A nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5' splice site

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The mechanism by which intron-containing RNAs are recognized by the splicing machinery is only partly understood. A nuclear cap-binding complex (CBC), which specifically recognizes the monomethyl guanosine cap structure carried by RNA polymerase II transcripts, has previously been shown to play a role in pre-mRNA splicing. Using a combination of splicing complex and psoralen cross-linking analysis we demonstrate that CBC is required for efficient recognition of the 5' splice site by U1 snRNP during formation of E (early) complex on a pre-mRNA containing a single intron. However, in a pre-mRNA containing two introns, CBC is not required for splicing of the cap distal intron. In this case, the presence of an intact polypyrimidine tract in the cap-proximal intron renders splicing of the cap-distal intron independent of CBC. These results support models in which the splice sites in a pre-mRNA are originally recognized by interactions spanning exons. The defects in splicing and U1 snRNP binding caused by CBC depletion can be specifically reversed by recombinant CBC. In summary, efficient recognition of the cap-proximal 5' splice site by U1 snRNP is facilitated by CBC in what may be one of the earliest steps in pre-mRNA recognition. Data in Colot et al. (this issue) indicate that this function of CBC is conserved in humans and yeast.

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Removal of introns from pre-messenger RNAs (pre-mRNAs) occurs in a dedicated nucleoprotein complex termed the spliceosome. The spliceosome consists of U1, U2, and U4/U6.U5 snRNP particles together with a large number of nonsnRNP splicing factors, most of which are poorly characterized (for review, see Lamm and Lamond 1993; Moore et al. 1993; Madhani and Guthrie 1994). The *cis*-acting elements which define introns, the 5' splice site, branchpoint sequence, polypyrimidine tract, and 3' splice site, have quite degenerate sequences in higher eukaryotes. This poses the problem of how pre-mRNAs are efficiently recognized by the splicing machinery in spite of the limited amount of sequence information.

In vitro, several stable intermediate complexes in the spliceosome assembly pathway have been observed. In mammalian nuclear extracts, the first prespliceosomal complex to be observed is the early (E) complex, which forms in the absence of ATP (Michaud and Reed 1991). The E complex contains U1 small nuclear ribonucleoprotein (snRNP) and the splicing factor U2AF (U2 snRNP auxiliary factor; Zamore and Green 1989) bound at the 5' splice site and polypyrimidine tract, respectively.

Additionally, other less well characterized proteins are found associated with E complex (Bennet et al. 1992a). This complex is probably the mammalian equivalent of the yeast commitment complex (Séraphin and Rosbash 1989; Abovich et al. 1994) whose formation represents the first definitive commitment of a pre-mRNA to the splicing pathway in *Saccharomyces cerevisiae* (for review, see Rosbash and Séraphin 1991; Hodges and Beggs 1994). Next, U2 snRNP is added to the E complex, in a step that requires ATP, to form the A complex. The U4/U6.U5 tri-snRNP then joins to form the mature spliceosome within which the two transesterification reactions take place to produce the mature mRNA and intron lariat (for review, see Moore et al. 1993).

In recent years much has been learned of the contributions the U snRNPs in defining the 5' and 3' splice sites (for review, see Madhani and Guthrie 1994; Newman 1994). However, the role of non-snRNP splicing factors in pre-mRNA recognition is less well understood (Lamm and Lamond 1993). It has been demonstrated previously that the presence of 5' cap structure plays an important role in efficient splicing of pre-mRNAs in mammalian cell extracts (Konarska et al. 1984; Krainer et al. 1984; Edery and Sonenberg 1985; Ohno et al. 1987; Patzelt et al. 1987) and also in vivo (Inoue et al. 1989). A nuclear cap-binding complex (CBC) had been characterized and cloned previously. CBC specifically recognizes the cap structure (Izaurralde et al. 1994, 1995) and con-

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sists of two subunits, cap-binding proteins 80 and 20 (CBP80 and CBP20), both of which are required for specific binding to capped RNAs in vitro (Izaurralde et al. 1995; Kataoka et al. 1995). Depletion of CBC from splicing extracts or injection of anti-CBP20 antibodies into *Xenopus* oocytes was found to markedly reduce splicing of a pre-mRNA containing a single intron derived from the adenovirus major late transcription unit, leading to an accumulation of unspliced pre-mRNA. Analysis of the kinetics of spliceosome assembly showed a major defect in A complex formation in depleted extracts compared to control extracts, suggestive of a role for CBC at, or prior to, A complex assembly (Izaurralde et al. 1994, 1995).

In this report we have characterized the defect in splicing and splicing complex assembly caused by CBC depletion from HeLa cell nuclear extracts. In addition, we examine the effects of CBC depletion on the splicing of cap distal introns. We find that E complex assembly, and more specifically, the association of U1 snRNP with the cap-proximal 5' splice site, occurs inefficiently in extracts depleted of CBC. The defects in CBC-depleted extracts can be fully complemented by recombinant CBC (rCBC). This suggests an important role for CBC in the

earliest steps of pre-mRNA recognition. Other results (Colot et al., this issue; J. Lewis et al., unpubl.) indicate that CBC carries out this function not only in mammalian cells but also in yeast.

Results

We have previously characterized and cloned cDNAs encoding both subunits, CBP80 and CBP20, of nuclear CBC from HeLa cells. Using antibodies raised against the individual subunits it has been possible to demonstrate a role for this complex in both pre-mRNA splicing and nuclear export of the RNA polymerase II transcribed spliceosomal U snRNAs (Izaurralde et al. 1994, 1995). Recombinant CBC purified from *Escherichia coli* lysates is able to bind to the cap structure with a specificity which is indistinguishable from CBC present in HeLa cell extracts (Fig. 1A). The HeLa CBC activity and rCBC produce a similar mobility shift when bound to the capped RNA probe. Both activities are efficiently competed with m⁷GpppG and et⁷GpppG (Fig. 1A, lanes 4,7,12,15) but not, for example, with m^{2,2,7}GpppG (lanes 6,14). m^{2,7}GpppG has an intermediate competitive efficiency (lanes 5,13). This demonstrates that a complex of CBP80 and CBP20 is sufficient to bind specifically to the

Figure 1. Cap specificity of rCBC and complementation a CBC-depleted extract. (A) Complexes formed between a m⁷GpppG-capped RNA probe and either HeLa nuclear extract (0.8 µg) or recombinant CBC (50 ng) were fractionated by native gel electrophoresis. Competitor dinucleotide analogs of the cap structure, as indicated at the top of each lane, were added to a final concentration of either 100 µM (to the HeLa extract); or 200 µM (to the CBC). (Lanes 1,9) Probe alone; (lanes 2,10) control. (B) The adenovirus pBSAd1 pre-mRNA was spliced in mock-depleted (lanes 1,8–13) or CBC-depleted (lanes 2–7) extract. Extracts were supplemented with buffer (lanes 2,8), increasing amounts of recombinant CBP20 (rCBP20, lanes 3,4,9,10), or increasing amounts of rCBC (lanes 5–7, 11–13). Splicing reactions were allowed to proceed for 2 hr. Products of the splicing reaction were then resolved on a 10% denaturing urea-polyacrylamide gel.

cap, although neither subunit binds detectably on its own [E. Izaurralde, J. Lewis, and I. Mattaj, unpubl.].

Depletion of CBC from nuclear extracts inhibits splicing of uniformly labeled m⁷GpppG capped Ad1, a pre-mRNA derived from Adenovirus 1, by ~90% [Izaurralde et al. 1994; and Fig. 1B, lanes 1,2]. Previously we have shown that addition of CBC purified from HeLa nuclear extracts could restore splicing to a depleted extract, whereas recombinant CBP80 alone had no stimulatory activity. We were interested to determine whether rCBC or rCBP20 would be able to restore splicing to CBC depleted extract. rCBC and rCBP20 were prepared from *E. coli* lysates as described previously [Izaurralde et al. 1995]. Addition of rCBP20 alone to extracts that had been depleted of CBC by passage over an anti-CBP80 antibody affinity column had no stimulatory effect on splicing either in depleted extract (Fig. 1B, lanes 3,4) or in mock-depleted extract (lanes 9,10). In contrast, addition of increasing amounts of rCBC to the depleted extract (lanes 5–7) restored splicing to levels similar to those observed in control reactions (lanes 1,8). Importantly, the same amounts of rCBC added to mock-depleted extract did not cause any stimulation of splicing (lanes 11–13), demonstrating that rCBC was replacing the depleted activity rather than causing a general increase in splicing efficiency. These results demonstrate that rCBC can functionally substitute for the endogenous complex in pre-mRNA splicing.

CBC is required for early steps in prespliceosome complex assembly

Spliceosome assembly proceeds in a defined order. The first specific complex to be observed, formed in the absence of ATP, is the E complex. This complex contains U1 snRNP, U2AF, and several other proteins (see introductory section). In the next step U2 snRNP is added, in a process that requires ATP, to form A complex. We previously demonstrated that CBC depletion caused a large reduction in the efficiency of assembly of the ATP-dependent A complex [Izaurralde et al. 1994]. Because complex formation was assayed by native gel electrophoresis, in which E complexes are not resolved [Michaud and Reed 1991], we were unable to determine whether CBC was required at the stage of, or before, A complex formation.

To further analyze the role of CBC, E complex assembly was analyzed by gel filtration [Michaud and Reed 1991]. Reactions were set up using extracts depleted of ATP (see Materials and methods). Parallel samples from either CBC-depleted or mock-depleted extracts were examined. Uniformly labeled Ad1 pre-mRNA was added to each reaction and incubated for 15 min to allow E complex formation, then fractionated by gel filtration. A complex with the mobility characteristic of E complex forms both in mock-depleted and CBC-depleted extract (Fig. 2A). These complexes were not observed using an antisense Ad1 transcript as a control (data not shown). The level of complex assembly observed with depleted extracts was calculated from the distribution of labeled

RNA between the E and H complexes, and was consistently on the order of 50% lower than that observed in the control reaction (a decrease in the amount of E complex leads to a corresponding increase in the height of the H peak; the efficiency of assembly is calculated from the ratio of these two peaks). This suggested that CBC might be involved in E complex assembly. However, the 50% reduction did not correlate quantitatively with the strong inhibition of splicing seen in depleted extracts (on the order of 80%–90%; e.g., Fig. 1). Two explanations of these data seemed possible. First that CBC might affect the efficiency of more than one step in complex assembly, and second that the E complex observed in the depleted extract might not be identical to that in the control extract.

It has been shown that E-like complexes can form independently on RNAs containing only a 3' splice site or a 5' splice site [Michaud and Reed 1993]. These are named the E3' and E5' complexes, respectively. The former complex is enriched in U2AF, which binds to the polypyrimidine tract, and the latter is enriched in U1 snRNP, however, both have a similar composition to the E complex formed on complete pre-mRNAs. To determine how the assembly of E3' or E5' complexes would be affected in CBC-depleted extracts, RNAs containing only a 3' splice site (Ad3') or a 5' splice site (Ad5') were transcribed and used in complex assembly reactions. Analysis of E3' complex assembly in depleted versus mock-depleted extracts (Fig. 2B) showed a similar profile to that observed in the complete E complex assembly reaction on the entire pre-mRNA. E3' complex assembly as again inhibited to ~50% compared with the mock-depleted control reaction. In contrast, the amount of E5' complex assembled was severely reduced compared to the control reaction (Fig. 2C). Although the small quantity of E5' complex remaining in the depleted extract makes accurate quantitation difficult, the level of inhibition of E5' complex formation was measured to be ~75%–80% in several experiments. This level of inhibition could account for most, if not all, of the splicing defect observed in depleted extracts. In summary, interactions involving factors interacting with the 5' splice site are more affected by CBC depletion than those at the 3' splice site. This, together with the similar quantitative reduction in E complex formation seen in Figure 2, A and B, suggests that the complex seen in Figure 2A probably corresponds to E3' rather than to complete E complex, and the relatively small reduction in this complex indicates that depletion of CBC has only a minor effect on the association of most components of E complex with the pre-mRNA. These results focus the role of CBC on the 5' end of the intron, and thus on the interaction between U1 snRNP and the 5' splice site.

CBC is required for efficient 5' splice-site U1 snRNP interaction

To address more directly whether CBC was required for the efficient association of U1 snRNP with the 5' splice

Figure 2. [See facing page for legend.]

site, a series of psoralen cross-linking experiments were undertaken. Either the full length Ad1 pre-mRNA or the 5' half of the Ad1 pre-mRNA (Ad5') were used as substrates. Similar results were obtained with either substrate (Fig. 3, A, lanes 5–8, and B, lanes 1–4), but because intramolecular cross-links in the full-length pre-mRNA led to the production of a smear of cross-linked products that ran close to the U1 snRNA–pre-mRNA species (Fig. 3B), mainly the Ad5' data are shown. As a control RNA, we used the 5' half of a mutant precursor RNA (CX5'), which contains a G → A mutation at intron position 5 of the 5' splice site that effectively inhibits U1 snRNP binding and splicing (Hamm and Mattaj 1990). RNAs were capped in vitro with either m⁷GpppG or ApppG for Ad5' or m⁷GpppG for CX5'. Reaction conditions were the same as used to analyze E complex formation.

One major psoralen cross-link species (labeled X in Fig. 3A, lane 5) was observed when the m⁷GpppG-capped wild-type substrate was used. This cross-link was dependent on the presence of psoralen in the reaction mixture and UV irradiation (data not shown). That its formation was attributable to a U1 snRNA–5' splice site interaction was demonstrated by two experiments. First, RNase H directed cleavage, using an oligonucleotide complementary to U1 snRNA, efficiently destroyed the cross-linked product (Fig. 3A, lane 6); and second, the U1 snRNA cross-link to the 5' splice site was abolished when the 5' splice site CX mutant RNA was utilized (Fig. 3A, lanes 1–4).

The efficiency of U1 snRNA cross-linking to m⁷G–Ad1 was greatly reduced in CBC-depleted extract (Fig. 3A, lane 7). Quantitation of this and three other independent experiments based on measurement of the amount of cross-linked species in relation to total RNA showed that the U1 snRNA cross-link was reduced by ~80%–85% in depleted extracts compared to the mock-depleted controls. Similar results were obtained with the full-length precursor. The effect of CBC depletion with this substrate in the presence and absence of the U1-complementary oligonucleotide are shown (Fig. 3B, lanes 1–4). Again, X is used to indicate the specific cross-linked product.

Interestingly, when the Ad5' precursor with the wild-type 5' splice site sequence but carrying an ApppG cap structure was used as a substrate, it cross-linked to U1 snRNA with an efficiency similar to that seen when m⁷GpppG-capped Ad5' RNA was cross-linked in CBC-depleted extracts (Fig. 3A, cf. lanes 7 and 9). The level of cross-linking to the ApppG-capped substrate was not detectably reduced further by CBC depletion (lane 11). The degree of inhibition of cross-linking in lanes 9 and 11 compared to the control reaction in lane 5 was ~80% in both cases. In summary, these results show directly that CBC depletion causes a large reduction in U1 snRNA

cross-linking to the 5' splice site, and that this reduction can be mimicked by using an RNA carrying a cap structure that does not efficiently interact with CBC. Note that the reduction in U1 cross-linking is similar for both the truncated and full length pre-mRNA substrates (Fig. 3, A, lanes 5, 7, and B, lanes 1, 3). Thus, despite that fact that the full length substrate still forms an E-like pre-splicing complex (see above), the U1 snRNA in this complex (Michaud and Reed 1993) does not associate normally with the 5' splice site. This indicates that although CBC depletion only has a small effect on the association of most E complex components with the pre-mRNA, its absence prevents normal U1 snRNP–5' splice site interaction.

To determine whether rCBC could reverse the defect in U1 snRNP association, rCBC was added to depleted or, as a control, mock-depleted extract. Although the levels of cross-linking in mock depleted controls (Fig. 3C, lanes 1, 2) were unaffected, rCBC efficiently restored the U1 snRNA cross-link in depleted extracts (cf. lanes 3 and 4). The magnitude of the reduction in U1 snRNA cross-linking following CBC depletion, and its restoration by rCBC, suggest that most, if not all, of the splicing defect in CBC depleted extracts can be attributed to the reduction in U1 snRNP binding to the 5' splice site.

SR proteins can overcome the effects of CBC depletion but do not mediate the CBC effect on U1 snRNP

Aside from CBC, the SR protein family of splicing factors has been shown to play a role in U1 snRNP recruitment to the 5' splice site (Fu and Maniatis 1992; Fu 1993; Kohtz et al. 1994; Jamison et al. 1995; see Discussion). We therefore examined the effects of adding SR proteins to CBC-depleted extract. The addition of increasing quantities of a purified mixture of SR proteins (which do not contain significant quantities of CBC; data not shown) to CBC-depleted or mock-depleted extract increased the splicing efficiency of both extracts to a point where no difference between the two extracts could be seen (Fig. 4A, lanes 3–10). Addition of CBC to SR protein-supplemented splicing reactions such as those in lanes 7–10 had no detectable further effect on splicing efficiency (data not shown). Cross-linking of U1 snRNP to the Ad5' substrate was measured in parallel, and also increased differentially in both depleted and mock-depleted extracts to a level where both extracts exhibited similar levels of the cross-linked product (Fig. 4B, lanes 3–10). These results were consistent with two possibilities. First, SR proteins might mediate the effect of CBC on U1 snRNP–5' splice site interaction. In this case, addition of excess SR proteins would have obviated the need for CBC. The second possibility was that SR proteins and CBC affect U1 snRNP–5' splice site interaction

Figure 2. CBC depletion affects early steps of presplicing complex assembly. The effect of CBC depletion was assayed on E (A), E3' (B), and E5' (C) complex formation in splicing reactions lacking ATP and MgCl₂ using either CBC-depleted or mock-depleted extracts. The complexes were allowed to form for 15 min and were then fractionated by gel filtration. A representative result is shown from each class.

Figure 3. CBC is required for the efficient cross-linking of U1 snRNA to the 5' splice site. (A) Splicing reactions were assembled under the same conditions used for E complex assembly using m⁷GpppG-capped CX5' (lanes 1–4) m⁷GpppG-capped Ad5' (lanes 5–8) and ApppG-capped Ad5' (lanes 9–12) in either depleted (D) or mock-depleted (M) extracts. After cross-linking the samples were digested with RNase H in the presence of either buffer (–) or an oligonucleotide complementary to U1 snRNA (+). Cross-linked products (x) were resolved from non-cross-linked probe (P) by 6% denaturing PAGE. (B) Reactions as described in A, lanes 5–8, except that the full length pBSAd1 substrate was used. (C) rCBC restores U1 snRNA cross-linking to the 5' splice site in depleted extract. rCBC was added to either depleted or mock-depleted extract (+) during the preincubation. As a control, buffer was added to both reactions (–).

by separate mechanisms. To distinguish between the two, we made use of highly purified U1 snRNP, recombinant CBC and purified SR proteins and tested their activity in the U1 snRNP cross-linking assay.

The amount of U1 snRNA cross-linked to the Ad5' substrate was measured over a range of U1 snRNP concentrations (Fig. 5A, lanes 1–4). Addition of CBC to these reactions was without effect on the efficiency of cross-linking (Fig. 5B, lanes 5–8) indicating that the effect of CBC on U1 snRNP–5' splice site interaction requires mediating factors lacking from this purified system. To test whether these factors were in the SR protein prepa-

ration, we added SR proteins to the reactions. Over a wide range of U1 snRNP, CBC and SR protein concentrations (Fig. 5B, lanes 1–4; and data not shown) we observed that whereas SR proteins increase U1 snRNP–5' splice site cross-linking, the addition of CBC to the mixture was without effect. In conclusion, the effect of CBC on U1 snRNP binding to the 5' splice site is not observed when only these components are present, pointing to the existence of an additional factor required for this step. Although SR proteins can also, as previously demonstrated, increase U1 snRNP–5' splice site interaction, they cannot mediate the CBC effect.

Figure 4. SR proteins restore splicing to a CBC-depleted extract by recruiting U1 snRNP to the 5' splice site. (A) Increasing amounts of SR proteins [0.7 μ g, lanes 3 and 4; 1.5 μ g, lanes 5 and 6; 3 μ g, lanes 7 and 8; 6 μ g, lanes 9 and 10] were added to either depleted (D) or mock-depleted (M) extracts. In the control reactions (C), buffer was added. The splicing reactions were incubated for 2 hr, and RNA was recovered and products resolved by denaturing PAGE. (B) Reactions were set up as for A above, with the exception that no ATP or creatine phosphate was added and m⁷GpppG-capped Ad5' was used as a substrate. The reactions were incubated for 15 min and then cross-linked [see Materials and methods]. RNAs were recovered and resolved by denaturing PAGE.

CBC facilitates splicing of cap-proximal introns

Having demonstrated that CBC exerts its influence via an effect on U1 snRNP–5' splice site interaction, we wished to examine whether this effect was in some way position dependent. Previous studies in which the splicing of precursors containing two introns and carrying either an m⁷GpppG or an ApppG cap structure had led to the conclusion that the effect of the cap structure was limited to the cap proximal intron (Ohno et al. 1987; Inoue et al. 1989). However, the basis for this effect was not clear. For example, it was possible to imagine that either the distance along the RNA between the cap structure and the distal 5' splice site, or the sequence context of the proximal versus the distal 5' splice site, could have led to the difference observed.

To investigate this, we constructed a hybrid double intron pre-mRNA called 004Ad. This contained the fourth intron of the *Xenopus* ribosomal protein L1a gene (Lorenz et al. 1985) at a cap proximal position and the Ad1 intron cap distal (see Fig. 6A). Uniformly labeled 004Ad pre-mRNAs were synthesized in vitro, primed

with m⁷GpppG. Splicing of both the single Ad1 intron (Fig. 6B, lanes 1,2), as previously shown, and the 004 intron [lanes 3,4] was sensitive to CBC depletion. Quantitation of the level of inhibition showed it to be reproducibly between 80% and 90% for both introns. The 004Ad pre-mRNA was efficiently spliced in mock-depleted extracts (Fig. 6B, lane 5, the positions of products and intermediates are shown). In CBC-depleted extracts there was a reduction in the level of splicing of the cap proximal 004 intron similar to that observed with the single intron constructs but much less effect on the cap distal adenovirus intron [Fig. 6B, lane 6]. Note that the 3' end of this precursor is not identical to that of the single Ad1 pre-mRNA, and thus the size of the Ad1 intron-containing lariat intermediate is altered.] This resulted in an accumulation of partially spliced pre-mRNAs containing the cap proximal intron. The inhibition can be observed most clearly by comparing the levels of the 004 intron product in the mock-depleted and depleted extracts (lanes 5,6). Quantitation of the efficiency of splicing showed that the cap proximal intron

Figure 5. A mixture of rCBC, U1 snRNP, and SR proteins does not reconstitute cap-dependent association of U1 snRNP with the 5' splice site. (A) Increasing amounts of purified U1 snRNP (30 ng, lanes 1 and 5; 62 ng, lanes 2 and 6; 125 ng, lanes 3 and 7; 250 ng, lanes 4 and 8) was incubated with m⁷G-capped Ad5' in the absence or presence of a constant amount of rCBC. The reactions were irradiated under standard conditions, and the cross-linked products resolved by 6% denaturing PAGE. (B) An SR protein-enriched fraction was assayed for its ability to stimulate U1 association with the 5' splice site. A constant amount of U1 snRNP and SR protein fraction was added to either ApppG-capped (lanes 2,4) or m⁷GpppG-capped Ad5' (lanes 1,3) either in the absence (lanes 1,2) or the presence (lanes 2,4) of rCBC. The reactions were incubated, and UV cross-linked products resolved by 6% denaturing PAGE [see Materials and methods].

was inhibited to levels comparable to that of the single intron, whereas the inhibition of splicing of the cap distal intron varied between 20% and 30%. This demonstrated that the cap dependence for efficient splicing of a cap-proximal intron (Ohno et al. 1987; Inoue et al. 1989) was mediated by the CBC.

To differentiate between various possibilities for the insensitivity of the splicing of the more distal intron to CBC depletion, we created a series of mutant versions of the 004Ad construct, containing mutations at the 5' splice site ($\Delta 5'$ ss), 3' splice site ($\Delta 3'$ ss), or the polypyrimidine tract (Δ PT) of the 004 intron (Fig. 6A). A second set of double mutants was then constructed where the $\Delta 5'$ ss mutation was combined with the $\Delta 3'$ ss or Δ PT

mutations. These constructs were then transcribed and assayed for their ability to splice in mock-depleted or depleted extract. Mutation of the 5' splice site inhibited splicing of the cap proximal 004 intron but did not significantly affect the level of splicing of the downstream intron (Fig. 6C, lane 7). The level of Ad intron splicing from this mutant pre-mRNA in depleted extract was not greatly reduced compared to the mock-depleted control reaction [cf. lanes 7 and 8]. Splicing of the Δ PT or $\Delta 3'$ ss pre-mRNAs was reasonably efficient in mock-depleted extracts (Fig. 6C, lanes 13,15) giving rise mainly to products that resulted from the skipping of exon 2 and ligation exons of 1 and 3 to produce the mature message. The resulting lariat product and lariat-exon intermedi-

Figure 6. CBC is required for efficient splicing of the cap proximal intron. (A) The double-intron constructs used are shown diagrammatically, together with the sequences mutated. (B) Splicing in either mock-depleted extracts (M) or CBC-depleted extracts (D) of the following m⁷GpppG-capped pre-mRNAs; Ad1 (lanes 1,2), 004 (lanes 3,4), and 004Ad (lanes 5,6). Splicing products were resolved on a 15% denaturing urea-polyacrylamide gel. The splicing products and intermediates are cartooned. (C) The polypyrimidine tract (ΔPT) insulates the cap distal intron from the effects of CBC depletion. The pre-mRNAs were synthesized with a m⁷GpppG cap and spliced in CBC-depleted extract (D) or mock-depleted extract (M). 004Ad (lanes 5,6) is the wild-type double intron pre-mRNA, mutations at the 5' splice site (Δ5'ss) (lanes 7,8) has a deletion at the 5' splice site, ΔPT (lanes 13,14) has a deletion of polypyrimidine tract sequences, Δ3'ss (lanes 15,16) is an AG→UU mutant at the 3' splice site, and Δ5'ssΔ3'ss (lanes 9,10) and Δ5'ssΔPT (lanes 11,12) combine these mutations. Splicing reactions were incubated for 90 min, and the splicing products resolved on either a 15% or a 6% denaturing urea-polyacrylamide gel.

ate contain the skipped exon. When assayed in depleted extracts both constructs behaved like the single-intron constructs, their splicing being inhibited by ~80% (lanes 14,16). Thus, the effect of CBC on utilization of the proximal 5' splice site was maintained even when this 5' splice site was being joined to the distal 3' splice site.

Next, the 5' splice site and Δ PT or Δ 3'ss double mutant constructs were assayed in mock-depleted or CBC-depleted extracts. The Δ 5'ss Δ 3'ss construct showed only a minor difference in downstream exon splicing, depending on whether CBC was present or not (Fig. 6C, lanes 11, 12). In contrast, although the Δ 5'ss Δ PT mutation allowed splicing of the adenovirus intron in mock-depleted extracts, this mutant was unable to splice efficiently in CBC-depleted extracts (lanes 9,10). The effect of the CBC on the efficiency of splicing, and thus presumably of U1 snRNP-5' splice site interaction, is therefore relatively insensitive to distance and to sequence context. Rather, an intact polypyrimidine tract in the cap-proximal intron appears to functionally substitute for CBC in stimulating the use of the cap-distal 5' splice site.

CBC is associated with the pre-mRNA during the splicing cycle

All of the above results are consistent with the function of CBC in splicing being at an early stage of the process. We wished to determine whether CBC was associated with the pre-mRNA only transiently early during splicing or was more stably associated with the spliceosome. Splicing reactions were therefore carried out in untreated splicing extracts and subjected to immunoprecipitation using either preimmune serum or anti-CBP80 serum (Fig. 7A). The anti-CBP80 serum specifically, although inefficiently, precipitated the pre-mRNA, the mature mRNA and the intermediates of splicing (lane 3). The lariat product however, which does not have a cap, was not precipitated at levels over those observed with pre-immune serum (lane 2). This suggested that CBC might associate early with the pre-mRNA and remain bound throughout the splicing cycle. To obtain independent evidence for this, a second method of examining the interaction of CBC before and during splicing was sought.

In recent years techniques of purifying splicing and presplicing complexes assembled on biotinylated pre-mRNA by gel filtration and streptavidin affinity selection have been established and refined (Reed et al. 1988; Reed 1990; Bennet et al. 1992a,b). Various pre-mRNA complexes have been defined in this way including the H complex (Bennet et al. 1992b), a nonspecific complex that forms immediately on addition of essentially any RNA to HeLa nuclear extract, the E and A complexes described above, and the B complex, or active spliceosome. To address the question of whether CBP80 and CBP20 were present in the two extreme cases, that is, the H and B complex fractions, streptavidin selection was carried out. Buffer conditions were chosen (250 mM salt) so as to isolate only those proteins which were stably associated with the pre-mRNA in the B complex frac-

tion, that is, the spliceosome associated proteins or SAPs (Bennet et al. 1992a). A large-scale splicing reaction was fractionated by gel filtration and the spliceosome fraction (B) and H complex identified. Selected proteins from the H or the B fraction were then resolved on an SDS-PAGE gel (Fig. 7B) and either silver stained (left panel) or blotted to a nitrocellulose membrane. The blot was first probed with anti-CBP80 antiserum then stripped and probed with serum raised against CBP20. Both proteins are recognized by their respective antibodies in the H complex and also in the B complex (lanes labeled α -CBP80 and α -CBP20, respectively). As can be seen in the silver-stained gel of the isolated fractions, which was run in parallel, the protein composition of the B fraction is quite complex. However, the arrowed band that is an abundant constituent of both H and B complexes corresponds in mobility to CBP80 and is therefore likely to be CBP80. It was not possible to identify CBP20 in the silver-stained gel as several closely spaced bands migrate in this region of the gel. Control precipitations using non-biotinylated pre-mRNA did not result in enrichment of either CBP80 or CBP20 in the bound fraction (data not shown). In conclusion, CBC appears to associate not only with the H complex but also to be stably associated with the B complex. Thus, CBC seems to associate with the pre-mRNA early in splicing and to remain bound to the cap when the mature mRNA leaves the spliceosome.

Discussion

The mode of action of the nuclear CBC in stimulating pre-mRNA splicing has been investigated. CBC binds to capped RNAs in vitro in a way that is independent of the presence of splicing signals in the RNA (Izaurralde et al. 1992, 1994, 1995). Consistent with this, immunoprecipitation and purification of splicing complexes showed that CBC was associated with the H complex that forms immediately on adding a substrate pre-mRNA to nuclear extract in a way that is independent of ATP or of splicing signals on the RNA. CBC, however, does not dissociate when specific splicing complexes form, as it was also found stably associated with the intermediates of splicing in the B complex, or spliceosome, as well as with the mature mRNA.

In spite of this, the major, and perhaps only, detectable influence of CBC on splicing is on the efficiency with which U1 snRNP interacts with the 5' splice site in the E, or early, splicing complex. Simply measuring E-like complex formation on a complete pre-mRNA showed only a twofold reduction upon CBC depletion. However, further analysis showed that whereas the formation of E3' complex (Michaud and Reed 1993) on the 3' portion of the intron was also not greatly affected by CBC depletion, the formation of the corresponding E5' complex (Michaud and Reed 1993) was reduced strongly. Further analysis of E complexes formed either on an entire pre-mRNA or on the 5' fragment of the intron showed that even when E-like complexes were formed, U1 snRNP-5' splice site interaction did not occur efficiently in CBC-depleted extracts. Thus, CBC depletion clearly does not

Figure 7. CBC remains associated with the pre-mRNA throughout the splicing cycle. (A) Splicing intermediates and mRNA can be specifically precipitated by anti-CBP80 serum. m⁷GpppG-capped Ad1 pre-mRNA was allowed to splice under standard conditions for 90 min and then immunoprecipitated using serum raised against CBP80 or preimmune serum. The immunoprecipitated products were recovered and resolved on a 10% denaturing urea-polyacrylamide gel. (Lane 1) Input reaction; (lanes 2,4) the precipitate and supernatant fractions for the preimmune serum; (lanes 3,5) the precipitate and supernatant fractions for the anti-CBP80 serum. (B) CBC is stably associated with B and H complex. Spliceosomes were assembled on m⁷GpppG-capped biotinylated Ad1 pre-mRNA and fractionated by gel filtration. The B and H fractions were selected in 250 mM salt, and the proteins resolved by 7.5%–20% gradient SDS-PAGE and silver stained. In parallel, samples were blotted to nitrocellulose, probed with anti-CBP80 serum, stripped, and probed with anti-CBP20 serum. The immunoreactive bands corresponding to CBP80 and CBP20 in the H and B complexes are marked as is the position of CBP80 in the selected proteins.

affect association of most of the components of E complex with the pre-mRNA but has a specific effect on U1 snRNP–5' splice site interaction within this complex.

U1 snRNP binding to the 5' splice site is a critical early step in intron recognition (see introductory section). CBC interacts with pre-mRNAs rapidly after their addition to splicing extract (see above) and is associated with nascent transcripts *in vivo* (Visa et al. 1996). This and the other data in this manuscript support the hypothesis that CBC binding to a nascent transcript, which will be capped after it reaches a length of 20–30 nucleotides (Rasmussen and Lis 1993), can help to define an RNA as a potential splicing substrate. If there is a 5' splice site in the transcript, CBC will help U1 snRNP to

bind there, thereby increasing the probability that an intron in the nascent transcript will be recognized and defined. This could potentially represent the first specific step of pre-mRNA recognition *in vivo*.

The equivalent earliest step in spliceosome assembly in yeast occurs during formation of the commitment complex (for review, see Rosbash and Séraphin 1991). Like mammalian E complexes, commitment complexes form in the absence of ATP and contain U1 snRNP and MUD2 in addition to the pre-mRNA, MUD2 being the probable yeast homolog of U2AF (Séraphin and Rosbash 1989; Abovich et al. 1994). It is therefore of considerable interest that the yeast equivalent of CBP20 has been identified as the product of the MUD13 gene, a mutant

form of which is synthetically lethal with a viable mutant allele of the gene encoding U1 snRNA (Colot et al., this issue). Yeast extracts lacking the CBP20 homolog form commitment complexes with very low efficiency, and this phenotype can be reproduced in wild-type yeast extracts by using pre-mRNA substrates that are uncapped or by immunodepleting the yeast CBC (Colot et al., this issue; J. Lewis et al., unpubl.). This indicates that yeast and human CBC carry out a similar, conserved function early in pre-mRNA recognition.

Limitation of CBC function to the cap-proximal intron

The effect of the cap structure on intron removal has previously been reported to be limited to the cap-proximal intron in pre-mRNAs containing more than a single intron (Ohno et al. 1987; Inoue et al. 1989). It is demonstrated here that this effect of the cap structure is mediated by the CBC. Extending the previous data, we were also able to demonstrate that CBC-independent splicing of the cap-distal intron was conferred by the polypyrimidine tract of the cap-proximal intron. When both the 5' splice site and the polypyrimidine tract of the cap-proximal intron were mutant, the cap-distal intron became CBC dependent, demonstrating that neither the distance from the cap nor the sequence context of the distal intron were important for its CBC independence. Interestingly, when only the 3' splice site or the polypyrimidine tract of the proximal intron were mutant, major products of splicing that involved exon skipping were seen, that is, splicing from the cap-proximal 5' splice site to the cap distal 3' splice site. These splicing events were again CBC-dependent. In total these results support the conclusion that it is the cap-proximal 5' splice site that is the target of CBC function, and that this interaction does not depend on a strict spacing between the cap and the affected 5' splice site. Moreover, they raise the possibility that the effect of CBC may be analogous to the effect, across the exon, of the polypyrimidine tract on the next downstream 5' splice site.

CBC and exon definition

Both of these conclusions about the function of CBC in splicing fit very nicely into the model of splice site recognition that has been called exon definition (Robberson et al. 1990). The model proposes, and is supported by evidence which shows, that stabilizing interactions can occur across the exon between the upstream 3' splice site and downstream 5' splice site (for review, see Berget 1995). In one of the best-studied cases, that of exon 4 of the rat preprotachykinin gene, there is evidence that U1 snRNP binding to the 5' splice site immediately downstream of exon 4, through an indirect mechanism, stimulates binding of U2AF to the polypyrimidine tract of the 3' splice site upstream of exon 4 (Hoffman and Grabowski 1992).

One problem posed by exon definition is how the first and last exons in a pre-mRNA are defined. Evidence ex-

ists that cleavage and polyadenylation signals may increase the efficiency of recognition of the 3'-most intron (e.g., Niwa and Berget 1991; for review, see Berget 1995), although this effect is apparently not universal (Nesic et al. 1995). This presumably reflects direct or indirect interactions between components of the 3' end formation and splicing machineries analogous to those between splicing factors bound to sites flanking an exon discussed above. Clearly, the CBC-mediated stimulation of the binding of the cap-proximal 5' splice site by U1 snRNP provides a mechanism for efficient recognition of this splice site. Because it is likely that this is an early event in pre-mRNA packaging, it is also plausible that this unique interaction helps to define which is the cap-proximal 5' splice site, and thus ensures that splice site pairing across the intron occurs in the correct register, resulting in accurate splice site definition along the entire length of the pre-mRNA transcript.

Which proteins might be involved in mediating the interactions between factors bound either to splice sites or to the extremities of a pre-mRNA? An extreme case of bridging interactions resulting in pairing between a 5' and a 3' splice site was reported recently (Chiara and Reed 1995). Here, interactions leading to *trans*-splicing between 5' and 3' splice sites located on two different RNAs was observed, provided that the 3' splice site RNA also contained, downstream of the 3' splice site, sequences encoding either a 5' splice site or an exon enhancer (Chiara and Reed 1995). The exon enhancer used has previously been proposed to bind both to U1 snRNP and to other proteins (Watakabe et al. 1993; Tanaka et al. 1994). Analysis of diverse exon enhancers (e.g., Tian and Maniatis 1993; Heinrichs and Baker 1995) show that among the critical proteins that bind to these elements are members of the SR protein family. This and other data (Fu 1993; Kohtz et al. 1994; Jamison et al. 1995) suggest that SR proteins play a role in the recognition of introns by helping to establish stable interactions between splicing factors like U1 snRNP and U2AF and their recognition sequences on the pre-mRNA, as well as potentially, presumably at a later step, mediating interactions between these factors across the intron once they are bound to the 3' or 5' splice site.

How then might the CBC participate in facilitating the interaction between U1 snRNP and the 5' splice site? Neither of the components of CBC, CBP80, or CBP20 (Izaurralde et al. 1994, 1995) contain the dipeptide repeats that are characteristic of the SR protein family (e.g., Zahler et al. 1992), and thus CBC is perhaps unlikely to act directly on U1 snRNP in the way proposed for some SR proteins (see, e.g., Fu and Maniatis 1992; Fu 1993; Kohtz et al. 1994; Jamison et al. 1995). Although we observed a large decrease in U1 snRNP-5' splice site interaction in extracts depleted of CBC, we were unable to see any positive effect on U1 snRNP-5' splice site cross-linking when utilizing highly purified U1 snRNP and recombinant CBC. Addition of SR proteins to CBC-depleted extract resulted in stimulation of splicing and of U1 snRNP-5' splice site interaction. However, in a highly purified reconstituted system SR protein addition

had no effect on the dependence of U1 snRNP–5' splice site interaction on CBC. These results argue against SR proteins being the mediators of the CBC effect on U1 snRNP. In addition, other work (Colot et al., this issue; J. Lewis et al., unpubl.) shows that yeast CBC plays a similar role in commitment complex assembly to that of human CBC in E complex formation. Because no SR proteins have thus far been identified in yeast, the likelihood of their mediating the CBC effect in vertebrates is further reduced. Thus, although either readdition of CBC or the addition of excess SR proteins to CBC depleted extracts could stimulate U1 snRNP–5' splice site interaction, the mechanism of these effects is likely to be different (see also Fu 1993; Kohtz et al. 1994; Jamison et al. 1995). The factor or factors that mediate the interaction between CBC and the U1 snRNP remains to be identified. It is even possible that the effect of the CBC is an antirepression effect, in that it might help to hinder the action of factors whose binding to the pre-mRNA would have a negative influence on the subsequent interaction of U1 snRNP with pre-mRNA. Further study will be required to distinguish between these possibilities.

The role of CBC in nuclear export

CBC remains associated with the cap throughout the splicing reaction and is released together with the mRNA. Studies on the nuclear export of RNA polymerase II transcripts have shown that CBC plays an important role in mediating U snRNA nuclear export (Izauralde et al. 1995). Although the cap structure does not have such a critical influence on mRNA export as it does on U snRNA export, the presence of a 5' cap stimulates the kinetics of mRNA export by approximately twofold (Jarmolowski et al. 1994). Although the basis of this effect is unknown, CBC might, for example, increase the efficiency with which a messenger RNP docks with the nuclear pore in a specific orientation that is favorable for export. Data from Dancho and co-workers have shown that in *Chironomus tentans* salivary gland cell nuclei, the Balbiani ring messenger RNP always docks with the nuclear pore complex 5' end first and is translocated through the pore to the cytoplasm in a 5' → 3' orientation (Mehlin et al. 1992, 1995). Recent data indicate that CBC is associated with Balbiani ring mRNPs during these docking and translocation steps (Visa et al. 1996), consistent with the hypothesis that CBC could have a role at these stages of RNA nuclear export.

Materials and methods

DNA constructs

The following pre-mRNA plasmids were used, transcribed by T3 RNA polymerase: pBSAd1 (Konarska and Sharp 1987) linearized by *Sau*III; Ad5' was made by cleaving pBSAd1 with *Fnu*4HI; pAd1DHindIII was linearized by *Sau*III; pBS004 (derived from the *Xenopus* L1a gene (Lorenz et al. 1985) encompassing exons 4 and 5) was linearized by *Eco*RI; pBS004Ad and

its mutagenized derivatives, pΔ5'ss, ΔPT, Δ3'ss, Δ5'ssΔ3'ss, and Δ5'ssΔPT were linearized by *Eco*RI.

pBS004Ad was constructed by cloning the *Pvu*II–*Eco*RI fragment of pBSAd1 into the pBS – vector (Stratagene) between the *Sma*I and *Eco*RI sites. Then the *Bam*HI fragment containing the Ad1 intron was recloned into the *Bam*HI site of pBS004. This double intron construct contains the 004 intron (194 nucleotides) at a cap-proximal position, the Ad1 intron (223 nucleotides) cap-distal and three exons: E1, E2, and E3 (124, 65, and 130 nucleotides, respectively). The derivatives of pBS004Ad were created by site-directed mutagenesis. pΔ5'ss has a deletion (GGTAA) at the 5' splice site of the 004 intron, ΔPT has a deletion (ATGCACTTTGCTTT) of sequences at and near the 004 polypyrimidine tract, and Δ3'ss has the last two nucleotides of the 004 intron changed from AG to TT. The double mutants combine these changes.

pCX (Hamm and Mattaj 1990), which is a mutant derived from pBSAd1, where position 5 of the intron is altered from G to A, was linearized by *Sau*III and transcribed by T7 RNA polymerase.

CBC depleted extracts

Fresh HeLa cells were purchased from the Computer Cell Culture Centre (Mons, Belgium). Nuclear splicing extracts were prepared essentially as described by Dignam et al. (1983). Extracts were depleted of CBC using anti-CBP80 serum coupled to Fast Flow Protein A 4 as described previously (Izauralde et al. 1994).

Splicing reactions

Splicing reactions were in a final volume of 20 μl as described in Izauralde et al. (1994). Substrates were transcribed using T3 RNA polymerase. The reaction was preincubated in the absence of ATP, creatine phosphate, or pre-mRNA (3–5 ng for single-intron constructs and 6–8 ng for double-intron constructs) for 15 min at 30°C. The splicing reaction was started by addition of these components and incubated for either 90 or 120 min. The spliced products were recovered and resolved by 10% denaturing PAGE for the Ad1 pre-mRNA. The double-intron constructs were resolved by 15% denaturing PAGE or, in the case of ΔPT and Δ3'ss, by 6% denaturing PAGE.

Recombinant CBC and CBP20 and gel mobility shift assay

rCBC was produced as described in Izauralde et al. (1995), with the following modification. Prior to elution with imidazole, the bound protein was washed in PGK buffer containing 0.05% Triton X-100 then eluted with 500 mM imidazole in PGK buffer, 0.05% Triton X-100. The peak fractions were dialysed against buffer D (100 mM KCl, 20 mM Hepes at pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM DTT). The gel mobility shift assay was as described previously (Izauralde et al. 1992).

Immunoprecipitation of splicing reactions

Splicing reactions were incubated for 90 min, then diluted 20-fold in IPP250 (250 mM NaCl, 20 mM Tris at pH 7.5, 0.1% Triton X-100). This mixture was then divided into two aliquots and 10 μl of either anti-CBP80 serum or preimmune serum was added and incubated on ice for 1 hr. To this was added 40 μl of a 50% suspension of protein A sepharose beads in IPP250 and the mixture was rotated at room temperature for 30 min. The bound antibody complexes were then pelleted by centrifugation and washed three times with 1 ml of IPP250. To extract the RNA,

the pellets were digested in a final volume of 50 μ l of RNA extraction buffer containing 1 mg/ml of proteinase K and 1% SDS. Carrier tRNA (2.5 μ g) was added, and the mixture was phenol extracted and ethanol precipitated. In parallel, the RNA from the supernatant was recovered. The precipitated products and supernatants were then resolved by 10% denaturing PAGE.

Purification of splicing complexes

Purification of splicing complexes was carried out essentially as described by Reed (1990). An 11-ml splicing reaction was assembled and aliquoted into 0.5-ml fractions. These were incubated for 20 min at 30°C in the presence of biotinylated m⁷GpppG-capped pre-mRNA (~30 μ g). Under these conditions, the vast majority of assembled spliceosomes have not undergone the first step of splicing and are predominantly B splicing complexes as determined by native gel electrophoresis. The only modification to the published protocol was that B complexes were resolved from H complex by gel filtration on a Sephacryl S-500HR XK26/70 column (Pharmacia) at a flow rate of 0.4 ml/min. Fractions (1.6 ml) were collected and 1/16 of each was counted by liquid scintillation. Peak fractions were pooled, and the salt concentration adjusted to 250 mM by addition of 5M NaCl prior to selection. Selected proteins were resolved by 7.5%–20% SDS-PAGE and silver stained (Wray et al. 1981).

E complex analysis

E, E', and E3' complex analysis was performed essentially as described (Michaud and Reed 1991, 1993). Splicing reactions (300 μ l) were set up using mock-depleted or depleted extracts in the absence of ATP or creatine phosphate, and preincubated at 30°C for 20 min to deplete endogenous ATP. After the preincubation, the m⁷GpppG-capped transcript (30–50 ng) was added and incubated for an additional 15 min to allow complex assembly. Complexes were then resolved by Sephacryl S-500HR gel filtration (XK16/70 Pharmacia) at a flow rate of 0.15 ml/min, and 0.6-ml fractions were collected and counted by liquid scintillation.

Psoralen cross-linking and RNase H digestion

Reactions were assembled under conditions similar to those used for E complex assembly, except that AMT-psoralen (HRI Associates) was added to a final concentration of 50 ng/ml. Probes were in vitro transcribed under conditions that result in a high specific activity in the presence of 80 μ Ci of [α -³²P]UTP (Wassarman and Steitz 1992), primed with either an m⁷GpppG or ApppG cap as indicated in the legend to Figure 3. The complexes were allowed to assemble for 15 min, then the samples were irradiated for 10 min at 360 nm in an HRI photocell that was precooled to 4°C. After cross-linking, proteins were digested at 50°C for 20 min by addition of a proteinase K/SDS mix. The samples were then extracted with phenol/chloroform (1:1 vol/vol) and ethanol precipitated. RNA pellets were either dissolved in formamide loading dye and heat denatured or dissolved in 4 μ l of TE for RNase H digestion. RNase H digestions were carried out for 60 min at 30°C in a final reaction of 10 μ l in a buffer containing 40 mM Tris (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 2.5 mg of tRNA, and 2 units of RNase H (GIBCO BRL). The anti-U1 snRNA oligonucleotide was complementary to nucleotides 64–75 of the U1 snRNA. After the digestion, reactions were phenol/chloroform extracted, ethanol precipitated, and dissolved in formamide loading dye. The cross-linked products were resolved from non-cross-linked probe on a 6% denaturing urea-polyacrylamide gel.

Psoralen cross-linking in purified system

For psoralen cross-linking by use of purified and recombinant components, the reactions were set up as described above with the following modifications. The salt concentration of the final reaction was adjusted to 35 mM KCl by addition of buffer D (see above). Acetylated BSA (NEB) was added to a final concentration of 100 μ g/ml to help stabilize the protein components. The reaction was assembled without the RNA and preincubated for 15 min at 30°C. To start the reaction, RNA was added and incubated for an additional 15 min. The samples were then irradiated in a HRI photocell that had been precooled to 4°C for 10 min. The samples were then processed as described above.

Purification of U1 snRNP

U1 snRNP was purified essentially as described by Bach et al. (1990). Nuclear splicing extracts were prepared (Dignam et al. 1983) and passed over an immobilized trimethyl-guanosine antibody (Oncogene Science) affinity column. The bound snRNPs were eluted overnight with methyl-7-guanosine (Sigma). The enriched snRNP eluate was then purified further using mono Q chromatography with a linear 25-ml gradient from 50–1000 mM KCl in mono Q buffer (20 mM Tris-Cl at pH 7.0, 1.5 mM MgCl₂, 0.5 mM DTT, 0.1 mM PMSF). U1 snRNP was localized by Northern blotting, and the purity of the peak fractions checked by SDS-PAGE. Protein concentration was estimated using the Bio-Rad Protein Assay.

Purification of SR proteins

SR proteins were prepared from HeLa cells using the method described by Zahler et al. (1992) and were a kind gift from Drs. Cinzia Calvio and Angus Lamond (EMBL, Heidelberg, Germany).

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References

- Abovich, N., X.C. Liao, and M. Rosbash. 1994. The yeast MUD2 protein: An interaction with PRP11 defines a bridge between commitment complexes and U2 addition. *Genes & Dev.* 8: 843–854.
- Bach, M., P. Bringmann, and R. Lührmann. 1990. Purification of small nuclear ribonucleoprotein particles with antibodies against modified nucleosides of small nuclear RNAs. *Methods Enzymol.* 181: 232–257.
- Bennet, M., S. Michaud, J. Kingston, and R. Reed. 1992a. Protein components specifically associated with prespliceosome and spliceosome complexes. *Genes & Dev.* 6: 1986–2000.

- Bennet, M., S. Piñol-Roma, D. Staknis, G. Dreyfuss, and R. Reed. 1992b. Differential binding of heterogeneous nuclear ribonucleoproteins to mRNA precursors prior to spliceosome assembly *in vitro*. *Mol. Cell. Biol.* **12**: 3165–3175.
- Berget, S.M. 1995. Exon recognition in vertebrate splicing. *J. Biol. Chem.* **270**: 2411–2414.
- Chiara, M. and R. Reed. 1995. A two-step mechanism for 5' and 3' splice-site pairing. *Nature* **375**: 510–513.
- Colot, H.V., F. Stutz, and M. Rosbash. 1996. The yeast splicing factor Mud13p is a commitment complex component and corresponds to CBP20, the small subunit of the nuclear cap-binding complex. *Genes & Dev.* [this issue].
- Dignam, J., R. Lebowitz, and R. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from mammalian nuclei. *Nucleic Acids Res.* **11**: 1475–1589.
- Ederly, I. and N. Sonenberg. 1985. Cap-dependent RNA splicing in a HeLa nuclear extract. *Proc. Natl. Acad. Sci.* **82**: 7590–7594.
- Fu, X.-D. 1993. Specific commitment of different pre-mRNAs to splicing by SR proteins. *Nature* **365**: 82–85.
- Fu, X.-D. and T. Maniatis. 1992. The 35-kDa mammalian splicing factor SC35 mediates specific interactions between U1 and U2 small nuclear ribonucleoprotein particles at the 3' splice site. *Proc. Natl. Acad. Sci.* **89**: 1725–1729.
- Hamm, J. and I.W. Mattaj. 1990. Monomethylated cap structures facilitate RNA export from the nucleus. *Cell* **63**: 109–118.
- Heinrichs, V. and B. Baker. 1995. The Drosophila SR protein RBP1 contributes to the regulation of doublesex alternative splicing by recognising RBP1 RBA target sequence. *EMBO J.* **14**: 3987–4000.
- Hodges, P. and J. Beggs. 1994. U2 fulfils a commitment. *Curr. Biol.* **4**: 264–267.
- Hoffman, B.E. and P.J. Grabowski. 1992. U1 snRNP targets an essential splicing factor, U2AF65, to the 3' splice site by a network of interactions spanning the exon. *Genes & Dev.* **6**: 2554–2568.
- Inoue, K., M. Ohno, H. Sakamoto, and Y. Shimura. 1989. Effect of the cap structure on pre-mRNA splicing in *Xenopus* oocyte nuclei. *Genes & Dev.* **3**: 1472–1479.
- Izaurrealde, E., J. Stepinski, E. Darzynkiewicz, and I. Mattaj. 1992. A cap binding protein that may mediate nuclear export of RNA polymerase II-transcribed RNAs. *J. Cell Biol.* **118**: 1287–1295.
- Izaurrealde, E., J. Lewis, C. McGuigan, M. Jankowska, E. Darzynkiewicz, and I. Mattaj. 1994. A nuclear cap binding protein complex involved in pre-mRNA splicing. *Cell* **78**: 657–668.
- Izaurrealde, E., J. Lewis, C. Gamberi, A. Jarmolowski, C. McGuigan, and I.W. Mattaj. 1995. A cap-binding protein complex mediating U snRNA export. *Nature* **376**: 709–712.
- Jamison, S.P.Z., J. Wang, C. Will, R. Lührmann, J. Manley, and M. Garcia-Blanco. 1995. U1 snRNP-ASF/SF2 interaction and 5' splice site recognition: Characterisation of required elements. *Nucleic Acids Res.* **23**: 3260–3267.
- Jarmolowski, A., W.C. Boelens, E. Izaurrealde, and I.W. Mattaj. 1994. Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.* **124**: 627–635.
- Kataoka, N., M. Ohno, I. Moda, and Y. Shimura. 1995. Identification of the factors which interact with NCBP, an 80kDa nuclear cap binding protein. *Nucleic Acids Res.* **23**: 3638–3641.
- Kohtz, J., S. Jamison, C. Will, P. Zuo, R. Lührmann, M. Garcia-Blanco, and J. Manley. 1994. Protein-protein interactions and 5'-splice-site recognition in mammalian mRNA precursors. *Nature* **368**: 119–124.
- Konarska, M. and P. Sharp. 1987. Interactions between small ribonucleoprotein particles in the formation of spliceosomes. *Cell* **49**: 763–774.
- Konarska, M., R. Padgett, and P. Sharp. 1984. Recognition of cap structure in splicing *in vitro* of mRNA precursors. *Cell* **38**: 731–736.
- Krainer, A., T. Maniatis, B. Ruskin, and M. Green. 1984. Normal and mutant human β -globin pre-mRNAs are faithfully and efficiently spliced *in vitro*. *Cell* **36**: 993–1005.
- Lamm, G.M. and A.I. Lamond. 1993. Non-snRNP protein splicing factors. *Biochim. Biophys. Acta* **1173**: 247–265.
- Loreni, F., I. Ruberti, I. Bozzoni, P. Pierandrei-Amaldi, and F. Amaldi. 1985. Nucleotide sequence of the L1 ribosomal protein gene of *Xenopus laevis*: Remarkable sequence homology among introns. *EMBO J.* **4**: 3483–3488.
- Madhani, H. and C. Guthrie. 1994. Dynamics in RNA-RNA interactions in the spliceosome. *Annu. Rev. Genet.* **28**: 1–26.
- Mehlin, H., B. Daneholt, and U. Skoglund. 1992. Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. *Cell* **69**: 605–613.
- . 1995. Structural interaction between the nuclear pore complex and a specific translocating RNP particle. *J. Cell Biol.* **129**: 1205–1216.
- Michaud, S. and R. Reed. 1991. An ATP-independent complex commits pre-mRNA to the mammalian spliceosome assembly pathway. *Genes & Dev.* **5**: 2534–2546.
- . 1993. A functional association between the 5' and 3' splice sites is established in the earliest prespliceosome complex [E] in mammals. *Genes & Dev.* **7**: 1008–1020.
- Moore, M., C. Query, and P. Sharp. 1993. Splicing of precursors to messenger RNAs by the spliceosome. In *The RNA world* (ed. R. Gesteland and J. Atkins), pp. 303–357. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Nesic, D., J. Zhang, and L.E. Maquat. 1995. Lack of an effect of the efficiency of RNA 3'-end formation on the efficiency of removal of either the final or the penultimate intron in intact cells. *Mol. Cell. Biol.* **15**: 488–496.
- Newman, A. 1994. Small nuclear RNAs and pre-mRNA splicing. *Curr. Opin. Cell Biol.* **6**: 360–367.
- Niwa, M. and S.M. Berget. 1991. Mutation of the AAUAAA polyadenylation signal depresses *in vitro* splicing of proximal but not distal introns. *Genes & Dev.* **5**: 2086–2095.
- Ohno, M., H. Sakamoto, and Y. Shimura. 1987. Preferential excision of the 5' proximal intron from mRNA precursors with two introns as mediated by the cap structure. *Proc. Natl. Acad. Sci.* **84**: 5187–5191.
- Patzelt, E., E. Tahlmann, K. Hartmuth, D. Blaas, and E. Kuechler. 1987. Assembly of pre-mRNA splicing complex is cap dependent. *Nucleic Acids Res.* **15**: 1387–1399.
- Rasmussen, E. and J. Is. 1993. *In vivo* transcriptional pausing and cap formation on three Drosophila heat shock genes. *Proc. Natl. Acad. Sci.* **90**: 7923–7927.
- Reed, R. 1990. Protein composition of mammalian spliceosomes assembled *in vitro*. *Proc. Natl. Acad. Sci.* **87**: 8031–8035.
- Reed, R., J. Griffith, and T. Maniatis. 1988. Purification and visualisation of native spliceosomes. *Cell* **53**: 949–961.
- Robberson, B.L., G.J. Cole, and S.M. Berget. 1990. Exon definition may facilitate splice site selection in RNAs with multiple exons. *Mol. Cell. Biol.* **10**: 84–94.
- Rosbash, M. and B. Séraphin. 1991. Who's on first? The U1 snRNP-5' splice site interaction and splicing. *Trends Biochem. Sci.* **16**: 187–190.

- Séraphin, B. and M. Rosbash. 1989. Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. *Cell* **59**: 349–358.
- Tanaka, K., A. Watakabe, and Y. Shimura. 1994. Polypurine sequences within a downstream exon function as a splicing enhancer. *Mol. Cell. Biol.* **14**: 1347–1354.
- Tian, M. and T. Maniatis. 1993. A splicing enhancer complex controls alternative splicing of *doublesex* pre-mRNA. *Cell* **74**: 105–114.
- Visa, N., E. Izaurralde, J. Ferreira, B. Daneholt, and I.W. Mattaj. 1996. A nuclear Cap Binding Complex binds Balbiani Ring pre-mRNA co-transcriptionally and accompanies the ribonucleoprotein particle during nuclear export. *J. Cell Biol.* **133**: 5–15.
- Wassarman, D.A. and J.A. Steitz. 1992. Interactions of small nuclear RNA's with precursor messenger RNA during *in vitro* splicing. *Science* **257**: 1918–1925.
- Watakabe, A., K. Tanaka, and Y. Shimura. 1993. The role of exon sequences in splice site selection. *Genes & Dev.* **7**: 407–418.
- Wray, W., T. Bouliskas, V.P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**: 197–203.
- Zahler, A., W. Lane, J. Stolk, and M. Roth. 1992. SR proteins: A conserved family of pre-mRNA splicing factors. *Genes & Dev.* **6**: 837–847.
- Zamore, P.D. and M.R. Green. 1989. Identification, purification, and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor. *Proc. Natl. Acad. Sci.* **86**: 9243–9247.

A nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5' splice site.

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References

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