Resolution of the mammalian E complex and the ATP-dependent spliceosomal complexes on native agarose mini-gels

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RNA 1999 5: 1504-1508

RNA (1999), *5*:1504–1508. Cambridge University Press. Printed in the USA.

METHOD

Resolution of the mammalian E complex and the ATP-dependent spliceosomal complexes on native agarose mini-gels

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ABSTRACT

A great deal of progress in elucidating the mechanisms of spliceosome assembly has been achieved by analyzing the A, B, and C spliceosomal complexes on native polyacrylamide gels. In contrast, progress in understanding the earliest spliceosomal complex E has been hampered because this complex dissociates on native gels and is difficult to detect by other methods. Here we report conditions for resolving the spliceosomal complex E using a native horizontal agarose mini-gel system. This system also provides a simple alternative to polyacrylamide gels for resolving the ATP-dependent spliceosomal complexes.

Keywords: E complex; native gel; spliceosome; spliceosome assembly; splicing

INTRODUCTION

Spliceosomal complexes assemble on pre-mRNA in a stepwise pathway in vitro. In mammals, the nonspecific complex H assembles first and consists of hnRNP proteins (Konarska & Sharp, 1986; Bennett et al., 1992). H complex formation does not require splice sites or ATP and occurs at 4 °C (Konarska & Sharp, 1986). The relationship between the H complex and the spliceosomal complexes is not clear, but at least a portion of the H complex cannot serve as a substrate for spliceosome assembly (Michaud & Reed, 1993). The ATP-independent E complex is the first discrete functional spliceosomal complex (Reed, 1990; Michaud & Reed, 1991). E complex formation requires incubation at 30 °C and assembles more efficiently on pre-mRNAs containing both a 5′ splice site and the pyrimidine tract at the 3′ splice site (Jamison et al., 1992; Michaud & Reed, 1993). Complexes related to the E complex, the E5′ and E3′ complexes, assemble on "half-substrate" RNAs containing only a 5′ or a 3′ splice site, respectively (Michaud & Reed, 1993). The E and E3′ complexes are precursors to the ATP-dependent A and A3′ complexes (Michaud & Reed, 1993). In contrast to the E complexes, the A complexes require the branchpoint sequence (BPS) for assembly (Champion-Arnaud et al., 1995). The B complex assembles after the A complex, and the two catalytic steps of splicing take place in the C complex (Konarska & Sharp, 1986, 1987; Lamond et al., 1987). Complexes analogous to the mammalian spliceosomal complexes are also detected in yeast, with the yeast commitment complex being the apparent counterpart of the mammalian E complex (Séraphin & Rosbash, 1989).

Several methods have been used to isolate and characterize the spliceosomal complexes in both yeast and mammals, including density-gradient sedimentation (Brody & Abelson, 1985; Frendewey & Keller, 1985; Grabowski et al., 1985), native gel electrophoresis (Konarska et al., 1985; Konarska & Sharp, 1986; Pikielny et al., 1986), and gel filtration (with or without an affinity-purification step) (Reed et al., 1988; Reed, 1990). The advantage of native gels is that multiple parameters can be readily tested on a single gel. The yeast and mammalian A, B, and C complexes, as well as the yeast commitment complex, are well-resolved on native gels (Konarska et al., 1985; Konarska & Sharp, 1986; Pikielny et al., 1986; Séraphin & Rosbash, 1989). In contrast, the mammalian E complex dissociates on native gels and has only been detected on density gradients and by gel filtration (Reed, 1990; Michaud & Reed, 1991; Jamison et al., 1992). Because these dif-

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ficult fractionation methods have hampered progress in characterizing the E complex, we have now established conditions for resolving the E complex on native agarose gels. This method is significantly easier than the native polyacrylamide gels previously used for analyzing the spliceosomal complexes and also provides a simple alternative for resolving the A, B, and C complexes.

RESULTS AND DISCUSSION

The most commonly used method for resolving the H, A, B, and C complexes is electrophoresis on a vertical, 4% nondenaturing polyacrylamide gel (Konarska & Sharp, 1986, 1987). The buffer contains Tris-glycine, and heparin is usually added to the splicing reaction to dissociate nonspecific interactions. However, under these conditions, the E complex comigrates with the H complex (Michaud & Reed, 1991, 1993). To determine whether we could resolve the E complex using different conditions, we tried the same horizontal agarose minigel and Tris-borate buffer (TBE) normally used for fractionation of DNA (see Materials and Methods).

AdML pre-mRNA was incubated under E complex conditions (i.e., −ATP) for the times indicated, and then heparin was either added or not added to an aliquot of each reaction prior to fractionation on a 1.5% TBE agarose gel. Consistent with previous work (Konarska & Sharp, 1986, 1987; Michaud & Reed, 1991, 1993), only one band was detected in the reactions containing heparin (Fig. 1A, +heparin). Significantly, however, a slow-mobility complex was also detected in the reactions lacking heparin (Fig. 1A, −heparin). This complex is not present at the zero time point and accumulates throughout the time course. By systematically varying conditions, we found maximal resolution of the two complexes when the gel was made with low-melting-point agarose and run at 4 °C. In addition, the complexes entered the gel better with Tris-glycine buffer instead of TBE (Fig. 1B). We have designated these complexes the E and H complexes (Fig. 1) based on the series of studies carried out below.

The putative E complex detected on the agarose gel requires incubation at 30 °C for assembly (Fig. 2A) as is the case for the E complex detected by gel filtration (Michaud & Reed, 1991). Moreover, as shown in Figure 2B, the putative E complex forms efficiently on wild-type pre-mRNA but significantly less efficiently on an isogenic pre-mRNA lacking either a 5′ splice site or the pyrimidine tract at the 3′ splice site. Significantly, the complex does not assemble at all on a pre-mRNA lacking both 5′ and 3′ splice sites (Fig. 2B). The temperature dependence, together with the splice-site requirements, are consistent with the conclusion that the slow and fast mobility complexes correspond to the E and H complexes, respectively (Michaud & Reed, 1991, 1993).

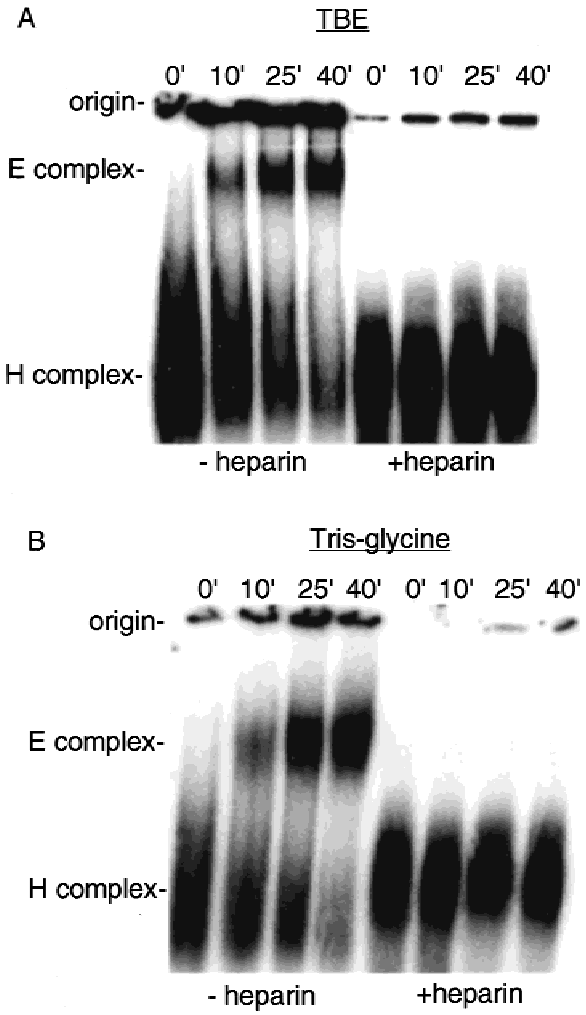

FIGURE 1. Native agarose gel analysis of ATP-independent spliceosomal complexes. **A**: AdML pre-mRNA was incubated in nuclear extract in the absence of ATP for the times indicated and then loaded directly (−heparin), or with heparin added (+heparin) prior to loading onto a 1.5% low-melting-point agarose gel. The gel running buffer was 0.5× TBE. Complexes were detected by phosphorimager. The gel origin and E and H complexes are indicated. **B**: Same as **A** except the gel running buffer was Tris-glycine.

Previous studies showed that the branchpoint sequence is not required for assembly of the E complex on AdML pre-mRNA (Champion-Arnaud et al., 1995). As shown in Figure 3, the putative E complex was detected on the native gel assembles about as efficiently in the absence of the BPS as on wild-type pre-mRNA (Fig. 3, lanes 1–4). This complex is heparin sensitive as expected for the E complex (Fig. 3, lanes 5, 6). In the presence of ATP (and heparin), the A complex assembles on normal pre-mRNA but not on the BPS mutant as shown previously (Fig. 3, lanes 7–10) (Champion-Arnaud et al., 1995).

To obtain further evidence that the heparin-sensitive complex detected on the agarose gel corresponds to

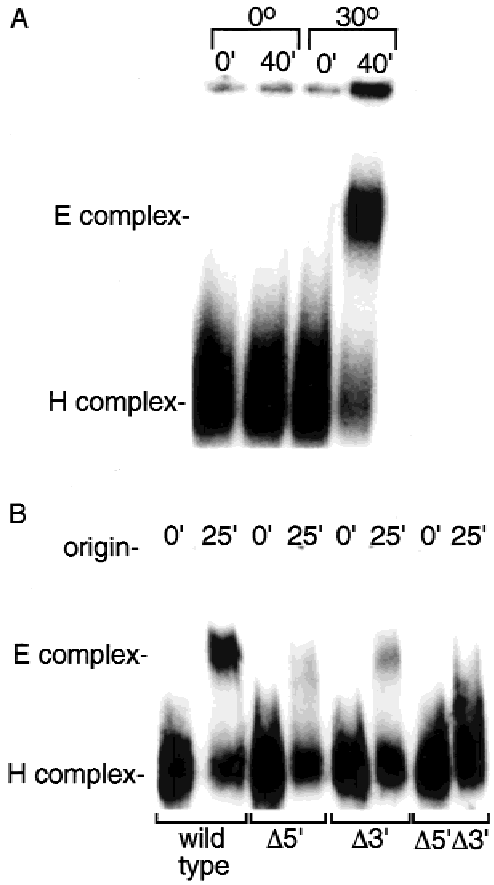

FIGURE 2. Temperature and splice-site dependence of the ATP-independent complexes. **A**: AdML pre-mRNA was incubated on ice or at 30 °C. An aliquot of each reaction was fractionated on a native agarose gel. **B**: The indicated pre-mRNAs were incubated in nuclear extracts in the absence of ATP and then fractionated on a native agarose gel.

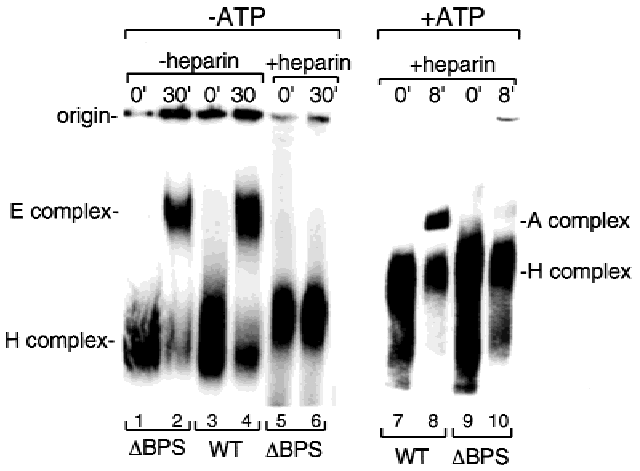

FIGURE 3. Analysis of a BPS mutation by native gel electrophoresis. Wild type (WT) pre-mRNA or a pre-mRNA containing a mutant BPS (Champion-Arnaud et al., 1995) was incubated in the presence or absence of ATP for the times indicated. Heparin was added to the indicated reactions prior to fractionation on a 1.5% (−ATP) or 2% agarose (+ATP) gel.

the E complex, we incubated pre-mRNA in nuclear extracts under E complex conditions, and then fractionated the reaction by gel filtration (Fig. 4A). Aliquots of fractions containing either the E or the H complex were then fractionated on a native agarose gel next to an aliquot of the total reaction loaded on the column. As shown in Figure 4B, the gel-filtration-isolated E and H complexes have about the same mobilities as the respective complexes in the total reaction. It is likely that the complexes from the total nuclear extract migrate slightly differently because they are present in the highly concentrated nuclear extract, whereas the gel-filtration-isolated complexes are very dilute.

Several observations reported here indicate that we have resolved the E complex by native agarose gel electrophoresis. Like the E complex characterized on gel-filtration columns, the complex that we have detected on agarose gels is ATP-independent, heparin-sensitive, requires 5′ and 3′ splice sites for efficient formation, requires incubation at 30 °C, and assembles independently of the BPS. Moreover, the E complex detected on native agarose gels is not contaminated with ATP-dependent spliceosomal complexes because the ATP-dependent complexes are detected on native gels after heparin treatment (see below), whereas the E complex is not (e.g., Fig. 1). Thus, in contrast to gel filtration, native agarose gels provide a rapid and simple assay for E complex formation.

In addition to the E complex, the ATP-dependent spliceosomal complexes are also readily resolved on native gels. For the analysis shown in Figure 5, we used ΔAG pre-mRNA that contains an AG-to-GG substitution at the 3′ splice site (Gozani et al., 1994). This mutation blocks step II of splicing and therefore allows accumulation of the C complex. (On wild-type pre-mRNAs, the catalytic steps of splicing occur so rapidly that the C complex is difficult to detect as a discrete complex.) As shown in the time course in Figure 5, heparin-resistant, ATP-dependent complexes are resolved on the native agarose gel. These complexes assemble with the same kinetics and have the same electrophoretic mobilities as the A, B, and C complexes on native polyacrylamide gels (Konarska & Sharp, 1987; Lamond et al., 1987). Another advantage of the low-melting-point agarose gels is that the complexes can be readily isolated from the gels for analysis of the labeled RNAs in these complexes. Such an analysis showed that the H, E, A, and B complexes contain unspliced pre-mRNA (Fig. 5B; data not shown). In contrast, the C complex contains the splicing intermediates, confirming the identity of this complex (Fig. 5B). Thus, native agarose gels provide a simple alternative to polyacrylamide gels for analysis of all of the spliceosomal complexes.

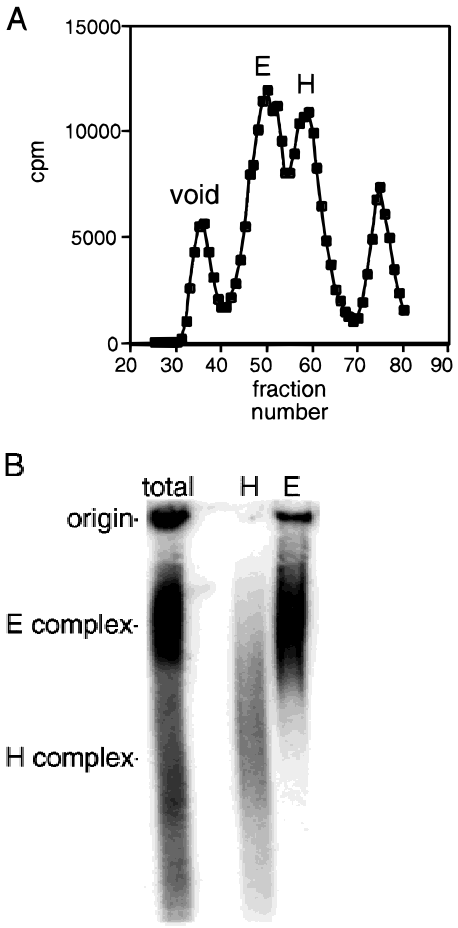

FIGURE 4. Fractionation of gel-filtration-isolated E complex on a native agarose gel. **A:** Pre-mRNA was incubated in nuclear extract in the absence of ATP for 25 min and then loaded onto a gel-filtration column. The void volume and E and H complexes are indicated. **B**: Aliquots (20 μL) of fractions containing the E and H complexes were fractionated on a 1.5% agarose gel next to an aliquot of the reaction loaded on the gel-filtration column.

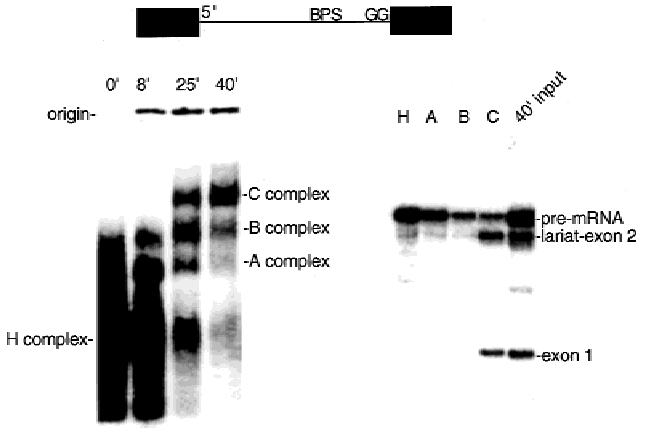

FIGURE 5. Resolution of the ATP-dependent spliceosomal complexes on a native agarose gel. **A**: ΔAG pre-mRNA was incubated in nuclear extract in the presence of ATP for the times indicated. Heparin was added to the reactions before loading on a 2% agarose gel. The spliceosomal complexes are indicated. **B**: Total RNA was extracted from the A, B, and C complexes and fractionated on an 8% denaturing gel. An aliquot of a splicing reaction incubated for 40 min was run as a marker.

MATERIALS AND METHODS

Plasmids and pre-mRNA synthesis

The plasmids encoding Δ5′, Δ3′, and the isogenic AdML control pre-mRNAs are described by Michaud & Reed (1993). A plasmid encoding Δ5′Δ3′ was constructed by combining the 5′ and 3′ portions of Δ5′ and Δ3′. ΔBPS (LUC mutant) and the isogenic AdML wild-type control are described by Champion-Arnaud et al. (1995). ΔAG is described by Gozani et al. (1994). Plasmids were digested with *Bam*H1 and transcribed with T7 RNA polymerase.

Spliceosome assembly reactions and analysis

For assembly of the E complex (Michaud & Reed, 1993), the indicated pre-mRNAs (5 ng) were incubated in 25 μL splicing reactions lacking ATP and $MgCl_2$. ATP was depleted from the extract by incubating it at room temperature for 20 min (Michaud & Reed, 1993). For assembly of the A, B, and C complexes, ΔAG pre-mRNA was incubated in nuclear extracts using standard splicing conditions (Krainer et al., 1984). Where indicated, 5 μL of 4 mg/mL heparin were added to 25-μL reactions prior to loading on gels. The 5× loading dye contains 1× TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA), 20% glycerol, 0.25% BPB and 0.25% XC. For native gel analysis, 3-μL aliquots of each reaction were loaded on 1.5% horizontal low-melting-point agarose (Gibco BRL) gels. The running buffer was 50 mM Tris, 50 mM glycine (Konarska & Sharp, 1987), except in Figures 1A and 4B, where 0.5× TBE was used. The gels were 7 cm × 8 cm and were run at 70 V for 3 h (Tris-glycine) or 4.5 h (TBE) at 4 °C. For the ATP-dependent complexes, gels were run at room temperature for 3.5 h. Gels were fixed in 10% acetic acid, 10% methanol for 30 min, and then dried down under vacuum at 80 °C. The E complex was fractionated by gel filtration as described (Michaud & Reed, 1993).

ACKNOWLEDGMENTS

We are grateful to M.-J. Luo, K. Chua, Z. Zhou, and N. Clouse for useful discussions. This study was supported by a National Institutes of Health grant to R.R.

Received July 13, 1999; returned for revision July 27, 1999; revised manuscript received August 18, 1999

REFERENCES

Bennett M, Pinol-Roma S, Staknis D, Dreyfuss G, Reed R. 1992. Differential binding of heterogeneous nuclear ribonucleoproteins to mRNA precursors prior to spliceosome assembly in vitro. *Mol Cell Biol 12*:3165–3175.

Brody E, Abelson J. 1985. The "spliceosome": Yeast pre-messenger RNA associates with a 40S complex in a splicing-dependent reaction. *Science 228*:963–967.

Champion-Arnaud P, Gozani O, Palandjian L, Reed R. 1995. Accumulation of a novel spliceosomal complex on pre-mRNAs containing branch site mutations. *Mol Cell Biol 15*:5750–5756.

Frendewey D, Keller W. 1985. Stepwise assembly of a pre-mRNA splicing complex requires U-snRNPs and specific intron sequences. *Cell 42*:355–367.

Gozani O, Patton JG, Reed R. 1994. A novel set of spliceosome-associated proteins and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. *EMBO J 13*:3356–3367.

Grabowski PJ, Seiler SR, Sharp PA. 1985. A multicomponent complex is involved in the splicing of messenger RNA precursors. *Cell 42*:345–353.

Jamison SF, Crow A, Garcia-Blanco MA. 1992. The spliceosome assembly pathway in mammalian extracts. *Mol Cell Biol 12*:4279–4287.

Konarska MM, Grabowski PJ, Padgett RA, Sharp PA. 1985. Characterization of the branch site in lariat RNAs produced by splicing of mRNA precursors. *Nature 313*:552–557.

Konarska MM, Sharp PA. 1986. Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. *Cell 46*:845–855.

Konarska MM, Sharp PA. 1987. Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. *Cell 49*:763–774.

Krainer AR, Maniatis T, Ruskin B, Green MR. 1984. Normal and mutant human beta-globin pre-mRNAs are faithfully and efficiently spliced in vitro. *Cell 36*:993–1005.

Lamond AI, Konarska MM, Sharp PA. 1987. A mutational analysis of spliceosome assembly: Evidence for splice site collaboration during spliceosome formation. *Genes & Dev 1*:532–543.

Michaud S, Reed R. 1991. An ATP-independent complex commits pre-mRNA to the mammalian spliceosome assembly pathway. *Genes & Dev 5*:2534–2546.

Michaud S, Reed R. 1993. A functional association between the 5′ and 3′ splice site is established in the earliest prespliceosome complex (E) in mammals. *Genes & Dev 7*:1008–1020.

Pikielny CW, Rymond BC, Rosbash M. 1986. Electrophoresis of ribonucleoproteins reveals an ordered assembly pathway of yeast splicing complexes. *Nature 324*:341–345.

Reed R. 1990. Protein composition of mammalian spliceosomes assembled in vitro. *Proc Natl Acad Sci USA 87*:8031–8035.

Reed R, Griffith J, Maniatis T. 1988. Purification and visualization of native spliceosomes. *Cell 53*:949–961.

Séraphin B, Rosbash M. 1989. Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. *Cell 59*:349–358.