of potential donor site sequences (21, 24), we have designated them α-like complexes (α′ complexes in the figures).

Requirements for the α or α-like complex assembly. To

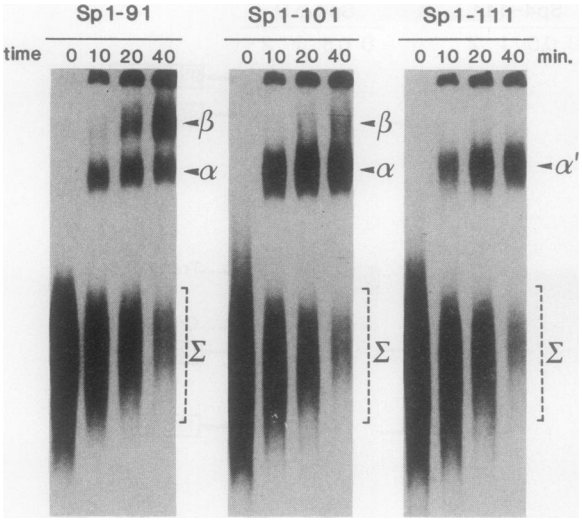

FIG. 3. Analysis of the splicing complex assembly by nondenaturing gel analysis. Labeled Sp1-91, Sp1-101, and Sp1-111 transcripts were incubated under splicing conditions in the presence of ATP. Samples of each reaction were removed at the indicated times and electrophoresed on a 4% polyacrylamide gel as described in Materials and Methods. The complex designations are those of Lamond et al. (26). The band α′ corresponds to the α-like complexes, produced from the mini-intron-containing transcript.

investigate whether the same sequence elements are required in the formation of complexes with normal intron- or mini-intron-containing transcripts, we next analyzed the assembly of other modified Sp1 transcripts. These new transcripts, Sp1-92 and Sp1-112 (see Materials and Methods for their detailed structure), contain the 5′ and 3′ splice sites of the 13S intron, separated by a sequence of 72 or 44 nt, respectively, but are deleted for the branch site sequence. By comparison with the Sp1-111 transcript, both these transcripts are defective in α or α-like complex assembly (Fig. 4A). Therefore, this suggests that the branch site of the mini-intron is effectively required for the formation of the α-like complexes with the Sp1-111 transcript. We have verified by Northern (RNA) blotting that the U2 snRNP is associated with the Sp1-111 transcript in the α-like complexes and that this binding is ATP dependent (data not shown).

In contrast to its involvement in the recognition of the donor site, the role of U1 snRNP in the subsequent interaction between the branch site and the U2 snRNP is not well understood. Therefore, it was interesting to determine with our E1A substrates whether U1 snRNP intervened in the α or α-like complex assembly. We subjected U1 snRNP of the nuclear extract to oligonucleotide-directed cleavage before the splicing assay, as previously described (3), and analyzed the subsequent complex formation by gel electrophoresis (Fig. 4B). Consistent with previous results of Zillmann et al. (54, 55), the cleavage of the 5′ end of U1 snRNA resulted in an almost complete absence of α complexes when the control transcript Sp1-91 was used (Fig. 4B). Therefore, this confirms that the stable interaction of U2 snRNP, or other related events, requires an initial interaction of U1 snRNP with the normal transcript, at least when a crude nuclear extract is used. A comparable inhibition in the assembly of complexes is observed with the mini-intron-containing transcript (Fig. 4B). Moreover, we have observed that two oligonucleotides complementary to U2 snRNA (U2 5′ and U2 loop) also allow inhibition of α- or α-like-complex assembly when present at a sufficient concentration to completely cleave U2 snRNA (data not shown). Therefore, it appears that α and α-like complexes are indistinguishable on the basis of the above criteria.

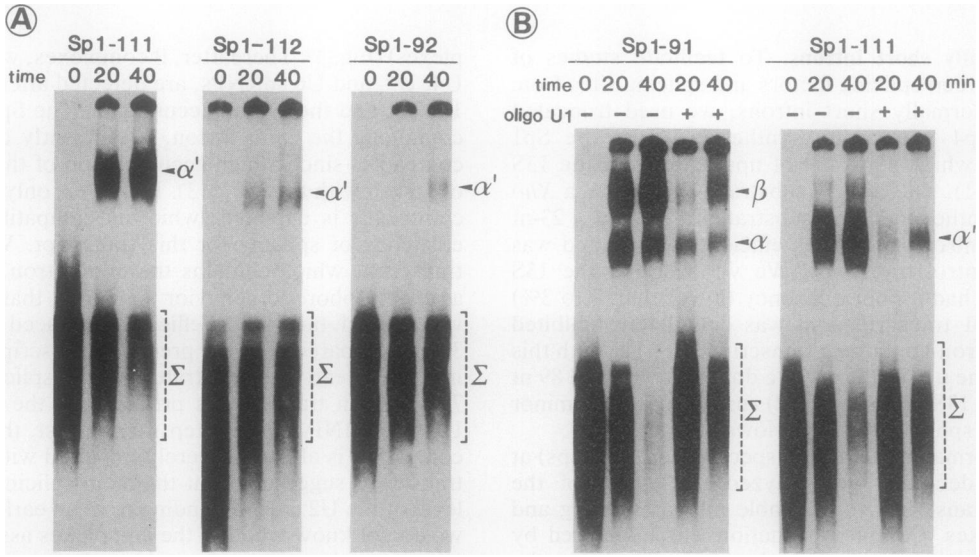

FIG. 4. Requirements for the α- and α-like-complex assembly. (A) *cis* requirements for complex assembly. The Sp1-92 and Sp1-112 transcripts, which contain introns of 72 and 44 nt, respectively, and the Sp1-111 transcript were incubated in a standard splicing reaction for the indicated times, and the complex assembly was analyzed as in Fig. 3. (B) U1 snRNP requirements for complex assembly. The Sp1-91 and Sp1-111 transcripts were incubated under splicing conditions, with nuclear extract pretreated with an oligonucleotide complementary to the 5′ end of U1 snRNA in the presence of RNase H. The autoradiograms in panels A and B were overexposed to clearly reveal weak complex formation.

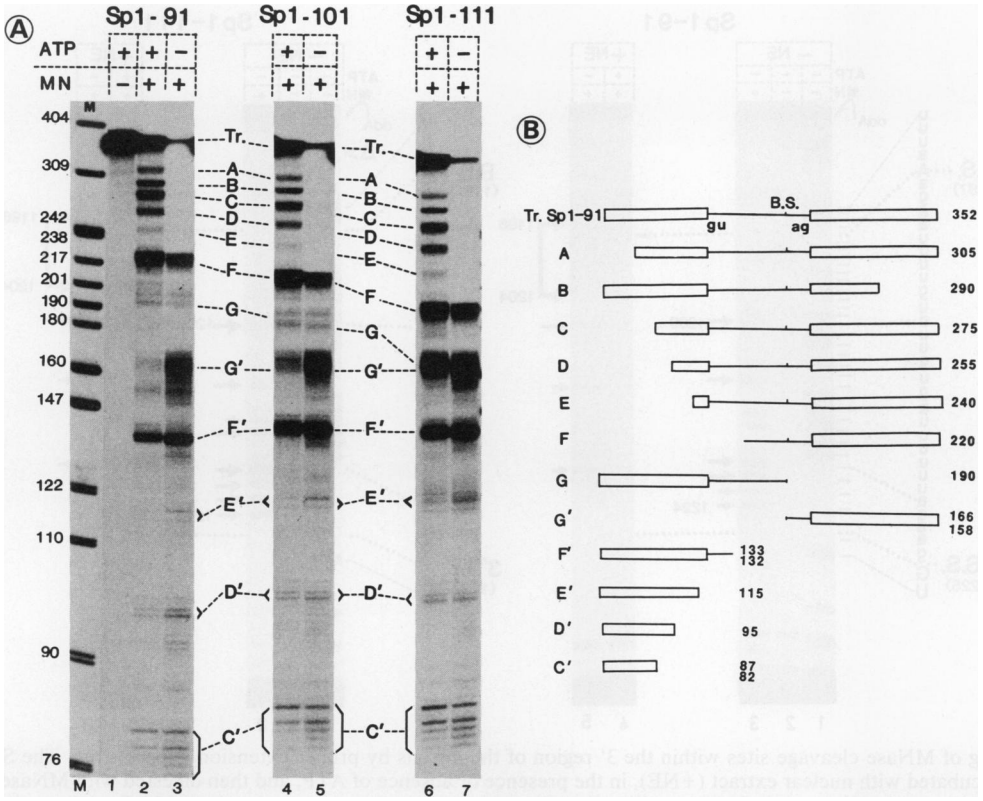

FIG. 5. Accessibility of the transcript sequence to digestion with MNase under mild conditions. (A) The uniformly labeled Sp1-91, Sp1-101, and Sp1-111 transcripts were incubated under splicing conditions in the presence (+) or absence (−) of ATP for 15 min at 30°C. The samples were treated with MNase, and the resulting RNA fragments were analyzed on a denaturing 8% polyacrylamide gel. Note that in the absence of MNase, no significant cleavage occurs in the presence of ATP (lane 1) or in its absence (not shown). The different digestion products resulting from a single cleavage of the substrates are denoted from A to G and their respective counterparts from A′ to G′ (see also panel B). An end-labeled *Msp*I digest of pBR322 is used as size markers in lane M. (B) Schematic representation of Sp1-91 digestion products. Symbols: ▭, exon sequences; ——, intron sequences. The size of the RNA fragments for the Sp1-91 transcript is given in nucleotides on the right.

Accessibility of E1A transcript sequences to nuclease digestion. To assess interactions between splicing factors and pre-mRNA sequences, we determined the accessibility of the RNA sequences to a mild nuclease digestion. This approach, used under conditions where statistically one or only a few cleavages occur on each pre-mRNA molecule, is similar to the footprinting analysis of DNA sequences. Under these conditions, in addition to stable interactions, even weak interactions can be deduced. The use of MNase was interesting because its sequence specificity for cleavage (X-A and X-U dinucleotides for RNA sequences with X representing A, U, or G nucleotides [see below]) includes motifs which are frequently found within the splice site and branch site sequences. MNase digestion was performed after a 15-min incubation of the transcript with nuclear extract, which allows primary interactions between splicing factors and pre-mRNA sequences as well as a sufficient accumulation of the presplicing complexes. The extent of the digestion and a rough localization of the cleavage sites were first assessed by a direct gel analysis of the RNA fragments, which were all detected by means of a uniform labeling of the transcripts (Fig. 5A). The fragments (5′ or 3′ parts) were denoted A, B, C, ..., according to their decreasing size, and their counterparts were denoted A′, B′, C′, ..., respectively. Their identity has been determined previously, based on determination of their size by using differently truncated E1A substrates and S1 mapping analyses (16a). The location of the cleavage sites has been confirmed by primer extension experiments (see Fig. 6 to 8).

The results of the gel analysis (Fig. 5A) of Sp1-91, Sp1-101, and Sp1-111 transcripts, treated by a mild MNase digestion after their incubation with a nuclear extract, can be summarized in essentially three observations. First, under splicing conditions, a limited set of major fragments is obtained with all transcripts. A subset of the cleavages, located in exons 1 and 2, generate fragments A to E as well as their counterparts. They are not related to the splicing process since they are observed under a variety of conditions including digestion of naked RNA (results not shown). The other cleavages, located within the intron near the donor site or within the branch region, generate fragments F-F′ and G-G′, respectively. We will show below that they are related to the complex assembly (see Fig. 6 and 8). Second, significant changes of the accessibility of the RNA were observed in the absence of ATP during the incubation, notably the very intense broad G′ band that appears after MNase digestion of the three substrates. The G′ band corresponds to their common 3′ part, resulting from a cleavage within the branch site region (Fig. 5B), which is not unexpected when ATP is omitted. The appearance of this highly accessible region and the slight increase in the number of cleavages in the exon sites, as seen by the increase in C′ to E′ fragment

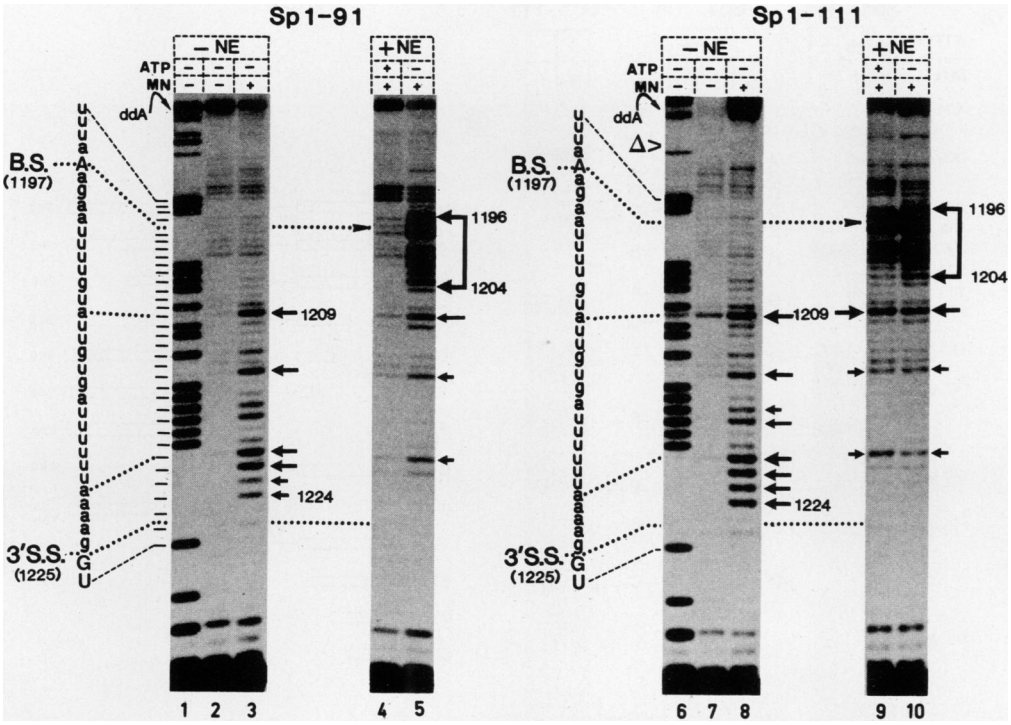

FIG. 6. Mapping of MNase cleavage sites within the 3′ region of the introns by primer extension experiments. The Sp1-91 and Sp1-111 transcripts were incubated with nuclear extract (+NE), in the presence or absence of ATP, and then digested with MNase (lanes 4, 5, 9, and 10) as in Fig. 5. The corresponding naked RNAs (−NE) were digested with a reduced concentration of MNase (lanes 3 and 8) to the same extent as RNA incubated under splicing conditions. The presence or absence of ATP and creatine phosphate has no effect on the digestion of naked RNA. Untreated samples (lanes 1, 2, 6, and 7) and MNase-treated samples were subjected to a primer extension analysis by using a 5′-end-labeled oligonucleotide complementary to the 5′ region of exon 2, from nt 10 to 31 downstream of the intron-exon 2 junction. Partial sequencing of transcripts in the presence of ddA is shown in lanes 1 and 6 and is used to position the nucleotide sequence alongside the Sp1-91 and Sp1-111 panels. The major cleavage sites are indicated by arrows, together with their position in the genome. The position of the deletion within the Sp1-111 transcript, compared with the Sp1-91 transcript, is indicated (Δ).

intensities, may explain, at least in part, the dramatic decrease in the amount of intact transcripts and the A to E fragments (Fig. 5A, lanes 3, 5, and 7). Equally, there is no significant accumulation of the G fragment, which might be expected given the dramatic increase of the G′ fragment (Fig. 5A, lanes 3 and 5). Third, a comparison of the accessibility of the three transcripts in the presence of ATP shows that the same digestion pattern was obtained with the control Sp1-91 and the minimally deleted Sp1-101 transcripts. This indicates that the interactions which lead to the formation of α complexes for both transcripts are indistinguishable on the basis of their accessibility to MNase. In contrast, the mini-intron-containing transcript Sp1-111 exhibits a high accessibility of its branch site, even under splicing conditions, as attested by the accumulation of G-G′ fragments in the presence of ATP (Fig. 5A, lane 6). Therefore, these data strongly suggest that shortening the donor site-branch site distance below 40 nt results in perturbations of the interactions between U2 snRNP and the branch site, or of an earlier step in complex assembly.

Interestingly, for all transcripts incubated in the presence or absence of ATP, the accessibility pattern around the donor site did not change significantly (Fig. 5A, fragments F-F′), suggesting that the U1 snRNP-donor site interactions are not modified by the larger deletion within the intron. We will show below (see Fig. 8) that these interactions create a hyperaccessible region 8 to 13 nt downstream from the 5′ splice site. We have also verified that the results obtained in Fig. 5 with the mini-intron-containing transcript do not change significantly when the time of incubation which precedes the MNase treatment is increased from 15 to 40 min. Therefore, the accessibility of the branch site region is not due to a delay in the assembly of the presplicing complexes, which is also in agreement with the data in Fig. 3.

We next analyzed the interactions at the branch site and 3′ splice site level by reverse transcription experiments, using Sp1-91 and Sp1-111 transcripts incubated with nuclear extract and subsequently treated with MNase (as in Fig. 5A) or MNase-treated naked transcripts. A primer complementary to the beginning of exon 2 was used, and the results of the analysis of the 3′ portion of the intron are shown in Fig. 6. With naked Sp1-91 or Sp1-111 RNA (lanes 3 and 8), the presence of reverse transcription stops between positions 1209 and 1224 on the genomic sequence indicates that the 3′ splice site, between positions −2 and −19 from the intron-exon 2 junction, is preferentially cleaved by MNase, whereas the branch site region is not particularly accessible. A better accessibility of the branch site to nuclease S1 and RNases T_2 and T_1 was obtained previously (28), but the analysis was not carried out under saline conditions compatible with splicing.

Dramatic modifications of the accessibility occur in the presence of nuclear extract. With the control Sp1-91 transcript, the 3′ region of the intron, including the branch site sequence, appears completely protected in the presence of

ATP (Fig. 6, lane 4). In the absence of ATP, the 3′ splice site sequence remains relatively protected, whereas very intense cleavages are revealed within the branch site sequence (lane 5). This highly accessible region extends from 2–3 nt upstream to 7 nt downstream of the branch acceptor, and the dinucleotide A-U, located 11 nt downstream of the branch acceptor, is also relatively well exposed. With the mini-intron transcript, however, the branch site region is already accessible to the MNase in the presence of ATP, and the accessibility of this region is only slightly increased in the absence of ATP (lanes 9 and 10). Therefore, the results in both Fig. 5 and 6 strongly suggest that a significant fraction of the mini-intron-containing pre-mRNA molecules are unable to form correct interactions with the U2 snRNP. These data are also consistent with the severe decrease in efficiency of mini-intron RNA in the assembly of α-like complexes (Fig. 3). In contrast, the accessibilities of the 3′ splice site sequence of the control and the shortest transcripts appear similar (Fig. 6 and 7, lanes 2, 3, 8, and 9), suggesting that the interactions which occur at this level are not significantly affected by the shortening of the intron.

Because the above results were obtained under conditions of limited MNase digestion, we verified that they remained valid under a larger range of conditions of MNase treatment. To do this, splicing assays with the Sp1-91 and Sp1-111 transcripts were subjected to treatments with increasing concentrations of MNase (20 to 75 U/ml of nuclear extract), which results in the progressive disappearance of the initial transcripts and A to E fragments (direct gel analysis not shown). These results are illustrated by the primer extension analysis of the RNA fragments (Fig. 7). For the control transcript, we observe that the region 8 to 13 nt downstream of the donor site is the most accessible whereas the branch site sequence is never significantly cleaved (lanes 2 to 5). However, for the mini-intron Sp1-111 transcript, the branch site and the dinucleotide A-U 11 nt downstream become the most exposed region as soon as the MNase concentration is higher than 20 U/ml (lanes 8 to 11), thus confirming the results in Fig. 6. In addition, the 3′ splice site region of both transcripts, which is just upstream of the position of the primer used for the extension analysis, is only weakly cleaved with the highest MNase concentration (Fig. 7, lanes 4, 5, 10, and 11), indicating that the 3′ splice site is relatively well protected, most probably by the factor(s) involved in the 3′ splice site recognition (14, 40, 51). This confirms that the interactions at this level are not disturbed in the case of the mini-intron transcript.

Finally, because our results could be biased by the cleavage specificity of the MNase, we tested another nuclease. By monitoring the accessibility to RNase T_1 of the G residues in the 3′ part of the intron, we found that the G residue located 2 nt downstream of the branch acceptor is much more exposed in the mini-intron than in the normal intron, whereas the three G residues at positions −13, −15, and −20 from the 3′ splice site display a similar accessibility within both introns (data not shown). Therefore, these results strengthen our conclusions based on the MNase accessibility of normal and mini-intron transcripts.

U1 snRNP-donor site interactions are not perturbed with the mini-intron-containing transcript. Our initial hypothesis was that U1 snRNP binding to the 13S donor site remains unchanged when we compare the normal and mini-intron transcripts. Therefore, it was crucial to verify that the absence of MNase cleavages within the 13S donor site sequence and their presence 8 to 13 nt downstream of the exon-intron junction of both transcripts (Fig. 6) truly result

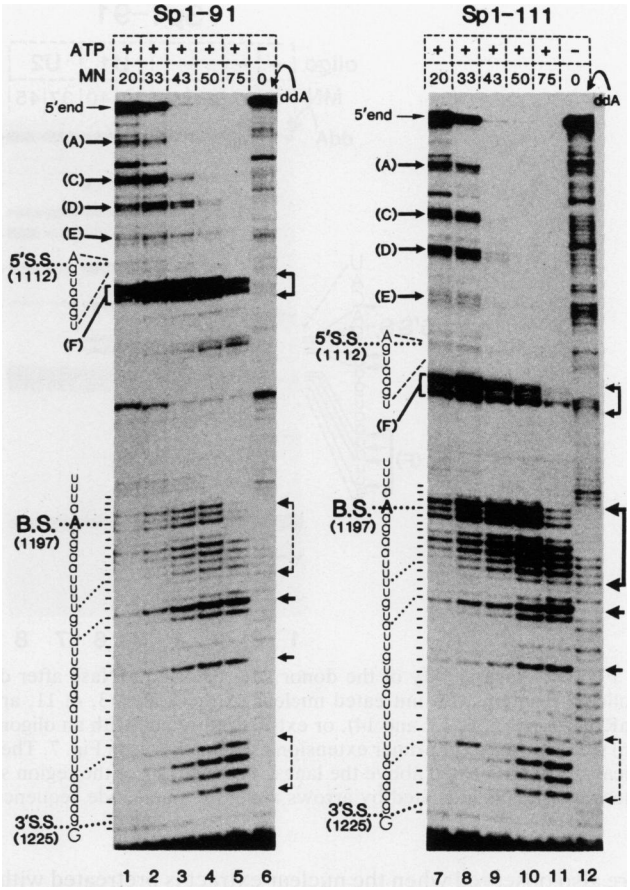

FIG. 7. Nuclease digestion pattern of the Sp1-91 and Sp1-111 transcripts subjected to increasing concentrations of MNase. Both transcripts were incubated under splicing conditions in the presence of ATP and subjected to increasing concentrations of MNase, as indicated above the lanes in units per milliliter of nuclear extract. Analysis of the cleavage sites from the 3′ splice site to the 5′ end of the transcripts was performed by primer extension experiments, by using an oligonucleotide complementary to nucleotides 3 to 25 downstream of the intron-exon 2 junction. The cDNAs corresponding to RNA fragments generated by cleavage in exon 1 and separated in Fig. 5A are indicated to the left of the panels. Partial sequencing of transcripts in the presence of ddA is shown in lanes 6 and 12.

from the binding of U1 snRNP. Consequently, the accessibility of the 13S donor site region was determined when both transcripts were incubated with nuclear extract after specific degradation of U1 or U2 snRNA. Figure 8 shows the analysis of the MNase accessibility by primer extension, focused on the donor site region. In agreement with the data in Fig. 7, we observe identical accessibilities for both control and deleted transcripts incubated with untreated nuclear extract (Fig. 8, lanes 3, 4, 11, and 12). When the U1-pretreated extract is used for the splicing assay, two concomitant features occur for both transcripts. First, MNase cleavages now occur within the donor site, 1 to 3 nt downstream from the exon 1-intron junction (lanes 5, 6, 13, and 14). In addition, a severe decrease in the accessibility of the sequence 8 to 13 nt downstream of the junction is also revealed, indicating that this last region represents a hyperaccessible spot when U1 snRNP is bound to the donor site. Similar changes in the accessibility of the 5′ splice site region

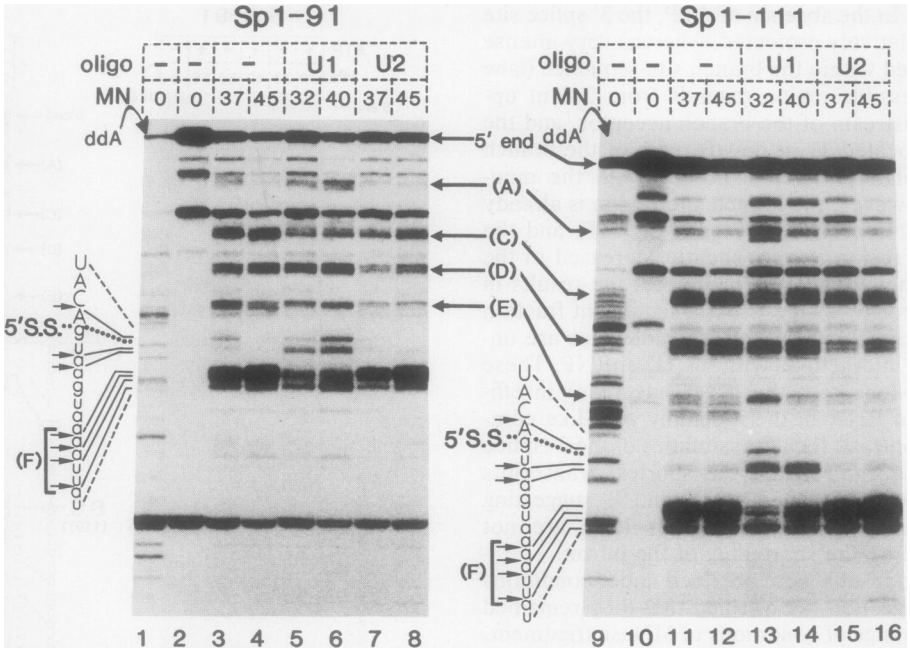

FIG. 8. Accessibility of the donor site region to MNase after directed U snRNA cleavage. MNase digestion products obtained from a splicing reaction with untreated nuclear extract (lanes 3, 4, 11, and 12), extract pretreated with an oligonucleotide complementary to U1 snRNA (lanes 5, 6, 13, and 14), or extract pretreated with an oligonucleotide complementary to the 5′ end of U2 snRNA (lanes 7, 8, 15, and 16) were subjected to primer extension experiments as in Fig. 7. The concentrations of MNase, which are adjusted to obtain equivalent overall cleavage are indicated above the lanes. The analysis of the region surrounding the donor site and above is given. The precise location of the cleavage sites is indicated by arrows along the nucleotide sequence in the region of the donor site.

are also observed when the nuclear extract is pretreated with monoclonal anti-RNP antibodies, which inactivate U1 snRNP (data not shown). Therefore, all of these results demonstrate that the primaray interactions at the level of the 13S donor site are not disturbed with the mini-intron transcript. Interestingly, when a U2-pretreated extract is used for the splicing assay, the accessibility of the donor site for both transcripts is not modified (lanes 7, 8, 15, and 16), indicating that intact U2 snRNP is not required for the primary recognition by U1 snRNP of the donor site.

DISCUSSION

It has been shown that the minimal donor site-branch site distance for efficient splicing of exons 2 and 3 of the rat α-tropomyosin gene (48) lies between 51 and 59 nt (43). This fits well with data obtained for other higher eukaryotic systems: between 47 and 55 nt in the β-globin pre-mRNA (38, 49), between 49 and 64 nt in the E1A pre-mRNA (46; see above) and 46 to 48 nt in the early simian virus 40 pre-mRNA (13). This size requirement implies that events linked directly or indirectly to the donor site and branch site are involved. We have studied the consequences of a reduction of intron size below the minimal functional size on the splicing factor-splice site interactions by monitoring the splicing complex assembly by gel electrophoresis analyses and the modifications in the accessibility of transcript sequences to mild nuclease treatment. The latter presented a series of advantages compared with the nuclease protection experiments: (i) a mild nuclease treatment does not result in the disruption of the presplicing complexes or in the significant hydrolysis of the U snRNP population (not shown), unlike the RNase protection method, as previously noted (55); (ii) as indicated in the Results, even weak interactions can be analyzed; (iii) comparison of the accessibility of a normal transcript in its naked form and under splicing conditions permit detection of all of the expected interactions. This includes the interactions occurring at the donor site with U1 snRNP (Fig. 8), at the 3′ splice site (Fig. 6 and 7), most likely with U2AF (40), and at the branch site with U2 snRNP (Fig. 5 and 6). Interestingly, the branch site region is not particularly accessible in the case of naked RNA, whereas, after incubation with nuclear extract in the absence of ATP, it becomes hyperaccessible (Fig. 5); this might result in the binding of the U2AF on the next 3′ splice site.

The stage at which inhibition of splicing occurs depends on the donor site-branch point distance. The analysis of complex formation by using transcripts containing introns of various lengths shows very clearly that inhibition occurs at different steps, depending on the distance separating the donor site and branch site. When this distance is ca. 50 nt long, as with the Sp1-101 transcript, blocking occurs just after U2 snRNP binding. This result is in agreement with that of Smith and Nadal-Ginard (43), who showed that a distance of 42 nt between the donor site and branch point allows an efficient accumulation of α complexes. The detection of a residual splicing efficiency with the Sp1-101 transcript implies that the architecture of the accumulating α complex (Fig. 3) is not highly modified when compared with that of the bona fide α complexes. The fact that the accessibility of RNA to MNase for this transcript (Sp1-101) is very similar to that for the control transcript (Fig. 5) supports this idea. The requirements for U4-U5-U6 snRNP binding to α complexes are not well known, but there are some indications that these snRNPs interact with the U1 and U2 snRNPs rather than with the transcript (2). However, we show clearly that the

binding of U1 and U2 snRNPs to the Sp1-101 transcript is not sufficient for the subsequent binding of the U4-U5-U6 complex (Fig. 3).

When the distance between the donor site and the branch site is reduced from 49 to 34 nt, formation of the α complex itself is strongly impaired. Similar results are also obtained with the yeast *MATa*1 transcript, in which a donor-branch point distance of 36 nt results in total inhibition of α-complex assembly (20). In agreement with these data, the analysis of the accessibility of the sequences of the mini-intron shows very clearly that a dramatic defect occurs at the level of interactions involving the branch site. The fact that the branch site of a mini-intron remains highly exposed to MNase indicates that the U2 snRNP is unable to recognize and/or to interact, even weakly, with the branch region of a large proportion of the corresponding RNA molecules. In contrast, our accessibility analysis revealed that the donor and acceptor sites are protected as in the control transcript, suggesting that the primary interactions linked to these regions are not disturbed.

Although we do not directly demonstrate that U1 snRNP is involved in the alteration of the branch site-U2 snRNP interactions with the mini-intron transcript, several features are consistent with this idea: (i) in the normal splicing process, the direct or indirect role played by U1 snRNP in the interaction between U2 snRNP and branch site implies that U1 snRNP might interact with the 3′ region of the intron or with factors bound to it and is compatible with the involvement of U1 snRNP in the splicing defect; (ii) the initial observation showing that the 13S donor site has a *cis*-inhibiting effect on the 12S and 9S mRNA splicing reactions is also in agreement with this involvement; and (iii) our data with mini-intron substrates strongly indicate that functional U1 snRNP-donor site interactions precede the interactions between U2 snRNP and the branch site and that the former are stable enough in splicing conditions to affect the subsequent steps involving U2 snRNP. In fact, two mechanisms at least may be proposed to explain the putative involvement of U1 snRNP. First, given its overall size of 13 to 14 nm, which is equivalent in length to an RNA sequence of ca. 40 nt (19), U1 snRNP itself might be unable to develop, after binding with the donor site, functional interactions with the 3′ region of the mini-intron. Second, it is possible that all the steps of recognition of the donor site and 3′ region of the mini-intron in which U1 snRNP is involved are normal. However, given the bulkiness of the U1 and U2 snRNPs, which are roughly equivalent (18, 19), the accessibility of the branch site to the U2 snRNP might be strongly inhibited as soon as U1 snRNP binds to the donor site of the mini-intron. Note that although the branch site is highly accessible to the MNase (Fig. 5 to 7), this can be easily explained by the small size of the enzyme relative to that of the U2 snRNP particle.

In contrast to these ideas, it has been suggested previously that the initial contact of U snRNPs with pre-mRNA might occur via a simultaneous binding of both U1 and U2 snRNP near the 3′ splice junction (55), a mechanism which is also proposed for the recognition of splicing sites of internal introns (36). If this was systematically the case, it would be expected that the subsequent binding of U2 snRNP to the branch site should be relatively insensitive to the size of the intron whereas the U1 snRNP binding with the donor site of a mini-intron might be inhibited. In fact, we observe exactly the opposite with the E1A pre-mRNA, which contains a single intron.

Involvement of the U1 snRNP factor in a modulation of alternative splicing in vivo. Because natural examples of alternative splicing exist in which a donor site (proximal) is located abnormally close to branch sites specifically used with an upstream (distal) donor site, it was interesting to consider whether this abnormal proximity might represent a sufficient constraint to modulate alternative splicing. An example concerns the simian virus 40 early pre-mRNA, for which small-t mRNA splicing involves the use of branch sites at positions −18 and −19 whereas the branch sites at positions −24 and −32 are too close to the small-t donor sites (35 and 43 nt, respectively) and are available only for the large-T splicing reaction (32). In agreement with our data, the results of Noble et al. (31) led them to propose the existence of steric hindrance so that U1 snRNP–small-t donor site interactions might disrupt U2 snRNP binding to the upstream branch site. First, they determined that the branch sites used predominantly for large-T splicing from wild-type pre-mRNA are the downstream branch sites at positions −18 and −19, whereas the upstream site is predominantly chosen when the small-t donor site is deleted. Second, an insertion which results in an increase of the small-t donor site–upstream branch point distance gives the same results.

We also asked whether the inhibition of 12S and 9S mRNA splicing obtained in vitro with the mini-intron E1A substrate (Fig. 2) was also detected in vivo (46). Although a precise determination of the amounts of spliced mRNA is not easy from transfection experiments, it seems clear that a severe inhibition of the 12S mRNA splicing was not detected in vivo with the mini-intron E1A substrate. However, the existence of a moderate inhibition (ca. 30%) of 12S and 9S mRNA splicing was not excluded from their results (46), and it is possible that, in vivo, a mechanism exists which relieves this inhibition at least partially. Finally, it should be noted that the importance of the U1 snRNP-donor site interactions that we observed may be a consequence of the E1A substrate, because the 13S donor site exhibits an intronic sequence identical to the consensus /GUAAGU, whereas the acceptor site sequence $(U)_6$AAAAG/ and the branch site sequence GUUUAAA are far from the corresponding consensus. Therefore, we cannot exclude that, during splicing of transcripts containing abnormally short introns, splicing defects occur through slightly different mechanisms depending on the relative strength of the splice sites and branch site.

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