Human step II splicing factor hSlu7 functions in restructuring the spliceosome between the catalytic steps of splicing

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The spliceosome catalyzes pre-mRNA splicing in two steps. After catalytic step I, a major remodeling of the spliceosome occurs to establish the active site for step II. Here, we report the isolation of a cDNA encoding hSlu7, the human homolog of the yeast second step splicing factor Slu7. We show that hSlu7 associates with the spliceosome late in the splicing pathway, but at a stage prior to recognition of the 3′ splice site for step II. In the absence of hSlu7, splicing is stalled between the catalytic steps in a novel complex, the $C_{\Delta hSlu7}$ complex. We provide evidence that this complex differs significantly in structure from the known spliceosomal complexes, yet is a functional intermediate between the catalytic steps of splicing. Together, our observations indicate that hSlu7 is required for a structural alteration of the spliceosome prior to the establishment of the catalytically active spliceosome for step II.

[*Key Words*: Pre-mRNA splicing; splicing factor; catalytic step II; spliceosome; hSlu7]

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The two catalytic steps of the pre-mRNA splicing reaction take place in the spliceosome, a dynamic complex of snRNAs and a large number of proteins (for reviews, see Moore et al. 1993; Kramer 1996; Reed and Palandjian 1997; Will 1997). In catalytic step I, the branch site adenosine carries out a nucleophilic attack on the 5′ splice site, generating the splicing intermediates (exon 1 and lariat–exon 2). In catalytic step II, the free hydroxyl group on exon 1 attacks the 3′ splice site to generate spliced mRNA and the excised lariat intron. Both transesterifications are thought to be mediated by snRNAs, but proteins play integral roles in configuring the active sites for catalysis.

The catalytic center of the spliceosome for step I is established after a series of spliceosomal complexes assembles on pre-mRNA in the order E, A, B, and C (for reviews, see Moore et al. 1993; Reed and Palandjian 1997). U1 snRNP recognizes the 5′ splice site in the E complex, and U2 snRNP recognizes the branchpoint sequence (BPS) in the A complex. In the B complex, a tri-snRNP particle consisting of U4, U5, and U6 snRNPs replaces U1 snRNP at the 5′ splice site. Prior to catalytic step I, U4 snRNP is released (Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988; Yean and Lin 1991) and an interaction between U2 and U6 snRNPs positions the branch site adenosine for attack at the 5′ splice site (Madhani and Guthrie 1994). The catalytically active spliceosome for both steps of the splicing reaction is referred to as the C complex, which is detected on native gels as a discrete band. However, different configurations of the C complex must exist because the two transesterifications involve different reactive groups (for reviews, see Moore et al. 1993; Staley and Guthrie 1998). In addition, between steps I and II, a rearrangement occurs that repositions U5 snRNP to contact the region between the BPS and the AG dinucleotide (Umen and Guthrie 1995a; Chiara et al. 1997). This interaction may play a role in specifying the AG that serves as the 3′ splice site. U5 snRNP is also thought to align exons 1 and 2 for ligation (for review, see Newman and Norman 1992). Here, we refer to the catalytically active step I spliceosome as the C_I complex and the catalytically-active step II spliceosome as the C_{II} complex.

A great deal of insight into the mechanism of step II has come from studies in yeast (for review, see Umen and Guthrie 1995c). The proteins Prp16 (Couto et al. 1987; Burgess et al. 1990; Schwer and Guthrie 1991), Prp17 (Vijayraghavan et al. 1989; Jones et al. 1995), Prp18 (Vijayraghavan and Abelson 1990; Horowitz and Abelson 1993), and Slu7 (Frank and Guthrie 1992; Ansari and Schwer 1995; Jones et al. 1995) function exclusively in step II, and Prp8 functions in both step I and step II (Brown and Beggs 1992; Teigelkamp et al. 1995; Umen and Guthrie 1995a,b). Prp22, a protein involved in the release of spliced mRNA, also plays a role in step II

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(Schwer and Gross 1998). In yeast, two different stages between the catalytic steps can be distinguished on the basis of their ATP requirements (for review, see Umen and Guthrie 1995c). After catalytic step I, Prp16 and Prp17 function first in a ATP-dependent stage (Schwer and Guthrie 1991,1992; Ansari and Schwer 1995; Jones et al. 1995), and Prp16 can be UV cross-linked to a 15-nucleotide RNase T1 digestion product containing the 3′ splice site (Umen and Guthrie 1995b). Subsequently, Slu7, Prp18, and Prp22 function in an ATP-independent stage (Horowitz and Abelson 1993; Ansari and Schwer 1995; Jones et al. 1995; Schwer and Gross 1998), and Slu7 and Prp8 can be UV cross-linked to the RNA at the 3′ splice site (Umen and Guthrie 1995b). Slu7, Prp18, and Prp22 are dispensable for use of 3′ splice sites located near the branch site (<12, 12, and 21 nucleotides, respectively), but are required for use of more distant 3′ splice sites (Frank and Guthrie 1992; Brys and Schwer 1996; Zhang and Schwer 1997). Therefore, these proteins are thought to play a role in bringing distant 3′ splice sites into the spliceosome's active site for catalytic step II. Although the UV cross-linking experiments indicate that Slu7 contacts the 3′ splice site, this region of the pre-mRNA is dispensable for the association of Slu7 with the spliceosome (Brys and Schwer 1996).

In humans, homologs of the step II factors Prp16, Prp17, and Prp18 have been identified shown to be general step II splicing factors (Horowitz and Krainer 1997; Ben Yehuda et al. 1998; Lindsey and Garcia-Blanco 1998; Zhou and Reed 1998). Thus, splicing factors important for step II are conserved from yeast to humans. To gain further insight into the mechanism of step II, we identified and characterized the human homolog of Slu7. We show that hSlu7 is first detected in purified spliceosomes concomitant with the products of catalytic step I. The association of hSlu7 with the spliceosome occurs before the AG dinucleotide and the adjacent pyrimidine tract at the 3′ splice site are recognized for step II. Significantly, extracts depleted of hSlu7 are blocked before step II and accumulate the products of step I in a novel spliceosomal complex, the $C_{\Delta hSlu7}$ complex. We provide evidence that this $C_{\Delta hSlu7}$ complex is a functional precursor to the catalytically active C_{II} complex. Thus, the $C_{\Delta hSlu7}$ complex defines a new stage between catalytic steps I and II of splicing.

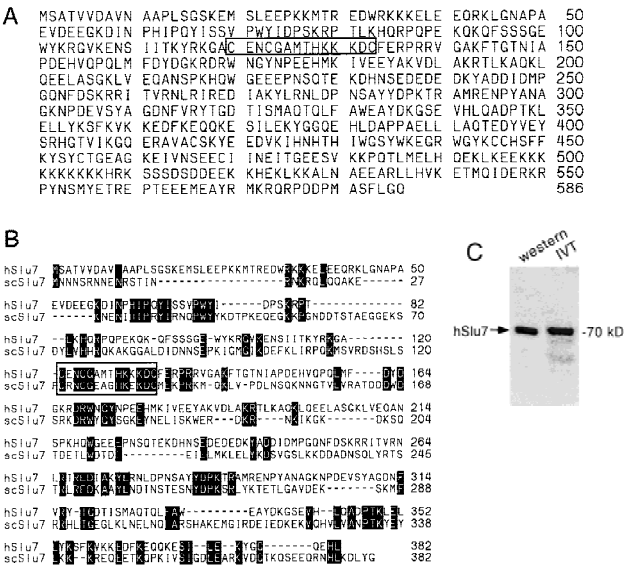

Figure 1. (*A*) Predicted amino acid sequence of hSlu7. The 1758-bp cDNA sequence of the hSlu7 gene has been deposited in the DDBJ/EMBL/GenBank database under accession no. AF101074. The zinc knuckle motif is boxed. (*B*) Alignment of the 382-amino-acid yeast Slu7 protein (scSlu7) with the amino-terminal 382 amino acids of hSlu7. Alignments were done by the Clustal method. Identical residues are shaded, and the zinc knuckles are boxed. (*C*) hSlu7 detected on a Western blot of nuclear extract comigrates with hSlu7 generated by in vitro translation of the hSlu7 cDNA.

Results

Isolation of a cDNA encoding hSlu7

A partial human cDNA with homology to Slu7 (Frank and Guthrie 1992) was identified in a BLAST search of expressed sequence tags (ESTs) and a full-length cDNA obtained by PCR strategies (see Materials and Methods). This cDNA (1758 bp) encodes a 586-amino-acid protein, with a predicted molecular weight of 68 kD (Fig. 1A). We have designated this protein hSlu7. To characterize hSlu7, we expressed a GST–hSlu7 fusion protein in *Escherichia coli* and raised rabbit polyclonal antisera against it. These antisera detect a major band of ~70 kD on Western blots of total nuclear extracts, and this band comigrates with in vitro-translated hSlu7 (Fig. 1C). This observation and the fact that an upstream stop codon precedes the designated initiator methionine indicate that the hSlu7 cDNA encodes a full-length protein. hSlu7 is 204 amino acids longer than yeast Slu7 (Fig. 1B). The amino-terminal two-thirds of the human protein aligns with Slu7, where the identity is 24% (Fig. 1B). The highest similarity is a zinc knuckle (boxed, Fig. 1A, B), a motif present in retroviral nucleocapsid proteins and several splicing factors (Frank and Guthrie 1992; Cavaloc et al. 1994; Arning et al. 1996; Abovich and Rosbash 1997).

The hSlu7 amino acid sequence was used to search the GenBank database. In addition to Slu7, this search identified a *Caenorhabditis elegans* and an *Arabidopsis thaliana* ORF, both of which are 41% identical to hSlu7 (data not shown). Because Slu7 is the only protein in the *Saccharomyces cerevisiae* genome with any significant homology to hSlu7 and because of the functional similarities between hSlu7 and Slu7 (see below), we conclude that the two proteins are orthologs.

hSlu7 joins the spliceosome late in the splicing pathway

To determine when hSlu7 associates with the spliceosome, we carried out a time course of spliceosome assembly. Spliceosomes were assembled on adenovirus

major late (AdML) pre-mRNA for 15, 25, 35, and 45 min and then isolated by gel filtration and affinity chromatography. Analysis of the RNA and protein components of the complexes is shown in Figure 2. The RNA products of catalytic step I are first detected at 25 min, and the step II products are detected at 35 min (Fig. 2A). Western blots of the spliceosomal complexes isolated at each time point were probed with antibodies to hSlu7. hSlu7 is first detected at 25 min, concomitant with the appearance of the splicing intermediates (Fig. 2, cf. B with A). In contrast, the U2 snRNP component SAP 130, which is known to first associate early in spliceosome assembly (Bennett et al. 1992), is detected at constant levels throughout the time course (Fig. 2B). This association of hSlu7 with late spliceosomal complexes occurs generally, because hSlu7 is detected in spliceosomes assembled on different pre-mRNA substrates, including AdML, α-tropomyosin (α-TM; Fig. 2C,D), and Fushi tarazu (Ftz; data not shown).

The kinetics and efficiency of splicing vary with different pre-mRNA substrates. In contrast to AdML pre-mRNA, the products of step I are not detected until the 45 min time point with α-TM pre-mRNA (Fig. 2C), and spliced mRNA is barely detectable by 60 min (Fig. 2C). Significantly, hSlu7 is not detected in these spliceosomes until the 45 min time point, concomitant with step I. Thus, on both AdML and α-TM pre-mRNAs, hSlu7 is first detected in purified spliceosomes when the products of step I first appear. Together, these observations suggest that the association of hSlu7 with the spliceosome is coupled to catalysis of step I.

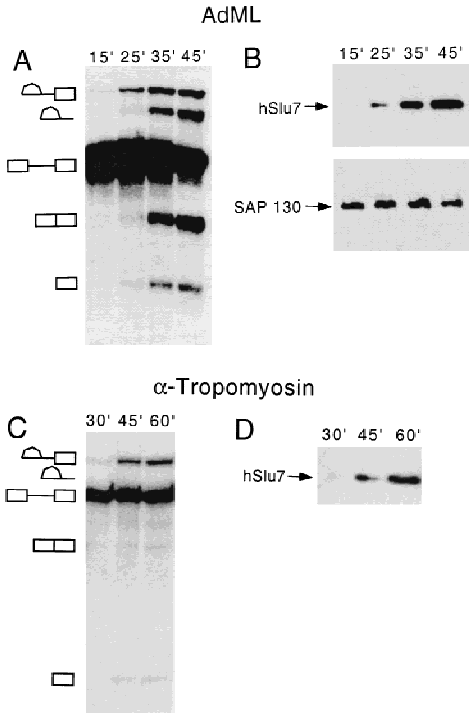

Figure 2. hSlu7 associates with the spliceosome late in the splicing pathway. Spliceosomes were assembled on AdML (*A*,*B*) or α-TM (*C*,*D*) pre-mRNA for the indicated times and then purified. (*A*,*C*) Total RNA isolated from an aliquot of the gel filtration fractions was fractionated on a 15% denaturing polyacrylamide gel. Splicing products and intermediates are indicated. (*B*,*D*) Western blots of total protein isolated from spliceosomal complexes in *A* and *C*, probed with antibodies to the indicated proteins.

The pyrimidine tract and AG dinucleotide are dispensable for association of hSlu7 with the spliceosome

The AG dinucleotide at the 3′ splice site functions in step II of splicing (Reed and Maniatis 1985; Aebi et al. 1986). To determine whether this sequence element is required for the association of hSlu7 with the spliceosome, we isolated spliceosomes assembled on an AdML pre-mRNA containing an AG → GG mutation at the 3′ splice site (Gozani et al. 1994). This mutation specifically blocks catalytic step II (Fig. 3B; cf. with wild type in Fig. 2A). However, as shown in Figure 3C, hSlu7 associates as efficiently with the mutant spliceosome (designated the C_{GG} complex) as with the wild type (cf. Figs. 3C and 2B). This result indicates that recognition of the AG dinucleotide for step II is not required for the association of hSlu7 with the spliceosome.

The pyrimidine tract is required for both catalytic steps of the splicing reaction (Reed 1989; Smith et al. 1989). The step II role for the pyrimidine tract can be analyzed by use of the pre-mRNAs designated pyJ and RanA in Figure 3A (Chiara et al. 1997). The presence of pyrimidines downstream of the BPS in both pre-mRNAs allows efficient step I (Fig. 3D, 30-min time points). Step II, however, occurs only in the presence of additional pyrimidines (pyJ) but not when random sequence (RanA) is adjacent to the 3′ splice site AG [Fig. 3D, 60 min time point; the band labeled with an asterisk is most likely a breakdown product (see legend)]. Significantly, hSlu7 associates equally well with spliceosomes on both mutant pre-mRNAs (Fig. 3E). Thus, recognition of a pyrimidine tract adjacent to the AG is required for catalytic step II, but not for the association of hSlu7. Together, our results demonstrate that, as in yeast (Brys and Schwer 1996), 3′ splice-site sequence elements are dispensable for the association of hSlu7 with the spliceosome.

hSlu7 is a general step II splicing factor

To investigate the function of hSlu7 in splicing, we immunodepleted hSlu7 from HeLa cell nuclear extracts. As shown in Figure 4A, hSlu7 is efficiently, though not completely, depleted. A control protein, the U2 snRNP component SAP 130, is present in equal amounts in ΔhSlu7 and mock-depleted extracts (Fig. 4A). These extracts were used to splice AdML pre-mRNA. Significantly, step II is inhibited, and the products of step I accumulate only in the ΔhSlu7 extracts (Fig. 4B, 45-min time point). This inhibition is specific to step II, because the first step is not inhibited in ΔhSlu7 extracts (Fig. 4B, cf. mock and ΔhSlu7, 25-min time point). Step II is also inhibited in ΔhSlu7 extracts when Ftz and β-globin pre-

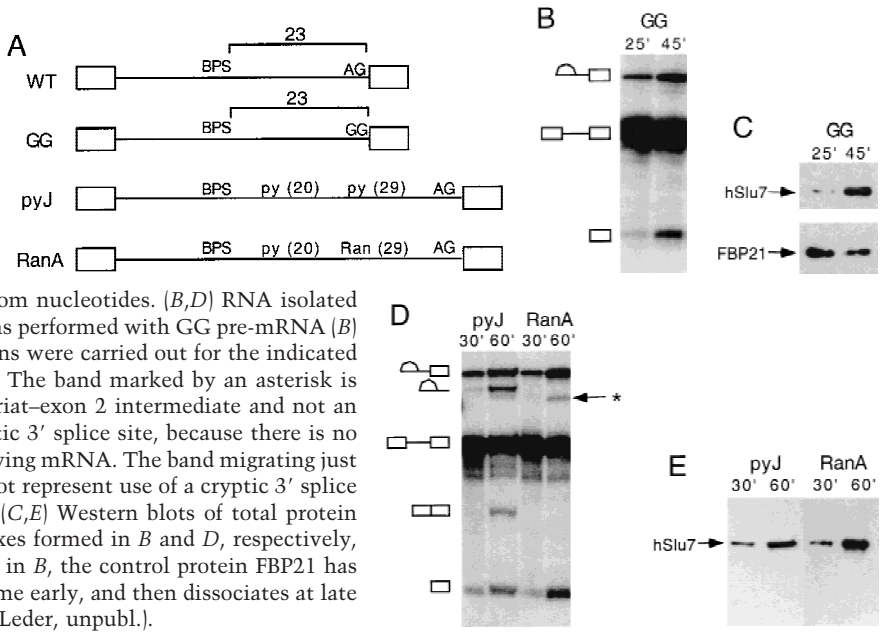

Figure 3. hSlu7 joins the spliceosome prior to recognition of the 3′ splice site for catalytic step II. (*A*) Schematic of wild-type AdML (WT) and derivative pre-mRNA substrates. The GG substrate contains an AG → GG substitution at the 3′ splice site, and the pyJ and RanA substrates contain insertions of pyrimidines (pyJ) and of random sequence (RanA) upstream of the AG. (BPS) Branchpoint sequence; (AG) wild-type AG dinucleotide at 3′ splice site; (GG) GG substitution at 3′ splice site; py (20), a 20-nucleotide pyrimidine stretch; py (29), insertion of 29 pyrimidines; Ran (29), insertion of 29 random nucleotides. (*B*,*D*) RNA isolated from aliquots of large-scale splicing reactions performed with GG pre-mRNA (*B*) or pyJ and RanA pre-mRNAs (*D*). Incubations were carried out for the indicated times, and the spliceosomes were purified. The band marked by an asterisk is most likely a breakdown product of the lariat–exon 2 intermediate and not an excised intron resulting from use of a cryptic 3′ splice site, because there is no band that could correspond to an accompanying mRNA. The band migrating just below the pre-mRNA in all four lanes cannot represent use of a cryptic 3′ splice site because its levels decrease over time. (*C*,*E*) Western blots of total protein from affinity-purified spliceosomal complexes formed in *B* and *D*, respectively, probed with antibodies to hSlu7. Note that in *B*, the control protein FBP21 has been shown to associate with the spliceosome early, and then dissociates at late time points (M.T. Bedford, R. Reed, and P. Leder, unpubl.).

mRNAs are used as substrates (Fig. 4C,D). Note that the low levels of spliced products detected in ΔhSlu7 extracts may be due to residual hSlu7 remaining after depletion (see Fig. 4A).

To determine whether recombinant hSlu7 can reconstitute the depleted activity, we expressed hSlu7 in insect cells (Fig. 5A). Addition of the recombinant protein (rhSlu7) to depleted extract restores step II activity with all three pre-mRNA substrates (Fig. 5B–D). However, we note that step II is less efficient than in mock-depleted extracts (see Fig. 4B). This may be due to loss of activity of rhSlu7 during isolation and renaturation (see Materials and Methods). As expected, recombinant hPrp16 (rhPrp16), which efficiently complements hPrp16-depleted extracts (Zhou and Reed 1998), cannot overcome the step-II block in ΔhSlu7 extracts (Fig. 5B–D). Together, our observations demonstrate that hSlu7 is a general step II splicing factor.

A novel spliceosomal complex accumulates in the absence of hSlu7

One approach to investigate the mechanism of spliceosomal remodeling between steps I and II is to isolate intermediates in this process. As depletion of hSlu7 from splicing extracts inhibits step II, we asked whether a spliceosomal intermediate accumulates in ΔhSlu7 extracts. First, we compared spliceosome assembly in ΔhSlu7 and mock-depleted extracts by native gel analysis (Fig. 6A). In mock-depleted extracts, the A complex is detected at 10 min, a mixture of A and B complexes is detected at 20 min, and the C complex is detected at 45 min. Strikingly, in ΔhSlu7 extracts, the wild-type C complex is not detected, and a novel spliceosomal complex migrating be-

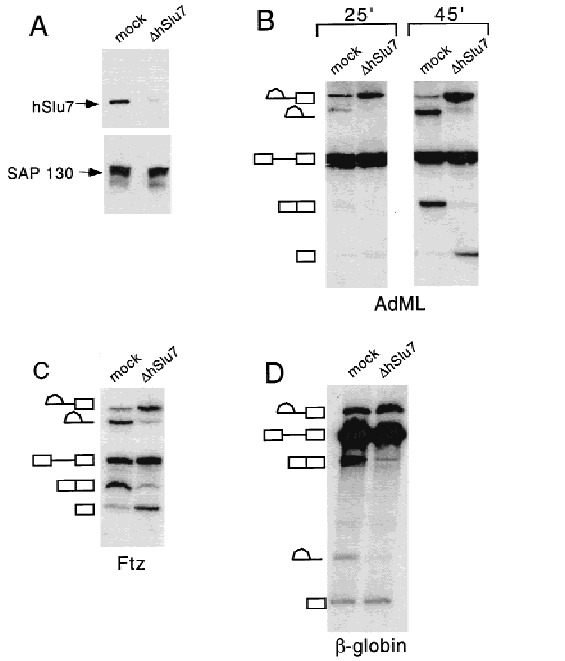

Figure 4. Splicing in ΔhSlu7 extracts is blocked between the two catalytic steps. (*A*) Western blots of ΔhSlu7 and mock-treated extracts probed with antibodies to hSlu7. The bands corresponding to hSlu7 and a control protein, SAP 130, are indicated. (*B–D*) Splicing in ΔhSlu7 and mock-treated nuclear extracts with pre-mRNA substrates AdML (*B*), Ftz (*C*), and β-globin (*D*). Uniformly labeled pre-mRNA was incubated under splicing conditions with ΔhSlu7 or mock-treated nuclear extracts for 60 min, and total RNA was fractionated on a 15% (*B*,*C*) or 8% (*D*) denaturing polyacrylamide gel. Splicing intermediates and products are indicated.

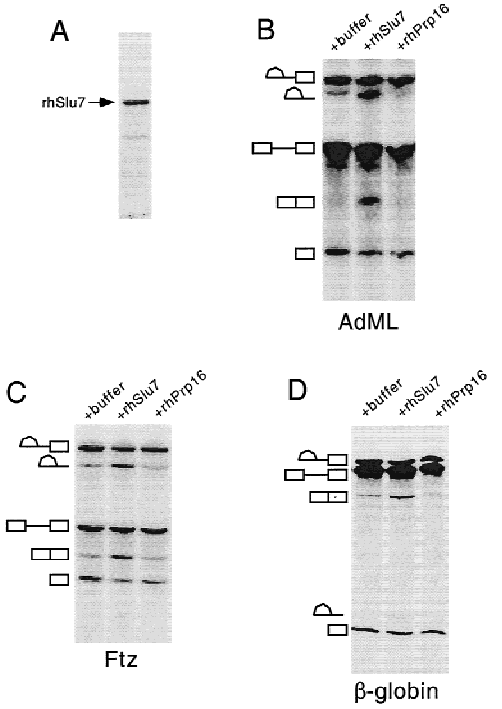

Figure 5. Recombinant hSlu7 reconstitutes step II activity to ΔhSlu7 extracts. (*A*) Recombinant hSlu7 (rhSlu7) expressed in Sf9 cells. (*B–D*) Reconstitution of step II activity with rhSlu7 on AdML (*B*), Ftz (*C*), and β-globin (*D*) pre-mRNAs. ΔhSlu7 extracts were used to splice the indicated pre-mRNAs in the presence of rhSlu7, rhPrp16, or buffer control. Splicing reactions were carried out for 60 min, and total RNA fractionated on a 15% (*B*), 13.5% (*C*), or 8% (*D*) denaturing polyacrylamide gel.

tween the A and B complexes is detected at 45 (Fig. 6A) and 60 min (Fig. 8A, below). We have designated this novel complex the $C_{\Delta hSlu7}$ complex. Analysis of the splicing intermediates and products in these reactions demonstrates that ΔhSlu7 and mock-depleted extracts differ only in step II (Fig. 6B). In addition, accumulation of the $C_{\Delta hSlu7}$ complex correlates with the accumulation of splicing intermediates (Fig. 6A,B).

To test directly whether the $C_{\Delta hSlu7}$ complex contains the products of catalytic step I, the native gel lane containing this complex (shown horizontally in Fig. 6C) was excised from the gel, cut into five fragments, and the RNA species isolated from each fragment (Fig. 6C). Splicing complexes formed in mock-depleted extracts (7.5- and 45-min incubations) are shown for comparison. As expected, pre-mRNA is detected in the A complex at the 7.5-min time point, and the lariat–exon 2 intermediate is detected in the C complex assembled on wild-type pre-mRNA at the 45-min time point. Significantly, the lariat–exon 2 intermediate is also detected in the $C_{\Delta hSlu7}$ complex, but not elsewhere in the native gel lane. Together, these observations identify the $C_{\Delta hSlu7}$ complex as a novel complex blocked between the two catalytic steps.

Both mutation of the 3′ splice-site AG dinucleotide to GG and depletion of hSlu7 from splicing extracts result in accumulation of spliceosomal complexes blocked before catalytic step II (Fig. 6; Gozani et al. 1994). The observation that hSlu7 is present in the C_{GG} complex (Fig. 3B and C) suggests that the $C_{\Delta hSlu7}$ complex is a precursor to the C_{GG} complex. To test this possibility, we incubated GG pre-mRNA in ΔhSlu7 or mock-depleted extracts (Fig. 7A). As expected, the C_{GG} complex, which comigrates with the wild-type C complex (Gozani et al. 1994), accumulates in mock-depleted extracts (Fig. 7B). In contrast, in ΔhSlu7 extracts, the $C_{\Delta hSlu7}$ complex accumulates, indicating that the $C_{\Delta hSlu7}$ complex is blocked prior to formation of the C_{GG} complex (Fig. 7B). Significantly, addition of rhSlu7 but not rhPrp16 allows formation of the C_{GG} complex (Fig. 7C). Thus, accumulation of the $C_{\Delta hSlu7}$ complex is due specifically to absence of the hSlu7 protein, and not to codepletion of an additional factor or to an irreversible side reaction occurring in the ΔhSlu7 extracts.

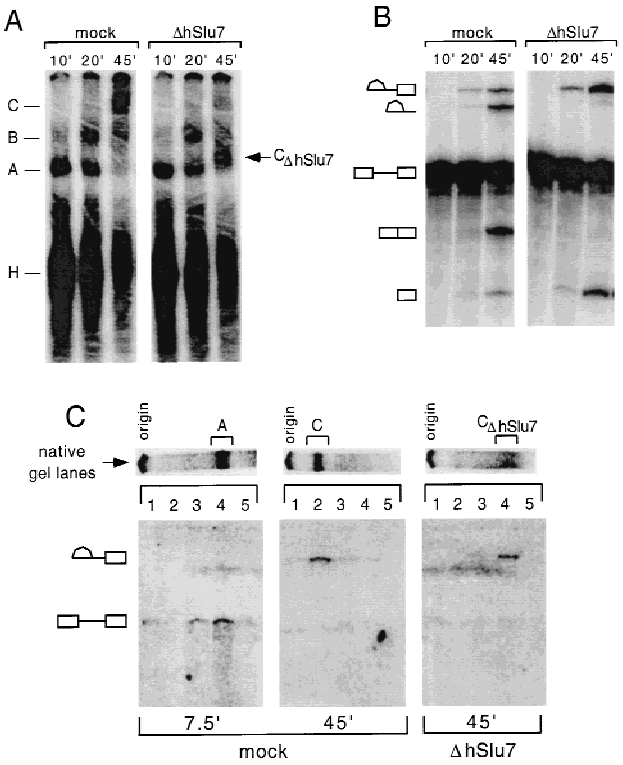

Figure 6. The $C_{\Delta hSlu7}$ complex accumulates between the catalytic steps. (*A*) Spliceosome assembly in mock and ΔhSlu7 extracts. Wild-type AdML pre-mRNA was incubated under standard splicing conditions for the indicated times in mock-treated or ΔhSlu7 extracts, and spliceosomal complexes detected by nondenaturing gel electrophoresis. (*B*) RNA isolated from aliquots of the splicing reactions used for native gels in *A*. (*C*) RNA isolated from spliceosomal complexes shown in *A*. Native gels were transferred to Whatman paper and spliceosomal complexes detected by PhosphorImager analysis. Lanes containing the indicated complexes were excised and cut into five fragments (lanes *1–5*). RNA was extracted from each fragment and run on a 15% denaturing polyacrylamide gel. The lanes containing the complexes are shown horizontally above the RNA gel.

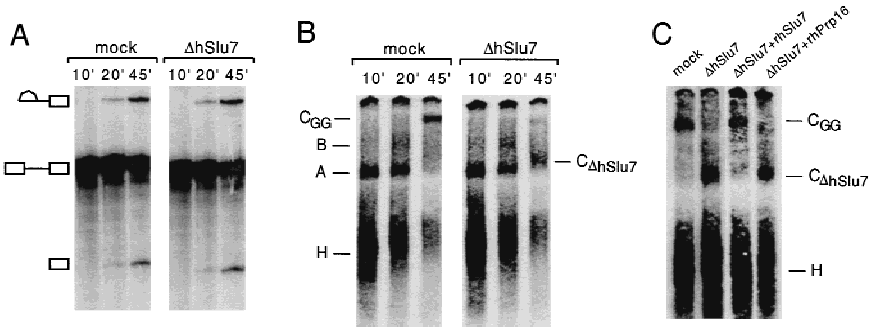

Figure 7. The $C_{\Delta hSlu7}$ complex is a precursor to the C_{GG} complex. (*A*) Splicing of GG pre-mRNA in mock or ΔhSlu7 extracts. GG pre-mRNA was incubated under standard splicing conditions for the indicated times in mock-treated or ΔhSlu7 extracts, and RNA was isolated and fractionated on a 15% denaturing polyacrylamide gel. (*B*) Spliceosomal complex formation in splicing reactions shown in *A*, detected by non-denaturing gel electrophoresis. (*C*) Reconstitution of the C_{GG} complex with rhSlu7. rhSlu7 or rhPrp16 (100 ng) was added to ΔhSlu7 extracts to splice GG pre-mRNA, and spliceosomal complexes were analyzed by native gels after 45-min incubations.

Evidence that the $C_{\Delta hSlu7}$ complex is a functional intermediate

To determine the relationship between the $C_{\Delta hSlu7}$ complex and the C_{II} complex, we asked whether the $C_{\Delta hSlu7}$ complex can be chased into the C_{II} complex with recombinant rhSlu7 (Fig. 8A). The $C_{\Delta hSlu7}$ complex was preformed by incubation of wild-type pre-mRNA in ΔhSlu7 extracts for 60 min (Fig. 8A, lane 2). Then, recombinant rhSlu7 or rhPrp16 was added and complex formation analyzed after brief incubations. Significantly, with rhSlu7 but not rhPrp16, the $C_{\Delta hSlu7}$ complex is converted into a band comigrating with the wild-type C_{II} complex after as little as 5 min (data not shown), and >50% of the $C_{\Delta hSlu7}$ complex is driven into this C complex by 12 min (Fig. 8A, cf. lanes 1–4). The C_{II} complex formed in the presence of rhSlu7 does not result from de novo complex formation because no C complex is detected even after a 20-min incubation in normal or mock-depleted extracts (see Fig. 6A, mock). These data indicate that rhSlu7 is necessary to convert the $C_{\Delta hSlu7}$ complex into the C_{II} complex, but do not address whether it is sufficient. Specifically, because the chase is performed in ΔhSlu7 extracts, it is possible that additional factors present in the extract are also required during this transition.

To address this possibility, $C_{\Delta hSlu7}$ complexes were preformed in large-scale reactions for 60 min and then fractionated by gel filtration. This procedure separates spliceosomal complexes from the nonspecific hnRNP complex H, as well as free proteins (Bennett et al. 1992). The $C_{\Delta hSlu7}$ complex was present in a single gel filtration peak (data not shown) in the size range expected for spliceosomal complexes (Bennett et al. 1992; Gozani et al. 1994). To determine whether rhSlu7 is sufficient to convert the isolated $C_{\Delta hSlu7}$ complex to the step II-competent spliceosome, an aliquot of the peak fraction was incubated with rhSlu7 or buffer control for 15 min in the presence of ATP (Fig. 8B, lanes 1,2). Notably, conversion of splicing intermediates to spliced products is not observed in the presence of rhSlu7 alone (Fig. 8B, lane 2). In contrast, spliced products are detected on incubation in a cytoplasmic S100 extract, which lacks SR proteins but contains all other essential splicing factors (Fig. 8B, cf. lanes 3 and 4; Krainer and Maniatis 1985; Ge et al. 1991; Krainer et al. 1991; Zahler et al. 1992, 1993). The splicing intermediates accumulated for 60 min in the $C_{\Delta hSlu7}$ complex could also be chased to spliced products after a 10-min incubation in normal splicing extracts (data not shown). In both S100 and normal extracts, it is unlikely that the spliced products derive from de novo splicing of pre-mRNA, as they are not normally detected after 15 min of incubation (see Fig. 2A). It is also unlikely that the spliced products are derived from A and B complexes, because these complexes are not detected in the 60-min ΔhSlu7 reactions used for the chase (see Fig. 8A, lane 2). Thus, our data indicate that the $C_{\Delta hSlu7}$ complex is a functional splicing intermediate, but is deficient in at least one step II activity in addition to hSlu7. This ac-

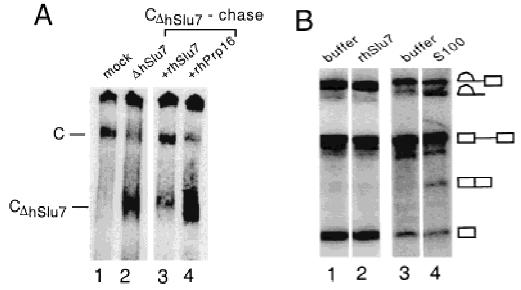

Figure 8. The $C_{\Delta hSlu7}$ complex is a functional precursor to the wild-type C_{II} complex. (*A*) Chase of pre-formed $C_{\Delta hSlu7}$ complexes into the C_{II} complex with recombinant hSlu7. $C_{\Delta hSlu7}$ complexes were preformed for 60 min in ΔhSlu7 extract (lane *2*), and incubated for 12 min in the presence of rhSlu7 (lane *3*) or rhPrp16 (lane *4*). A 60-min incubation in mock-treated extract is shown for comparison (lane *1*). (*B*) Chase of isolated $C_{\Delta hSlu7}$ complexes to complete splicing. $C_{\Delta hSlu7}$ complexes were preformed in ΔhSlu7 extract, and separated from free splicing factors by gel filtration. Aliquots from the peak fraction were chased for 15 min under splicing conditions with buffer control (lanes *1*,*3*), rhSlu7 (lane *2*), or S100 extract (lane *4*). Splicing intermediates and products are indicated. We note that the mRNA appears to be preferentially degraded in the S100 extract, most likely due to high levels of nuclease present in the extract (Abmayr et al. 1988).

tivity is due to a factor (or factors) that is present in both S100 and ΔhSlu7 extracts (Figs. 7C and 8A) but that is not stably associated with the $C_{\Delta hSlu7}$ complex. Together, these studies identify the $C_{\Delta hSlu7}$ complex as a novel precursor to the catalytically active C_{II} complex and a functional intermediate in the splicing pathway.

Discussion

During the first catalytic step of splicing, pre-mRNA is cleaved immediately upstream of the GU at the 5′ splice site. Prior to this reaction, numerous RNA–RNA, RNA–protein, and protein–protein interactions take place as the spliceosome assembles on pre-mRNA in a step-wise pathway (for reviews, see Moore et al. 1993; Reed and Palandjian 1997; Will 1997). These interactions are not only critical for establishing the active site for step I, but are also fundamental to the mechanism for achieving the high levels of fidelity required for catalysis (Madhani and Guthrie 1994; Nilsen 1994). The spliceosome undergoes extensive remodeling between steps I and II (for reviews, see Nilsen 1994; Umen and Guthrie 1995c; Staley and Guthrie 1998). It is likely that a similarly complex network of interactions is involved in this remodeling process and is necessary for achieving fidelity for catalytic step II. Therefore, the identification and characterization of proteins that function between steps I and II provide insights into the nature of the remodeling. In yeast, four splicing factors, Slu7 and Prp16, Prp17, and Prp18, are required specifically for step II (for review, see Umen and Guthrie 1995c), and in metazoans, the counterparts of three of these proteins (hPrp16, hPrp17, and hPrp18) have been identified (Horowitz and Krainer 1997; Ben Yehuda et al. 1998; Lindsey and Garcia-Blanco 1998; Zhou and Reed 1998). Here, we report the isolation of the human homolog of Slu7 and show that it too is required exclusively for step II. We provide evidence that this protein associates with the spliceosome after catalysis of step I and may trigger an essential step in the establishment of the catalytically active spliceosome for step II.

A novel spliceosomal intermediate between the catalytic steps

Four spliceosomal complexes, which assemble in the order E, A, B, and C, have been well characterized as discrete functional intermediates in the splicing pathway (for reviews, see Moore et al. 1993; Reed and Palandjian 1997). Although the C complex appears as a single discrete band on native gels, considerable evidence indicates that significant rearrangements take place between the two catalytic steps and that these reactions are catalyzed by distinct configurations of the C complex (for reviews, see Moore et al. 1993; Staley and Guthrie 1998). Minimally, during conversion of the C_I to the C_{II} complex, the chemical groups involved in the step I transesterification must be displaced from the active site, which then must be reconfigured around the reactive groups for the step II reaction. Despite the extensive remodeling that takes place between steps I and II, distinct complexes corresponding to the C_I and C_{II} complexes or intermediate stages in this process have not been distinguished by native gel analysis.

In this study, we have identified such an intermediate, a novel complex that accumulates in extracts specifically depleted of hSlu7. This complex, the $C_{\Delta hSlu7}$ complex, together with other previously identified step II events, can be used to define distinct stages in the transition from step I to step II. We demonstrate that the $C_{\Delta hSlu7}$ complex is blocked at a stage preceding a structural alteration of the spliceosome for which hSlu7 is required. Specifically, we observe a dramatic difference in mobility between the $C_{\Delta hSlu7}$ complex and the wild-type C complex on a native gel. Significantly, the $C_{\Delta hSlu7}$ complex can be chased to a complex that comigrates with the wild-type C_{II} complex, and this conversion is observed as a large shift in the spliceosome's electrophoretic mobility. These data indicate that the $C_{\Delta hSlu7}$ complex is a functional spliceosomal intermediate between steps I and II that is biochemically distinct from the wild-type C complex. Our identification of the $C_{\Delta hSlu7}$ complex highlights the fact that the kinetics of the rearrangements between the catalytic steps of splicing are extremely fast, and specific stages in this process can be discerned only when a transition is made rate limiting by depletion of an essential step II factor.

The large mobility shift detected on native gels during conversion of the $C_{\Delta hSlu7}$ complex to the wild-type C_{II} complex may reflect alterations in the spliceosome's composition, stability, or structure. The nature of this alteration is likely to be complex, as the dramatic shift in electrophoretic mobility cannot be accounted for by the size of the hSlu7 protein alone. Indeed, our observations indicate that conversion of the $C_{\Delta hSlu7}$ complex to the catalytically active step II spliceosome requires at least one additional step II splicing activity other than hSlu7 itself. This activity is present in cytoplasmic S100 and ΔhSlu7 extracts, but does not co-fractionate with the $C_{\Delta hSlu7}$ complex by gel filtration. The missing activity may be due to one or more factors, which function concomitant with or subsequent to hSlu7, or are required to activate hSlu7. These factors are likely to include hPrp18, because this protein (and its yeast homolog Prp18) is thought to function at a very late stage between catalytic steps I and II and is only transiently associated with the spliceosome (Horowitz and Krainer 1997). One possibility is that hSlu7 is required for the stability of one or more spliceosome components. We note, however, that we have been unable to detect a difference in the snRNP composition of the $C_{\Delta hSlu7}$ complex and that of the wild-type C complex (K. Chua and R. Reed, unpubl.). Our data are consistent with observations in *S. cerevisiae*, in which a ΔSlu7 spliceosome was purified by gradient sedimentation. Like the $C_{\Delta hSlu7}$ complex, this spliceosome is a functional splicing intermediate, but conversion to the catalytically active step II spliceosome requires factors in addition to Slu7 (Ansari and Schwer 1995).

In the absence of hSlu7, the products of catalytic step

I accumulate in the $C_{\Delta hSlu7}$ complex. It is possible that the $C_{\Delta hSlu7}$ complex corresponds to the configuration of the spliceosome immediately following catalysis of step I, and may thus be similar or identical in structure to the C_I complex. However, functional ordering of step II splicing factors in yeast has indicated that at least two proteins, Prp16 and Prp17, function after step I but before Slu7 (Ansari and Schwer 1995; Jones et al. 1995), and preliminary studies suggest that the human orthologs of these proteins function in the same order (Z. Zhou and R. Reed, unpubl.). These observations suggest that the $C_{\Delta hSlu7}$ complex is distinct from the C_I complex.

We have also demonstrated that the $C_{\Delta hSlu7}$ complex is blocked at a stage preceding formation of the C_{GG} complex (see Fig. 7C), which accumulates on a pre-mRNA containing an AG → GG mutation at the 3′ splice site and is arrested between catalytic steps I and II (Gozani et al. 1994). Although the C_{GG} complex is a dead end complex, characterization of its snRNA and protein composition suggested that the C_{GG} complex might represent the same spliceosomal configuration as the C_{II} complex assembled on wild-type pre-mRNAs (Gozani et al. 1994). At least one observation, however, indicates the C_{GG} complex is distinct from the wild-type C_{II} complex. Specifically, the crosslinking of an ~75 kD protein [designated AG^{75} (Chiara et al. 1996) and p70 (Wu and Green 1997)] to the RNA at the AG dinucleotide is detected in the wild-type C complex but not in the C_{GG} complex. However, the C_{GG} complex comigrates on native gels with the wild-type C complex (this study; Gozani et al. 1994), rather than with the $C_{\Delta hSlu7}$ complex. Thus, an AG dinucleotide at the 3′ splice site is not required for the decrease in mobility observed on native gels on addition of rhSlu7 to the $C_{\Delta hSlu7}$ complex.

Similarly, the AG dinucleotide is not required for a number of events that have been implicated previously in remodeling the spliceosome for step II, and these events may contribute to the large mobility shift detected on native gels between the $C_{\Delta hSlu7}$ and C_{GG} complexes. Fourteen spliceosome-associated proteins (SAPs) become stably associated with the spliceosome between the B and C complexes and are present in the C_{GG} complex (Gozani et al. 1994). Modification and repositioning of splicing factors that have roles in earlier stages of the splicing pathway are also detected in the C_{GG} complex. Specifically, the U2 snRNP protein SAP 155 becomes hyperphosphorylated (Wang et al. 1998), and the U5 snRNP proteins $U5^{110}$, $U5^{116}$, and $U5^{220}$ are repositioned to contact the pyrimidine tract at the 3′ splice site, where they can be UV cross-linked to the RNA (Chiara et al. 1997). Future studies may allow more precise delineation of the timing of these events.

A pyrimidine tract located immediately adjacent to the AG dinucleotide at the 3′ splice site is essential for step II and may function to specify the AG that serves as the site of exon ligation (Chiara et al. 1997). It has been proposed that the repositioning of U5 snRNP on the 3′ splice site may underlie this sequence requirement (Chiara et al. 1997), and consistent with this model, antibodies to the U5 snRNP proteins $U5^{116}$ (Fabrizio et al. 1997) and $U5^{200}$ (Lauber et al. 1996) can inhibit step II. As hSlu7, hPrp16, and hPrp17 are all stably associated with the spliceosome in the absence of such a pyrimidine tract (see Fig. 3; Zhou and Reed 1998), recognition of the pyrimidine tract for step II most likely occurs in a stage following the association of these proteins. Indeed, one possibility is that the conversion of the $C_{\Delta hSlu7}$ complex to the catalytically active C_{II} complex may involve the incorporation of the pyrimidine tract at the 3′ splice site into the catalytic center of the spliceosome.

This model fits well with studies of the step II splicing factors in *S. cerevisiae*. Specifically, the association of Slu7 with the spliceosome in *S. cerevisiae* occurs even when 3′ splice site sequences are removed by RNase digestion, suggesting that at least the initial association of Slu7 precedes incorporation of the 3′ splice site (Brys and Schwer 1996). However, Slu7 is thought to be positioned at or near the 3′ splice site at a time very close to catalysis of step II, because it (as well as Prp8) can be UV cross-linked to a 15-nucleotide RNase digestion product containing the 3′ splice site (Umen and Guthrie 1995b). Moreover, a differential step II requirement for Slu7 has been described, depending on the location of the 3′ splice site, suggesting that Slu7 may initiate the recruitment of distant 3′ splice sites (Brys and Schwer 1996; Frank and Guthrie 1992). Therefore, it has been proposed that Slu7 first associates with the spliceosome (possibly via protein–protein interactions) independent of the 3′ splice site and is subsequently repositioned along with Prp8 onto the 3′ splice site (Brys and Schwer 1996). Thus, both the $C_{\Delta hSlu7}$ complex in humans and the ΔSlu7 complex in yeast may represent stages in the transition between steps I and II, in which the spliceosome has not yet reconfigured itself around the 3′ splice site.

Materials and methods

Plasmids

Wild-type AdML pre-mRNA is encoded by pAdML described in (Michaud and Reed 1993). GG (previously designated GG–GG), PyJ, and RanA pre-mRNAs are derived from pAdML (Chiara et al. 1997). All AdML derivatives were linearized with *Bam*HI and transcribed with T7 RNA polymerase. Ftz pre-mRNA (Rio 1988) was linearized with *Xho*I and transcribed with T7 RNA polymerase. α-TM pre-mRNA (Smith and Nadal-Ginard 1989) was linearized with *Bam*HI and transcribed with SP6 RNA polymerase.

Isolation of hSlu7 cDNA

The BLAST program was used to search the GenBank database for sequences that resemble the Slu7 protein sequence (Frank and Guthrie 1992). A *C. elegans* ORF (GenBank accession no. Z71181) was identified as the most similar to Slu7. A second database search for sequences resembling this *C. elegans* sequence identified several human EST sequences. One of these (GenBank accession no. AA076462) was used to design oligonucleotide primers (5′-GAGGCTGGACATGTTCATCTGGAGC-3′ and 5′-TCTGGAGAATGGTACAAGAGGGG-3′) for PCR. Overlapping 5′ and 3′ RACE products were amplified from HeLa cell cDNAs by use of the Marathon RACE kit (Clontech).

On the basis of the sequences of these RACE products, a full-length cDNA was then amplified by PCR and subcloned into the pCR2.1 vector (Invitrogen).

Immunodepletion and reconstitution

hSlu7 fragments encoding amino acids 15–157 and 15–172 were subcloned into pGEX-2TK (Pharmacia), and GST fusion proteins were expressed in *E. coli*. Recombinant protein was affinity purified with glutathione–Sepharose 4B (Pharmacia) and eluted with reduced glutathione. Rabbit polyclonal antibodies were raised against a mixture of these fusion proteins (Covance Research Products, Denver, PA). For immunodepletions, 60 µl of hSlu7 or pre-immune antibodies were coupled to 250 µl of protein A–Sepharose CL-4B (Pharmacia), and used to deplete four volumes of nuclear extract in 700 mM KCl. Three sequential depletions were carried out for 1 hr each, rotating at 4°C. The resulting extracts were dialyzed against 20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.2 mM PMSF, and 0.5 mM DTT. Recombinant hSlu7 and hPrp16 were expressed using the Bac-to-Bac baculovirus expression system (Gibco/BRL). Sf9 cells were infected with recombinant baculovirus, harvested after 48 hr, and lysed by sonication in 20 mM Tris-HCl (pH 8.5), 10 mM 2-mercapthoethanol, 1 mM PMSF, and 1% NP-40. Centrifugation of total cell lysate produced an inclusion body pellet highly enriched in recombinant protein. Further purification was accomplished by sequential washes in 2 and 4 M urea. The final pellet was solubilized in 6 M urea, and the resulting protein renatured by dialysis into 20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM PMSF, 0.5 mM DTT. Recombinant protein (100 ng) was added to ΔhSlu7 splicing reactions (25 µl) to reconstitute splicing activity.

Analysis of spliceosomal complexes

Biotinylated, ^{32}P-labeled pre-mRNA (1.92 µg) was incubated in 2.4-ml splicing reactions. Spliceosomal complexes were isolated by gel filtration and affinity purification on avidin–agarose, and RNA or protein analyzed as described previously (Reed 1990; Bennett et al. 1992). For time-course experiments, equal amounts of purified complex were used for each time point. For Western analysis, proteins eluted from purified spliceosomal complexes assembled on 80–100 ng pre-mRNA were loaded in each lane.

Native gel analysis

^{32}P-Labeled wild-type AdML or GG mutant pre-mRNA (20 ng) was incubated in 25-µl splicing reactions for the indicated times. Three microliters of 6.5 mg/ml heparin/50% ficoll dye was added and the samples incubated at room temperature for 5 min. Four microliters of the samples were run on 4% Tris–glycine polyacrylamide gels (Konarska and Sharp 1987). Gels were transferred to Whatman paper, dried, and detected by PhosphorImager analysis. RNA was isolated from 20 µl of the reactions and run on 15% denaturing gels to analyze splicing in each sample.

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