Autonomous Splicing and Complementation of In Vivo–assembled Spliceosomes

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Abstract. We have used an in vivo system generating assayable amounts of a specific pre-mRNA to study the relationship between splicing and an operationally defined nuclear matrix preparation (NM). When NM is prepared by extraction of DNase I–treated nuclei with an approximately physiological concentration of KCl (0.1 M), a portion of NM-associated precursor can be spliced in vitro in the presence of ATP and Mg^{2+} and in the absence of splicing extract ("autonomous splicing"). We propose that the autonomous reaction, which does not exhibit a temporal lag and is half-complete in 5 min, occurs in fully assembled, matrix-bound ribonucleoprotein complexes (in vivo spliceosomes). Extraction of the NM with concentrations of KCl >0.4 M eliminates autonomous splicing but leaves behind preassembled complexes that can be complemented for splicing with HeLa cell nuclear extract. The splicing complementing factor, representing one or more activities present in the nuclear extract and also in the cytoplasmic S100 fraction, is relatively heat resistant, devoid of an RNA component, and does not bind to DEAE–Sepharose in 0.1 M KCl. It exists in the nucleus in two forms; bound to autonomous spliceosomes and free in the nucleoplasm. Biochemical features of the complementation reaction, and conditions for reversible uncoupling of the two splicing steps are described and discussed.

We have previously developed a model system that can be used to examine the potential involvement of the presumptive nucleoskeleton in nuclear pre-mRNA splicing (Zeitlin et al., 1987). The nucleoskeleton (or nuclear scaffold or in situ nuclear matrix) is thought to be composed of the peripheral lamina and an internal fibrogranular network that is poorly defined structurally and biochemically (reviewed by Hancock and Boulikas, 1982; Bouteille et al., 1983; Berezney, 1984; Nelson et al., 1986; Verheijen et al., 1988; see also Fey et al., 1986). Chromatin is organized in loops anchored to the scaffold at specific DNA regions (reviewed by Gasser and Laemmli, 1987), and heterogeneous ribonucleoproteins (HnRNPs) also appear to be connected with this structure (see Fey et al., 1986). Because of these associations, it has been proposed that the scaffold is not a simple structural element but a dynamic entity involved in DNA replication, transcription, pre-mRNA processing, and mRNA transport. These processes could occur at a solid state/solution interphase, and the latter three might be topologically and temporally linked (see Nelson et al., 1986; Cook, 1988). Consistent with part of this general hypothesis are data suggesting an involvement of the nucleoskeleton in DNA replication and transcription (see, for example, Jackson and Cook, 1985, 1986). In addition, a temporal linkage between transcription and processing is supported by electron microscopic analyses indicating that splicing occurs cotranscriptionally in a subset of nascent precursors (Osheim et al., 1986; Beyer and Osheim, 1988).

In vitro experiments (reviewed by Padgett et al., 1986; Green, 1986; Sharp, 1987; Krainer and Maniatis, 1988) have demonstrated that splicing can occur in solution after the assembly of a multicomponent complex, the spliceosome (Brody and Abelson, 1985; Grabowski et al., 1985; Frendeway and Keller, 1985). Thus, under in vitro conditions, binding of spliceosomes to a solid support is not a prerequisite for their function. However, in the absence of any evidence for the existence of free nucleoplasmic splicing complexes, direct extrapolations to the in vivo situation are not warranted.

In situ pre-mRNA splicing might be occurring in matrix-bound spliceosomes, representing at least a subset of the HnRNPs. Anchorage of spliceosomes to a solid structure could be advantageous for rapid and efficient splicing and/or coupling of pre-mRNA processing and mRNA transport. However, such a hypothesis lacks experimental support. Moreover, variability in results and the possibility of artifacts hamper the interpretation of data obtained with the matrix. In this regard, it is important to make a distinction between the presumptive "in situ nuclear matrix" and a biochemical "nuclear matrix preparation" (NM). The latter can be defined

1. *Abbreviations used in this paper*: HnRNP, heterogeneous nuclear ribonucleoprotein; HS-NM, nuclear matrix prepared by extraction with high-salt; LS-NM, nuclear matrix prepared by extraction with low-salt; NM, nuclear matrix preparation; PVA, polyvinyl alcohol; SCF, splicing complementing factor; snRNP, small nuclear ribonucleoprotein.

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operationally as the insoluble pellet remaining after salt and detergent extraction of interphase nuclei that have been treated with DNase I (see Bouteille et al., 1983; Berezney, 1984; Nelson et al., 1986; Verheijen et al., 1988). In such preparations the HnRNPs partition with the pellet, whereas they can be isolated from nuclei or from NM as free particles only by sonication. Nevertheless, arguments have been made that such complexes are found associated with the NM because of artifactual aggregation and precipitation in high salt of RNA and soluble proteins onto a subnuclear fraction (for discussion, see Bouteille et al., 1983; Berezney, 1984).

Because of these problems, we decided to examine the possible association of the splicing apparatus with the NM by applying a functional criterion. For this purpose, we developed a system that can be used to assay splicing events occurring in NM preparations under defined in vitro conditions (Zeitlin et al., 1987; see also Results). In such assays a NM pellet isolated after a 0.4 M KCl wash, was incubated in the presence of ATP and Mg^{2+} with a HeLa cell in vitro splicing extract (Dignam et al., 1983; Krainer et al., 1984). We observed that the amount of a matrix-associated specific pre-mRNA progressively decreased without a significant temporal lag, with a corresponding increase in free intron lariat (Zeitlin et al., 1987). From these results, we postulated that parts of preassembled splicing complexes had survived in the NM preparation and could be complemented for splicing with soluble factors in the extract. However, our data did not exclude the possibility that the functional (complementable) structures were localized in the NM pellet simply as a consequence of the method of preparation.

Here we show that if the high-salt extraction in the NM preparation protocol is substituted with a 0.1 M KCl wash (an approximately physiological concentration of monovalent cation), splicing of pre-mRNA occurs in the matrix pellet under in vitro conditions in the absence of added extract ("autonomous splicing"). This suggests that the precursor in the preparation is associated with all of the spliceosome components. In addition, we report studies on complementation reactions of preassembled, but not autonomous, splicing complexes with splicing extract, and define conditions under which the first and second splicing steps can be uncoupled. Finally, we describe some biochemical features of the complementing activities and their relationship to previously identified splicing factors other than small nuclear ribonucleoproteins (snRNPs).

Materials and Methods

Matrix Preparation

Our transfection/infection protocol (see Results), the plasmid construction ptkR2 used for transfection of HeLa cells, and the probes used for RNA blot hybridization, have been described previously (Zeitlin et al., 1987). Nuclear matrix was prepared as described (Gallinaro et al., 1983; Zeitlin et al., 1987) with the following modifications. After digestion of the nuclei with DNase I and centrifugation for 15 min at 600 g through a 30% sucrose cushion containing 10 mM Tris-HCl, pH 7.4, 0.1 M KCl, 0.2 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF, the matrix pellet was resuspended gently in ~10 vol of buffer D (20 mM Hepes, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 20% [vol/vol] glycerol; Dignam et al., 1983), and repelleted (10 min centrifugation). This low salt-matrix (LS-NM) was then resuspended in a small volume of buffer D (~10^5 nuclei equivalents per microliter) and divided into small aliquots that were quickly frozen in liquid N_2 and stored at −80°C. These preparations were stable for 1–2 mo. Freezing and thawing once or twice had no noticeable effect on autonomous splicing or complementation assays. For the preparation of derivative high-salt matrix (HS-NM), 10–20 μl aliquots of LS-NM were thawed on ice and, after the addition of 400 μl of high-salt wash buffer (10 mM Tris, pH 7.4, 0.6 M KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 0.25 M sucrose), incubated on ice for 15 min. The matrix was then pelleted by a 15-s spin in a microfuge at 4°C, washed with 200 μl of buffer D, repelleted, and finally resuspended in the original matrix volume of buffer D.

Extract Preparation

HeLa cell nuclear extract and S100 cytoplasmic fraction were prepared exactly as described by Dignam et al. (1983). The same procedure was used for sequential extraction of crude nuclei with increasing concentrations of KCl, as follows. Crude nuclei were resuspended in buffer C containing 0.1 M KCl (20 mM Hepes, pH 7.9, 0.1 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 25% glycerol; ~3×10^9 nuclei/ml) and stirred for 30 min at 4°C. After pelleting for 20 min at 25,000 g, the supernatant (0.1 M extract) was collected, and the nuclei were extracted sequentially by repeating the procedure with equal volumes of the same buffer containing KCl at final concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 M. All extracts were then dialyzed against buffer D and stored in small aliquots. To eliminate snRNPs by ultracentrifugation (Ruskin et al., 1988), NP-40 at a final concentration of 2% was added to the extract, which was then centrifuged for 2 h at ~360,000 g in an ultracentrifuge (model TL-100; Beckman Instruments Inc., Palo Alto, CA) using the TL100.2 rotor. (NP-40 at a final concentration of 1.2% does not affect matrix complementation assays.)

Complementation Reactions

A typical complementation reaction (25 μl) contained 80 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM ATP, 5 mM creatine phosphate, 1 mM DTT, 20 U placental RNase inhibitor (RNasin; Promega Biotech, Madison, WI), 2 μl of derivative HS-MX (~2×10^5 nuclei equivalents), and 60% (vol/vol) of HeLa cell nuclear extract or S100 (15 μl). Buffer was supplied by the extract (12 mM final concentration of Hepes, pH 7.9). Polyvinyl alcohol (PVA) at a final concentration of 0.9% was included in some assays. In some experiments (see text) we used extract that had been desalted by gel filtration on a Sephadex G-25 column (300 μl of extract per prepacked NAP-5 column; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with a buffer containing 25 mM triethanolamine acetate, pH 7.9, and 0.1 M KCl. Reactions were incubated at 30°C for various times as indicated in figure legends. The samples were deproteinized using proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) in the presence of SDS, as described (Krainer et al., 1984), and, after phenol extraction, the RNA was ethanol precipitated. For better electrophoretic resolution, the RNA was deadenylated for 20 min at 30°C in a 25 μl reaction containing 50 mM Tris, pH 7.4, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 200 U/ml RNasin, 10 μg/ml oligo(dT)$_{18}$, and 8 U/ml *Escherichia coli* RNase H (Pharmacia Fine Chemicals). After treatment with RNase-free DNase I (10 μg/ml) for 10 min at 30°C, phenol extraction, and ethanol precipitation, the RNA was subjected to electrophoresis on denaturing 5% urea-polyacrylamide gels and transferred electrophoretically onto nylon membranes (GeneScreen Plus; New England Nuclear, Boston, MA). Prehybridization and hybridization with high specific activity single-stranded probes synthesized on M13 templates (Hu and Messing, 1982) were as described (Zeitlin and Efstratiadis, 1984).

Autonomous Splicing Reactions

For autonomous splicing, the complementation reaction conditions described above were modified; the matrix was not washed, extract was substituted with buffer D, and PVA was omitted. To define optimal conditions promoting autonomous splicing, we tested various reaction parameters (data not shown).

ATP at concentrations of 0.5, 1.5, 2.0, or 5.0 mM, and Mg^{2+} at concentrations of 0.5, 1.0, 2.0, or 4.0 mM, were tested in all combinations. We observed that under all of these conditions the efficiency of autonomous splicing was invariant (data not shown). Although we did not examine ATP concentrations below 0.5 mM, we performed a finer titration for Mg^{2+} (using ATP at a fixed concentration of 1.5 mM; see text).

The autonomous splicing reaction exhibited a very broad optimum of KCl concentration between 40 and 150 mM; 80% of the maximal efficiency at 0.1 M was obtained at any concentration in this range. The reaction could still be sustained (at ~50% of the maximal efficiency) at concentrations between 8 and 20 mM, whereas concentrations of KCl >200 mM were inhibitory. We note that complementation reactions of HS-NM with nuclear

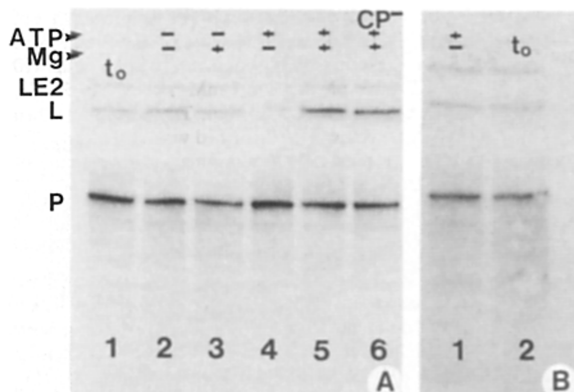

Figure 1. Autonomous splicing of nuclear matrix–associated pre-mRNA (*A*) Northern analysis of total RNA extracted from aliquots (2 μl per lane) of a low-salt nuclear matrix preparation (0.1 M KCl final wash). The matrix was prepared from HeLa cells that had been transfected with plasmid ptkR2. The insert of this plasmid is a fragment of the rabbit β globin gene that includes the second intervening sequence (see Results and Materials and Methods). In this and in subsequent experiments the blots were hybridized with a rabbit β globin IVS2 probe. The RNA was extracted either directly (time zero control, t_0; lane *1*) or after incubation of the matrix for 30 min at 30°C in splicing buffer, in the presence (+) or absence (−) of ATP and Mg^{2+} in all combinations (lanes *2–5*). Creatine phosphate (*CP*) was omitted from the reaction of lane *6*. The hybridizing bands, corresponding to the precursor (*P*), the lariat-exon2 intermediate (*LE2*), and the free lariat product (*L*), are indicated in this and in subsequent figures. (*B*) The Northern profile of RNA (lane *1*) from a reaction identical to that in lane *4 A*, but from an independent experiment, and the corresponding time zero control (lane *2*).

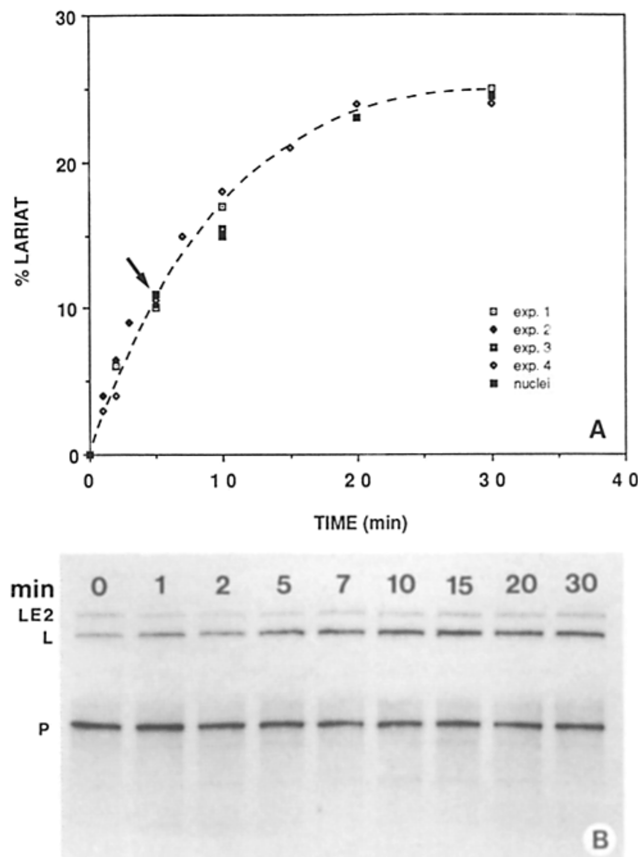

Figure 2. Kinetics of autonomous splicing. The corrected percentage of released intron lariat at each time point (calculated as described in Materials and Methods) is plotted in *A*. The results of four independent experiments using different low-salt matrix preparations incubated in splicing buffer without extract are shown. Also shown are the results from a single experiment using whole hypotonic nuclei instead of matrix. The arrow indicates the time required for half-completion of the reaction (~5 min) calculated from this graph. An example of the Northern analyses used to derive the plotted data (matrix experiment 4) is shown in *B*.

extract exhibited similar requirements for monovalent cation. KCl concentrations as low as 14 mM could still sustain complementation.

The pH optimum was also very broad. Autonomous splicing efficiencies were practically invariable throughout a pH range between 6.5 and 7.9. However, only 25% of this level was attained at pH 8.5. The reaction was rapid at the three temperatures that we examined (20, 30, and 37°C). However, reactions at 37°C longer than 10 min resulted in RNA degradation. Substitution of Hepes-KOH with triethanolamine-acetate buffer had no effect on the reaction.

DEAE-Sepharose Chromatography

Nuclear extract was fractionated on DEAE-Sepharose as described (Krämer et al., 1987). A 7-ml DEAE-Sepharose column (Fast Flow; Pharmacia Fine Chemicals) was equilibrated with 10-column volumes of buffer containing 0.1 M KCl, 20 mM Hepes-KOH, pH 7.9, 0.1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, and 10% glycerol. Nuclear extract (22 mg protein, adjusted to a concentration of 4 mg/ml with buffer D) was applied to the column at a flow rate of 2 ml/min. The flowthrough (20 ml) was collected, and the bound protein was eluted with 20 ml of equilibration buffer containing 0.5 M KCl. Fractions were frozen in liquid N_2 and stored at −80°C. For matrix complementation assays, aliquots of the two fractions were concentrated by ammonium sulfate precipitation (0.6 g/ml fraction). The precipitate was collected by centrifugation (20,000 *g*, 30 min), resuspended in a small volume of buffer D, and desalted on a NAP-5 column equilibrated with buffer D. The flowthrough and bound fractions were concentrated ~7.5- and 5.5-fold, respectively (the corresponding final concentrations of protein were 4.6 and 7.1 mg/ml). Protein concentrations were determined according to Bradford (1976).

Binding of SCF to the Matrix

We performed two types of experiments in which complementing factors in the nuclear extract were bound to the matrix. In the first type, matrix and extract were incubated in the presence of Mg^{2+} at low concentration, and then the matrix was purified away from the extract by centrifugation through a sucrose cushion, and incubated under conditions of autonomous splicing ("cushion experiment"). In the second type of experiment, the extract and the matrix were not separated after the first incubation, but the reaction volume was increased ("dilution experiment").

The first reaction of the "cushion experiment" (100 μl) contained 1.5 mM ATP, 5 mM creatine phosphate, 50 μM $MgCl_2$, 1 mM DTT, 400 U/ml RNasin, 80 mM KCl, 20 mM Hepes, pH 7.9, 13 mM triethanolamine, pH 8.3, 0.9% PVA, 55% (vol/vol) of desalted extract, and 15 μl of HS-NM (prewashed with 0.6 M KCl). After 30 min at 30°C, the reaction was layered on a 100-μl cushion in an Eppendorf tube, containing 30% (wt/vol) sucrose, 50 μM $MgCl_2$, 1 mM DTT, 12 mM each Hepes and triethanolamine, and 0.9% PVA, and centrifuged for 15 min in a microfuge (12,000 *g*). The matrix pellet was resuspended in 10 μl of cushion buffer without sucrose. A 3-μl aliquot was then incubated in a 25-μl reaction as described above, except that extract was omitted and the Mg^{2+} concentration was raised to 0.5 mM. A second equal aliquot was incubated as above, but without ATP and creatine phosphate, whereas a third aliquot (control) was deproteinized immediately.

The first reaction of the "dilution experiment" was the same as that of the "cushion experiment", except that it was performed in a volume of 25 μl containing 4 μl HS-MX and no PVA. To provide controls, an additional mock reaction was performed by omitting the desalted extract. After withdrawing (from the reaction or the mock reaction) half of the volume for immediate deproteinization, the other half was diluted by adding 1 ml buffer containing all of the ingredients of the first reaction, except that the concentration of

Mg^{2+} was raised to 0.5 mM. Incubation was then continued for 30 min at 30°C without any additions in the experimental reaction, whereas 6.75 μl of desalted extract were added to the diluted mock reaction.

In Vitro Splicing

SP6 transcription and splicing reactions were performed as described (Parent et al., 1987). The plasmid construct used to derive the SP6 precursor contains the first exon, the IVS1, and portion of the second exon of the rabbit β globin gene (construct WT; Parent et al., 1987).

Densitometric Scanning

Northern blots were autoradiographed using XAR-5 film (Eastman Kodak Co., Rochester, NY) at −80°C with an intensifying screen for 2-16 h. Exposures within the linear response range of the film were scanned with the Joyce-Loebl Chromoscan-3 densitometer, and the areas under the peaks (intensities) were calculated for the precursor (P), the lariat-exon2 (LE2), the free lariat (L'), and (whenever it appeared) the nicked-lariat (Y). For each reaction (or time point), we considered the sum $L' + Y = L$ as the intensity value for "lariat", and the sum $L + P + LE2 = S$ as the total intensity. The calculated percentages of each species were $\%L = (L/S) \times 100$, $\%LE2 = (LE2/S) \times 100$, and $\%P = (P/S) \times 100$. We call the %L and %P values of the zero time controls of autonomous reactions $\%L_0$ and $\%P_0$, respectively, and the corresponding values of the "autonomous splicing background" of complementation reactions $\%L_a$ and $\%P_a$, respectively. The corrected %L values, plotted in Figs. 2 and 4, were the ratios $\%L_{corr} = (\%L - \%L_0)/\%P_0$ and $\%L_{corr} = (\%L - \%L_a)/\%P_a$, respectively.

Results

In Vitro Splicing Assays of Matrix-associated Pre-mRNA

For splicing assays, we use the following system. A plasmid (ptkR2; Zeitlin et al., 1987) is first transfected into HeLa cells. This construct contains the inducible promoter of the herpes simplex virus tk gene linked to a segment of the rabbit β globin gene that includes the second intervening sequence flanked by two exons. After transfection, the promoter is transactivated by infecting the cells with a tk^- herpes simplex virus. Nuclear matrix is then prepared from the transfected/infected HeLa cells, and used for splicing assays under in vitro conditions. The pre-mRNA transcript and its splicing intermediates and products can be monitored by high resolution Northern analysis, using an IVS2 probe or an exonl probe. The IVS2 probe detects the pre-mRNA, the lariat-exon2 intermediate, and the free intron lariat. Linear intron (debranched lariat) and nicked lariat (an artifact of the in vitro manipulation of the RNA) are occasionally also detected. The exonl probe detects pre-mRNA, exonl intermediate, and mature mRNA. The majority of these RNAs (80–99%, depending on the species) are associated with the matrix pellet after a 0.4 M KCl wash. In this pellet, the mature mRNA is ∼20-fold more abundant than the precursor (Zeitlin et al., 1987). Thus, ligation of exons cannot be monitored in our assay system because even complete conversion of the pre-mRNA to spliced product would result in only a small increase in the amount of preexisting mRNA. For this reason, we measure in our assays the decrease in the level of precursor, the corresponding increase of free intron lariat, and any change in the level of lariat-exon2 intermediate. The assay monitors three splicing reactions; endonucleolytic cleavage at the 5′ splice site and intron branching (first splicing step), and cleavage at the 3′ splice site (one of the reactions of the second step). We assume that the fourth reaction (ligation of exons) is also occurring, since it is concomitant with the release of intron lariat (reviewed by Padgett et al., 1986; Green, 1986; Krainer and Maniatis, 1988).

Autonomous Splicing in Matrix-associated Complexes

Previously, we defined as matrix preparation the insoluble pellet that was obtained after a final 0.4 M KCl wash (HS-NM; Zeitlin et al., 1987). During complementation experiments using such pellets, we observed that the control reactions of some preparations (HS-NM incubated in buffer containing ATP and Mg^{2+}, but no extract) exhibited very low levels of splicing. We call this reaction "autonomous splicing" to indicate that it occurs in fully assembled and functionally self-sufficient complexes.

To examine in detail the splicing of NM-associated pre-mRNA in preparations extracted with different salt concentrations, we first prepared NM using for the final wash a buffer containing 0.1 M KCl (LS-NM). This is the approximate physiological concentration of K^+, the main nucleoplasmic cation (Palmer and Civan, 1975, 1977; reviewed by Civan, 1978; see also Alvarez-Leefmans, 1986).

An aliquot of the LS-NM was immediately deproteinized to serve as a time zero negative control (Fig. 1 *A*, lane *1*). Equal portions of the LS-NM were then incubated for 30 min at 30°C in the presence of splicing buffer alone (final KCl concentration of 80 mM; lane *2*), in buffer containing 1 mM Mg^{2+} (lane *3*), in buffer containing 1.5 mM ATP (lane *4*), or in buffer containing both ATP and Mg^{2+} (lane *5*). The concentrations of KCl, ATP, and Mg^{2+} were chosen on the basis of previous complementation assays with nuclear extracts.

We observed that only when both ATP and Mg^{2+} were present (Fig. 1 *A*, lane *5*; compare with lanes *2–4*), the structures associated with the LS-NM were capable of autonomous splicing according to our criterion (decrease of pre-mRNA with corresponding increase of free lariat, as compared with the time zero control; compare lanes *1* and *5* in Fig. 1 *A*). Omission of creatine phosphate from the complete reaction did not change the result (Fig. 1 *A*, lane *6*). In the reaction that was performed in the presence of ATP, but in the absence of Mg^{2+} (lane *4*), we observed some degradation of both the lariat-exon2 and the free lariat species, for unknown reasons. However, this result was not consistent (Fig. 1 *B*, lane *1*; compare with lane *2*). By varying the parameters of the reaction, we defined optimal conditions for autonomous splicing (see Materials and Methods).

Autonomous splicing complexes assembled in vitro on SP6 precursors have not been identified yet, despite the description of several forms of complementable spliceosomes (Grabowski et al., 1985; Lin et al., 1987; Cheng and Abelson, 1987; Reed et al., 1988; Abmayr et al., 1988). Thus, it could not be excluded that the splicing reactions depend on triggering by transiently associated factors. Such a hypothesis can now be eliminated.

Study of the kinetics of autonomous splicing (Fig. 2) indicated that the reaction is rapid and does not exhibit a temporal lag (in contrast to in vitro splicing reactions or matrix complementation; see below). An increase in free lariat was detected even at the earliest time point that we examined (1 min; Fig. 2 *B*), and the reaction was half-complete in ∼5 min (Fig. 2 *A*). These features provide additional evidence supporting our interpretation that the autonomous splicing

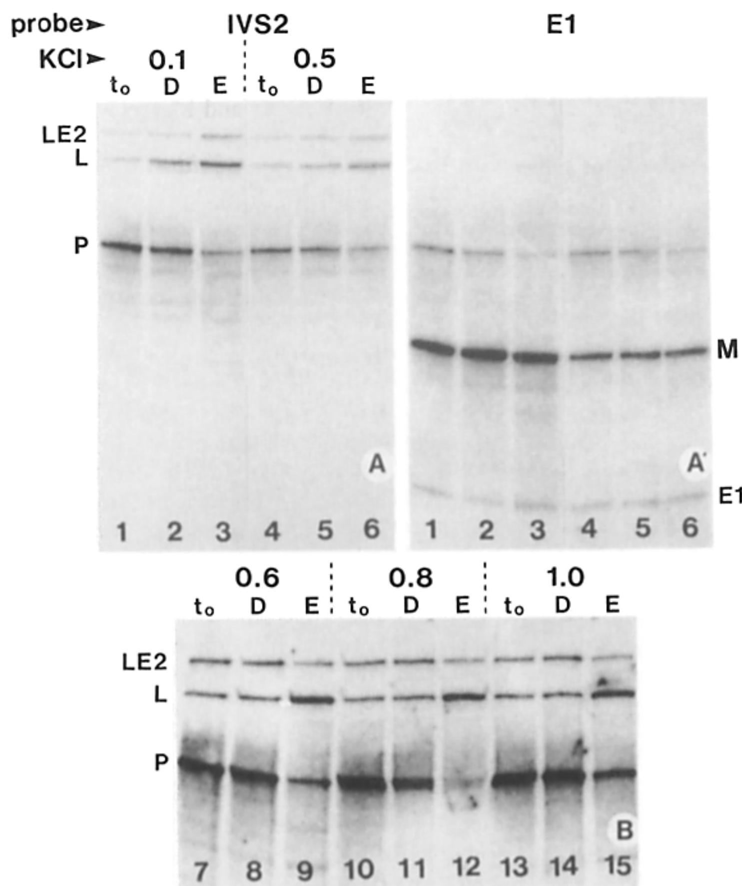

Figure 3. Salt resistance of in vivo–assembled splicing complexes. The Northern blots in *A* and *B* were hybridized with an IVS2 probe. The blot in *A′* is the same as in *A* after rehybridization with an exon1 probe without intermediate washing. It is shown as an example demonstrating the abundance and practically constant amount of mature mRNA (*M*). E1 is the first exon. For these experiments, low-salt matrix (0.1 M KCl wash) was prepared, and aliquots were washed again either with 0.1 M KCl or with higher KCl concentrations, as indicated. (The results from a 0.4 M KCl wash were practically identical to those obtained with the 0.5 wash, but are not presented because of low RNA recoveries.) RNA extracted directly from a portion of each washed aliquot (time zero negative controls; t_0) is displayed in lanes *1*, *4*, *7*, *10*, and *13*. Two other portions from each washed aliquot were incubated for 30 min at 30°C either in splicing buffer alone (*D*; lanes *2*, *5*, *8*, *11*, and *14*) or in buffer containing nuclear extract (*E*; lanes *3*, *6*, *9*, *12*, and *15*).

reactions occur in preassembled complexes that require for function only ATP and Mg^{2+}.

We note that the time course of the splicing reaction, monitored in whole hypotonic nuclei (washed with 0.1 M KCl), was indistinguishable from that occurring in LS-NM preparations (Fig. 2 *A*). This result can be minimally interpreted as indicating that the destruction of chromatin structure by DNase I to derive the matrix preparation does not perturb the splicing complexes. Variation in the nuclei isolation procedure did not affect (positively or negatively) autonomous splicing; the behavior of LS-NM prepared from nuclei that were obtained from isotonically lysed cells was identical to that derived from hypotonic nuclei (not shown). We also note that the matrix-associated splicing complexes cannot be solubilized in functional form under a variety of conditions that we have used (unpublished results). Complexes could be released from the matrix by sonication or by incubation at 30°C in very low salt (<5 mM), but they were incompetent for splicing either in autonomous reactions or in complementation assays. A drastic solubilization protocol using lithium diiodosalycilate (Mirkovitch et al., 1984) also released nonfunctional complexes from the matrix, containing only U2 and U5 snRNAs.

Our results indicate that the function of the apparently insoluble splicing complexes is not affected by potential experimental artifacts due to hypotonic lysis conditions, DNase I digestion, and high-salt effects during matrix preparation. However, a crucial question remains open: does autonomous splicing occur in complexes that are artifactually associated with the matrix preparation? To invoke artifacts, one has to suggest that the functional splicing complexes we study are in reality soluble nucleoplasmic entities, which, for unknown reasons, partition with the pellet during fractionation even under physiological concentrations of monovalent cation. We consider this an unlikely possibility because we cannot identify any plausible cause for such hypothetical behavior of the complexes. Nevertheless, further experimentation is necessary to resolve this issue.

Complementable Splicing Complexes

To examine the salt resistance of the autonomous splicing complexes, we washed equal portions of a LS-NM preparation with buffer containing KCl in concentrations ranging between 0.4 and 1.0 M, and then divided each sample into three equal aliquots. One of them was deproteinized (zero time control), a second was incubated under conditions of autonomous splicing, and the third was complemented with extract (Fig. 3). All of the washed samples were incapable of autonomous splicing. However, the leftover structures could still be complemented for splicing, regardless of the concentration of salt used for washing (Fig. 3, *A* and *B*, lanes *4–15*). We infer from these data that one or more splicing factors are removed by washing with KCl at concentrations >0.4 M. These components can be added back to functional complexes by complementation with extract. For simplicity, we call the complementing activity or activities SCF ("splicing complementing factor").

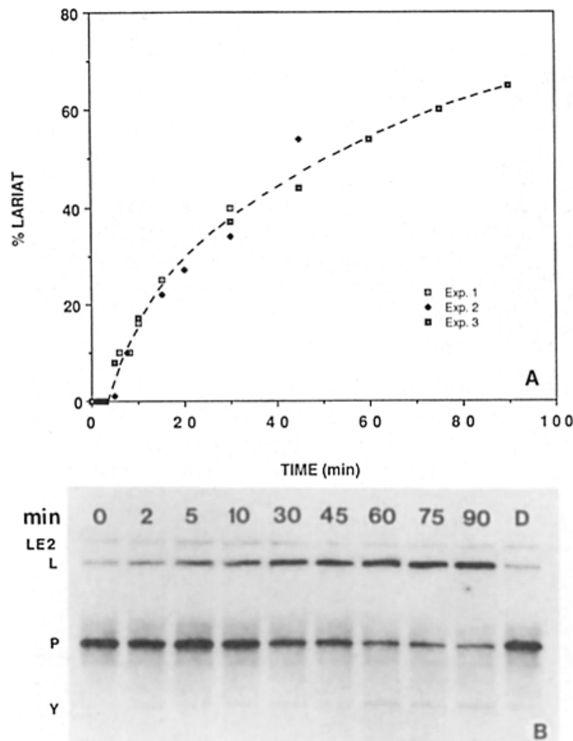

Figure 4. Kinetics of splicing by complementation. The corrected percentage of released intron lariat at each time point of a complementation reaction is plotted in *A*. The results are from three independent experiments using different matrix preparations washed with 0.6 M KCl and then complemented for splicing with nuclear extract. Because of clustering, it is not possible to discriminate in the graph that the lag period (0% lariat) is represented by six points (assays at 1, 2, and 3 min from two experiments). An example of the Northern analyses used to derive the plotted data (experiment 3) is shown in *B*. To provide a negative control, an aliquot of matrix was incubated for 60 min at 30°C in buffer alone (*B*, lane *D*). Y is the nicked-lariat species.

These results indicated that we could generate from a single, autonomously splicing, LS-NM preparation a second form of complementable HS-NM by washing with 0.6 M KCl. This treatment guarantees that the autonomous splicing, which is occasionally observed with some HS-NM preparations (and constitutes "background" for complementation reactions) can be effectively eliminated.

We used such derivative HS-NM to study the kinetics of complementation reactions. In contrast to the kinetics of autonomous splicing, complementation kinetics can vary between reactions. It is expected, for example, that the complementation kinetics with a fixed amount of matrix will change upon dilution or concentration of extract. In our particular examples (Fig. 4), the reactions exhibited a brief temporal lag of ~5 min (Fig. 4 *A*), apparently reflecting the time necessary for association of complementing factors. The in vitro splicing reactions with SP6 precursors usually exhibit a 30-min lag for the detection of splicing products (reviewed by Padgett et al., 1986; Green, 1986; Krainer and Maniatis, 1988), presumably corresponding to the time required for complete assembly.

Our complementation reactions did not reach completion; ~65% of the precursor was converted into product in 90 min (Fig. 4 *A*). We found it difficult to determine the exact time at which such reactions reach a plateau because of RNA degradation during prolonged incubations with unfractionated or undiluted extracts. However, on one occasion, when the RNA was left intact after 2 h of incubation, we observed that ~86% of the precursor was converted into free lariat. This result suggests that only a small minority of complexes, if any, are irreversibly damaged (for example, rearranged) during the matrix preparation procedure, including secondary washing with high salt.

Parallel examination of autonomous splicing and complementation using the same LS-NM preparation showed that the level of splicing attained in the autonomous reaction was augmented by addition of nuclear extract (Fig. 3 *A*, compare lane *2* with lane *3*). Quantitation by densitometry showed that ~25% of the precursor was converted to product during autonomous splicing, whereas an additional 40% could be spliced after complementation for 30 min. These numbers varied only slightly in repeats of such assays using different preparations incubated for the same amount of time. Although more precursor is chased into product in longer incubations (see above, and Fig. 4), autonomous splicing does not exceed 25%. Since complementable complexes are also present in the matrix, a corollary of this result is that SCF does not recycle under in vitro conditions, although such a possibility cannot be excluded for the in vivo situation. The absence of recycling is consistent with the results of mixing experiments (not shown); addition of excess nonspecific LS-NM (prepared from HeLa cells without transfection/infection) did not complement for splicing HS-NM from transfected/infected cells.

Uncoupling of the First and Second Splicing Steps

While testing the variables of the matrix complementation reaction, we identified two conditions under which splicing can be reversibly arrested after the first step, as evidenced by the accumulation of lariat-exon2 intermediate.

Uncoupling by Extract Dilution. Our transfection/infection protocol includes treatment of the cells with cycloheximide. Interestingly, this protein synthesis inhibitor does not impair splicing, even when the treatment is extended for as long as 6 h (Zeitlin et al., 1987). This observation is consistent with the existence of a significant factor reservoir (and/or with vigorous in vivo recycling of splicing components). Thus, we thought that SCF must be present in a large excess in the amount of extract (15–30 μl) used in our standard reactions to complement an aliquot of HS-NM (2 μl); these amounts correspond to 5×10^6–10^7 and 2×10^5 nuclei equivalents, respectively (assuming, for illustration purposes, that there are no losses during the preparation procedures).

To examine directly the validity of these estimates, we performed a titration experiment (Fig. 5 *A*) using a fixed amount of matrix (2 μl/50 μl reaction) and extract that was either undiluted (0.6 μl/μl of reaction) or serially diluted. We observed that the amount of released free lariat was progressively reduced (Fig. 5 *A*, lanes *3–5*), and practically abolished when the extract was diluted eightfold (lane *6*). In two additional experiments, that were performed using different preparations of matrix and extract, free lariat still

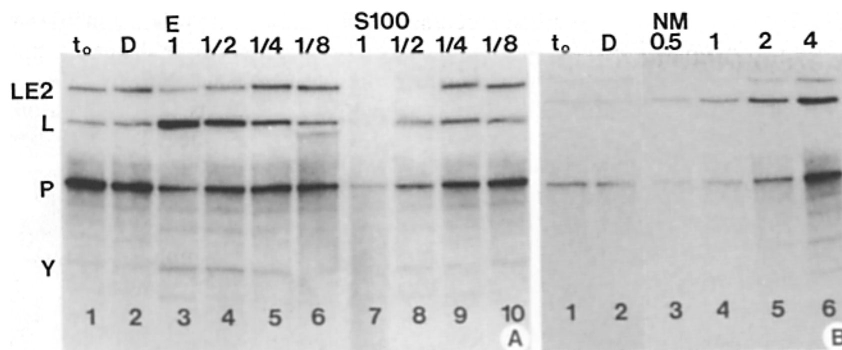

Figure 5. (*A*) Complementation reactions with diluted nuclear extract or S100 cytoplasmic fraction. A nuclear matrix preparation was washed with 0.6 M KCl and divided into 2-μl aliquots. RNA was extracted immediately from an aliquot for display (time zero control; lane *1*). A second aliquot was incubated under splicing conditions in buffer D, but without extract for 60 min at 30°C (control to demonstrate that the salt wash effectively eliminated autonomous splicing; lane *2*). Other aliquots were complemented in 50-μl reactions with 30 μl of nuclear extract, either undiluted (lane *3*) or diluted with buffer D, twofold (lane *4*), fourfold (lane *5*), or eightfold (lane *6*). Complementation reactions using S100 fraction (instead of extract), either undiluted or twofold diluted resulted in RNA degradation (lanes *7* and *8*, respectively). The results of complementation reactions with fourfold or eightfold diluted S100 fraction are shown in lanes *9* and *10*, respectively. (*B*) Complementation reactions with increasing amounts of matrix. The same matrix and nuclear extract preparations as in *A* were used in 25-μl complementation reactions, each containing 7.5 μl of extract and increasing amounts of matrix (0.5, 1.0, 2.0, or 4.0 μl; lanes *3*, *4*, *5*, and *6*, respectively). The controls in lanes *1* and *2* (RNA from 1 μl of matrix per lane) correspond to those in the same lanes of *A*.

appeared, despite its progressive decline, even after a 16-fold dilution of the extract (data not shown). These results apparently reflect the relative concentrations of factors in different extracts. We assume that during the fixed incubation time (1 h) the number of fruitful collisions (leading to association) between the SCF and a stationary constant number of complementable complexes on the matrix is reduced because of the dilution.

Unexpectedly, in all of these experiments we observed accumulation of the lariat-exon2 intermediate when the dilution of extract was fourfold or higher (see Fig. 5 *A*, lanes *5* and *6*). Additional experiments (not shown) indicated that the uncoupling of the two splicing steps upon extract dilution is a kinetic phenomenon; the second step also appeared in the presence of eightfold diluted extract by extending the incubation time beyond 2 h.

To examine whether uncoupling is due to a requirement for a limited component, we performed an additional titration experiment. This time we used in all of the reactions (25 μl each) a twofold dilution of extract (0.3 μl/μl of reaction), while increasing stepwise the amount of matrix between 0.5 and 4.0 μl. We observed that the extract was always in excess, and that accumulation of intermediate did not occur (Fig. 5 *B*, lanes *3–6*); in all four reactions, constant percentages of lariat-exon2 and free lariat were detected by densitometry, which, in absolute amounts, were exactly proportional to the matrix input in each reaction. Excess of factors was also demonstrated in an independent experiment (not shown) in which increasing amounts of nonspecific HS-NM were added to the same amount (2 μl) of HS-NM from transfected/infected cells. Competition for complementing factors present in the extract (0.3 μl/μl of reaction volume) was not observed even at a ratio of nonspecific/specific matrix of 15:1.

Biochemical Uncoupling. Uncoupling of the two splicing steps was also observed under the following conditions. The splicing extract was first desalted by gel filtration to remove free Mg^{2+}, and then used in a series of complementation reactions with HS-NM, performed under standard conditions, but with Mg^{2+} added at varying concentrations. A small increase of lariat-exon2 was observed even when Mg^{2+} was not added to the reaction (Fig. 6 *A1*, lane *2*, and Fig. 6 *A2*, lane *3*). However, detection of this first splicing step was abolished by addition of EDTA (Fig. 6 *A2*, lane *4*). Thus, it seems that after desalting of the extract some tightly bound Mg^{2+} is left behind that can propagate to some extent the first splicing step in the presence of ATP (Fig. 6 *A2*; compare lane *2* with lane *3*).

The amount of lariat-exon2 intermediate increased with increasing Mg^{2+} concentration, and reached a plateau at 50 μM Mg^{2+}, with the simultaneous appearance of some free lariat product (Fig. 6 *A1*, lane *4*). The ratio of intermediate to product decreased progressively with increasing Mg^{2+} concentrations above the 50-μM level (Fig. 6 *A1*, lanes *5–8*). When the Mg^{2+} concentration exceeded 200 μM, accumulation of the intermediate was no longer detectable (Fig. 6 *A1*, lanes *9–11*).

It is noteworthy that the autonomous complexes, which already contain SCF, behave differently than the complementable complexes in reactions with increasing Mg^{2+} concentrations. In a series of autonomous reactions of LS-NM we used 1.5 mM ATP and Mg^{2+} concentrations varying between 5 μM and 1 mM. The lariat product was barely detectable, if at all, at Mg^{2+} concentrations below 50 μM (Fig. 6 *B*, lanes *2–5*), whereas at an apparent threshold of 100 μM Mg^{2+} the reaction reached its maximal attainable efficiency (lane *6*; compare with lanes *7* and *8*). However, accumulation of lariat-exon2 was not observed in any of these reactions.

Binding of SCF to Complementable Spliceosomes

The identification of biochemical conditions to uncouple reversibly the two splicing steps suggested the following experiment to "pick-up" SCF from the extract by affinity.

Matrix was washed with 0.6 M KCl, and an aliquot was withdrawn for display (time zero control; Fig. 7 *A*, lane *1*). Another aliquot was incubated, in the presence of 1.5 mM ATP and 50 μM Mg^{2+}, with desalted extract. At the end of this reaction, the mixture was centrifuged through a sucrose

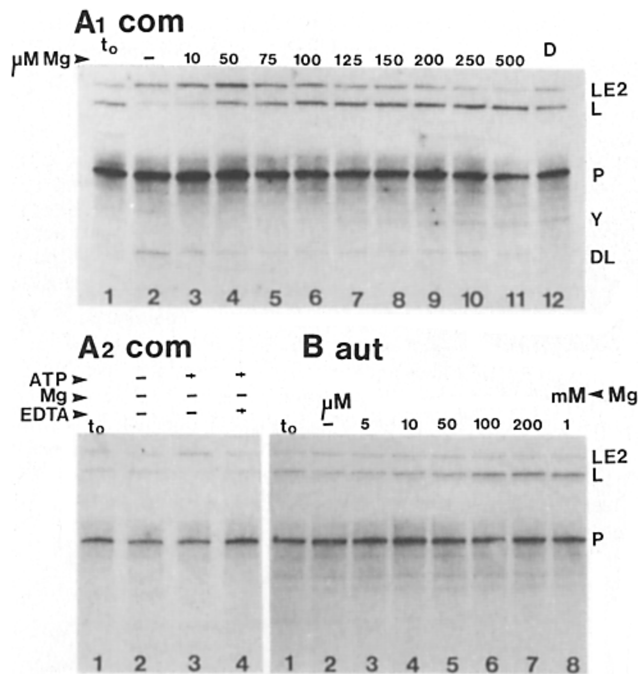

Figure 6. (*A*) Biochemical uncoupling of the first and second splicing steps in matrix complementation. (*A1*) Northern profiles of complementation reactions (*com*) performed with desalted extract in the absence of Mg^{2+} (lane *2*) or in the presence of Mg^{2+} at the indicated concentrations (lanes *3–11*). Lanes *1* and *12* (t_0 and *D*) are as in previous figures. *DL* denotes the debranched lariat. We have consistently observed that this species appears in increased amounts when the reactions are performed in the presence of very low Mg^{2+}. (*A2*) Lanes *1* and *3* are as lanes *1* and *2* of *A1*. In the reaction of lane *2*, ATP was omitted, whereas the reaction of lane *4* was performed in the absence of Mg^{2+} but in the presence of ATP and EDTA (5 mM). (*B*) Autonomous splicing reactions (*aut*) performed with Mg^{2+} at varying concentrations, as indicated on the top of the figure. Lane *1* is the time zero control.

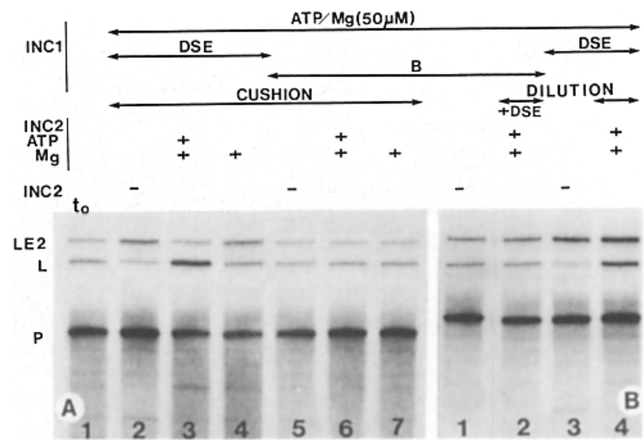

Figure 7. Binding of SCF to matrix-associated splicing complexes (*A*) Northern profiles of the time zero control (lane *1*) and of RNA from high-salt matrix aliquots that were processed as follows. After a first incubation (*INC1*) of reactions, performed in the presence of ATP and Mg^{2+} in low concentration (50 μM), either with desalted extract (*DSE*; lanes *2–4*) or with buffer (*B*; lanes *5–7*), the matrix was pelleted through a sucrose cushion. RNA was then extracted from aliquots for immediate display (without secondary incubation, *INC2*(−); lanes 2 and *5*), whereas other aliquots were incubated further (*INC2*) either with both ATP and (0.5 mM) Mg^{2+} (lanes *3* and *6*) or with Mg^{2+} alone (lanes *4* and *7*). (*B*) Northern profile of an aliquot of a reaction incubated with desalted extract in the presence of low Mg^{2+} (lane *3*). Another aliquot of the same reaction was diluted 80-fold with splicing buffer containing 0.5 mM Mg^{2+}, and then incubated further (lane *4*). Lane *1* is as lane *3*, except that the sample is from a parallel mock reaction that was first incubated with buffer, instead of desalted extract. Another aliquot of the mock reaction was diluted 80-fold and then desalted extract (at the same concentration as that in the experiment of lane *4*) was added before the secondary incubation (lane *2*).

cushion, and the pellet was resuspended and divided into three aliquots. One of them was processed directly for electrophoresis to show the accumulation of lariat-exon2 intermediate (Fig. 7 *A*, lane *2*). The other two aliquots were incubated for 30 min under conditions of autonomous splicing, one of them in the absence of ATP. The analysis showed that the intermediate in the latter reaction did not proceed through the second splicing step (Fig. 7 *A*, lane *4*). In contrast, when both 0.5 mM Mg^{2+} and 1.5 mM ATP were present, the amount of the previously accumulated lariat-exon2 intermediate decreased, while free lariat appeared (Fig. 7 *A*, lane *3*). Interestingly, in this particular experiment, the amount of lariat product was greater than that expected from the conversion of the intermediate alone, and was accompanied by reduction of the amount of precursor (Fig. 7 *A*; compare lane 2 with lane *3*). This suggests that under conditions of low Mg^{2+} SCF associates with the splicing complex, but can propagate to some extent only the first splicing step. When the Mg^{2+} concentration is then raised, SCF can chase the accumulated intermediates into products, while, at the same time, more precursor is processed through both splicing steps.

To provide controls, another aliquot of the salt-washed matrix was incubated for 30 min with buffer, instead of extract. An aliquot of this reaction was processed immediately for display (Fig. 7 *A*, lane *5*), and two other aliquots were incubated for an additional 30 min under conditions of autonomous splicing in the presence or absence of ATP (Fig. 7 *A*, lanes *6* and *7*, respectively). Comparison with the time zero matrix control (Fig. 7 *A*, lane *1*) demonstrated the absence of autonomous reactions in these controls (lanes *5–7*).

To eliminate the possibility that some residual extract was trapped during the centrifugation step through the sucrose cushion and propagated the second splicing step, we repeated the experiment described above, but with the following modification. After incubation with desalted extract for factor "pick-up" (experimental sample), or with buffer (control), aliquots were withdrawn for display (Fig. 7 *B*, lanes *3* and *1*, respectively). The remainder of each reaction was then diluted 80-fold with splicing buffer. Addition of extract to the control sample at this time and further incubation showed that SCF was unable to complement the splicing complexes when diluted 80-fold (Fig. 7 *B*, lane 2). In contrast, lariat product did appear in the experimental sample that was incubated in the dilute mixture for an additional 30 min with both ATP and Mg^{2+} (Fig. 7 *B*, lane *4*), in agreement with the results of the previous experiment (Fig. 7 *A*). These data provide additional evidence that SCF is a component of the autonomous spliceosomes.

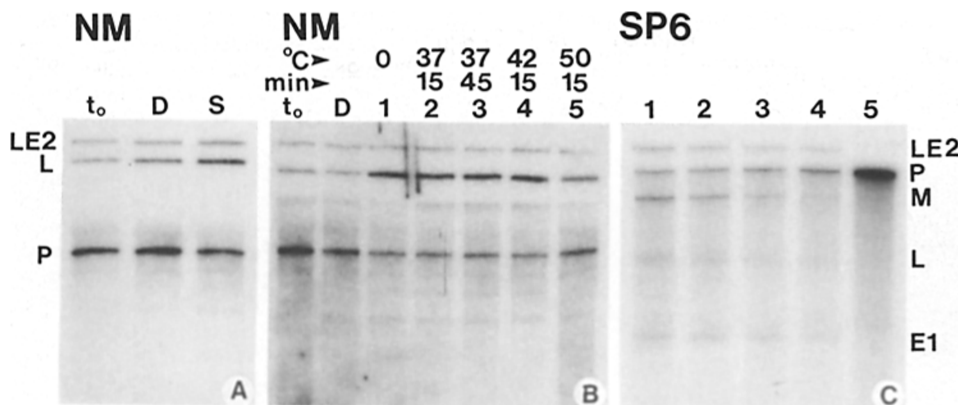

Figure 8. (*A*) Matrix complementation with supernatant after ultracentrifugation of extract. The controls in lanes t_0 and *D* are, respectively, the Northern profiles of time zero RNA and RNA after incubation of the matrix without extract (3 μl of high-salt matrix per lane). The profile in lane *S* is the result of a complementation reaction (50 μl) containing 3 μl of matrix and 30 μl of supernatant after ultracentrifugation of the nuclear extract to remove the snRNPs (see Materials and Methods). (*B*) Matrix complementation reactions with heat-treated extract. The controls in lanes t_0 and *D* (2 μl of high-salt matrix per lane) are the same as in *A*. The Northern profiles in lanes *1–5* show the results of matrix complementation reactions performed either with untreated extract (lane *1*) or with extract heated at the indicated temperatures for the indicated times (lanes *2–5*). (*C*) In vitro splicing reactions with heat-treated extract. Lanes *1–5*, corresponding to the same lanes of *B*, show the results of in vitro splicing reactions (controls) that were performed using an SP6 precursor containing the rabbit β globin gene IVS1 and aliquots of the same heat-treated extracts as in *B*.

Nucleoplasmic and Spliceosome-bound SCF

Using our complementation assay, we examined whether SCF is also present in the S100 cytoplasmic fraction. Fig. 5 *A* (lanes *7* and *8*) shows that the addition of undiluted or twofold-diluted S100 fraction to matrix complementation reactions resulted in RNA degradation. Upon fourfold dilution the nucleolytic activities were not as apparent, and the S100 fraction could complement the NM. However, accumulation of lariat-exon2 intermediate was again observed (Fig. 5 *A*, lane *9*), as with the addition of dilute nuclear extract (Fig. 5 *A*, lane *5*). This result suggested that SCF is a soluble nucleoplasmic factor that leaks out of the nuclei during extract preparation (although the possibility that there is also a cytoplasmic pool of the factor cannot be formally excluded).

Our previous demonstration of autonomous splicing had indicated that a compartment of spliceosome-bound SCF should also exist. To examine whether this compartment is a major source of the complementing activities present in the nuclear extract, we prepared a set of extract fractions by increasing sequentially the KCl concentration (between 0.1 and 0.6 M, in 0.1 M increments), instead of extracting the nuclei in one step with 0.42 M NaCl (Dignam et al., 1983). Assays of the fractions for SCF activity and quantitation of the results (not shown) demonstrated that <10% of the factor was released in the 0.1 M KCl wash. All fractions up to 0.5 M (inclusive) were active, but a sharp peak of SCF (∼70% of the total) was found in the 0.3 M wash. This salt-elution behavior of the complementing factor is in excellent correlation with that of matrix preparations extracted with increasing concentrations of salt. We conclude that very little of the free nucleoplasmic form of SCF is left in the hypotonic nuclei during their preparation, because, otherwise, most of the factor would have been found in the first 0.1 M KCl wash.

Features of SCF

A feature of the complementation reaction is that it does not depend on factors with an RNA component. Previously, we showed that nuclear extract that had been treated effectively with micrococcal nuclease could still sustain matrix complementation (Zeitlin et al., 1987). However, this result did not exclude the participation in the complementation reaction of the nuclease-resistant U5-snRNP (Chabot et al., 1985) or of unknown resistant snRNPs. To eliminate this possibility we removed the snRNPs from the nuclear extract by ultracentrifugation for 2 h at 356,000 *g* (see Ruskin et al., 1988), and showed that the high-speed supernatant was still complementation competent (Fig. 8 *A*, lane *S*; compare to the other two lanes). Comparative electrophoretic analysis (ethidium bromide staining) of the snRNAs in the supernatant and the pellet fractions showed that occasionally trace amounts of U1 and U2 snRNAs can be found in the supernatant, in addition to tRNA (data not shown). On other occasions, however, the supernatant was devoid of detectable snRNAs; gel electrophoresis of pCp 3′ end-labeled RNAs from deproteinized aliquots of supernatant and solubilized pellet demonstrated that only the pellet yielded labeled snRNA species (not shown).

To characterize SCF further, we examined its thermal stability. Aliquots of splicing extract were incubated on ice, or heated at 37°C for either 15 or 45 min, or at 42 or 50°C for 15 min. Examination of these heated extracts for their ability to splice in vitro an SP6 precursor (control reactions) showed that even the treatment at 37°C for 45 min impaired splicing (Fig. 8 *C*, lane *3*). In vitro splicing with extract heated at 42°C was substantially reduced (Fig. 8 *C*, lane *4*), and was abolished by heat treatment at 50°C (lane *5*). In contrast, when the same extracts were used for matrix complementation, we observed that treatment at 42°C did not impair complementation (Fig. 8 *B*, lane *4*), and that a significant portion of the activity survived even the 50°C treatment (lane *5*). Apparently SCF is relatively heat resistant, whereas other, heat-sensitive components exist which presumably participate in the in vitro assembly of spliceosomes, as previously suggested (see, for example, Abmayr et al., 1988). In the control experiments described above (Fig. 6 *C*) we observed reduction of both splicing steps. However, in other in vitro SP6 splicing reactions with heated extracts we had observed on occasion extract-dependent accumulation of intermediates,

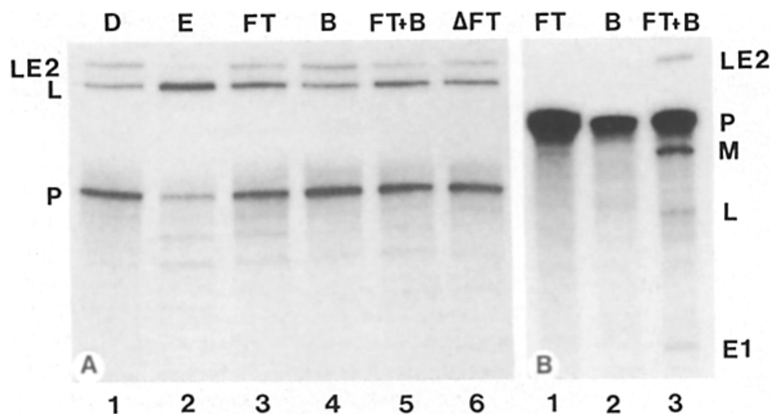

Figure 9. Assays of SCF activity in DEAE–Sepharose fractions. (*A*) High-salt matrix (2 μl/25 μl reaction without PVA) was complemented either with the flowthrough fraction (*FT*; 16 μl) or with the bound fraction (*B*; 4 μl) of DEAE–Sepharose column chromatography after concentration by ammonium sulfate precipitation (lanes *3* and *4*, respectively; see Materials and Methods for details). A combination of the two fractions was used for the reaction in lane *5*. The aliquot of flowthrough fraction used for the assay shown in lane *6* was first heated at 55°C for 10 min (*ΔFT*). The negative and positive control reactions in lanes *1* and *2*, respectively, were performed in the presence of either buffer alone (*D*) or unfractionated extract (*E*). (*B*) Lanes 1–3, corresponding to lanes *3–5* of *A*, show the results of in vitro splicing reactions (controls) that were performed using the same SP6 precursor as in Fig. 8 *C*. Each 50 μl reaction (performed in the presence of 2.5% PVA for 2 h at 30°C) contained aliquots of concentrated chromatographic fractions: flowthrough (24 μl; lane *1*), bound (6 μl; lane *2*), or a combination of the two (lane *3*).

in agreement with other reports (see Reed et al., 1988; Abmayr et al., 1988). For this reason, we examined additional extracts in matrix complementation reactions, but the results were invariable. The heat resistance of SCF was further documented in experiments using fractionated extracts (see below). Treatment of the extract with *N*-ethylmaleimide reduced significantly, but did not abolish, complementation (data not shown).

To correlate SCF with non-snRNP splicing factors that have been described (see Discussion), we initiated extract fractionations, using as a first step DEAE–Sepharose chromatography under conditions identical to those described in an advanced fractionation scheme (Krämer et al., 1987). We found that SCF was present in the flowthrough fraction of the column (Fig. 9 *A*, lane *3*), whereas the bound fraction was practically devoid of activity (lane *4*). Addition of bound fraction to the flowthrough did not augment the complementation level attained with the latter fraction alone (Fig. 9 *A*, lane *5*). The SCF activity in the flowthrough was relatively heat resistant at 55°C for 10 min (Fig. 9 *A*, lane *6*).

To demonstrate that the components in the DEAE–Sepharose-bound fraction were still active, we performed in vitro control reactions with an SP6 precursor. Fig. 9 *B* shows that neither the flowthrough nor the bound fraction were active in in vitro splicing (lanes *1* and *2*, respectively), but they could complement each other (lane *3*).

Discussion

We have described autonomous splicing reactions occurring under in vitro conditions in presumptive complexes associated with a nuclear matrix pellet prepared by extraction with a physiological concentration of salt. Using several different LS-NM preparations, we showed that ∼25% of the pre-mRNA can be processed autonomously. This level could reflect the percentage of complexes that are fully assembled and ready to initiate splicing at any time point of the steady-state, assuming that the particular complexes that we monitor are typical of all spliceosomes. The great majority of the remaining 75% of the complexes are also functional because they can be complemented for splicing with nuclear extract.

Based on our demonstration of functionality, we propose that at least a subset of matrix-bound HnRNPs correspond to in vivo spliceosomes of the in situ matrix. This view is consistent with a previously proposed general hypothesis linking HnRNP particles with pre-mRNA processing (reviewed by Pederson, 1983). Our interpretation relies on extrapolation from the description of in vitro–assembled spliceosomes because we did not examine directly the structural composition of the in vivo complexes.

The autonomous reactions occur in the matrix or in isolated nuclei without a temporal lag and are half-complete in 5 min. This rapid kinetics is a feature consistent with other information about the in vivo behavior of splicing complexes. In vivo results from electron microscopic analyses have indicated that cotranscriptional spliceosome assembly (formation of "committed presplicing complexes") might be quite common (Beyer and Osheim, 1988). In a subset of precursors splicing also occurs cotranscriptionally and presumably before polyadenylation. The overall time for intron removal is ∼3 min after the transcriptional synthesis of the 3′ splice site, regardless of intron length in the cases studied (Bayer and Osheim, 1988). This estimate reflects the time required for both spliceosome assembly (∼2 min; as evidenced by the formation of an intron loop) and for the splicing reactions (elimination of the loop ∼1 min after its appearance). By comparison, our kinetics of autonomous splicing, which measures only the latter, seems somewhat retarded. However, the precursor we study presumably belongs to a different category because it is spliced after polyadenylation (Zeitlin et al., 1987). We note that an earlier in vivo estimate (from a pulse–chase experiment) suggested that the half-time for removal of either of two introns that are eliminated first from the polyadenylated ovomucoid precursor is of the order of 10 min (Tsai et al., 1980). An in vivo time course study of the splicing intermediates and products

Table I. Splicing Factors

snRNP-containing fractions

SF1 (SF = splicing factor; Krainer and Maniatis, 1985)
II (Furneaux et al., 1985)
QU1 (U1 + U5), QU2 (U2 + U4/U6) (Krämer et al., 1987)

Non-snRNP ("protein") splicing factors

[SP6 assays]
SF2, SF3, SF4A, SF4B (Krainer and Maniatis, 1985)*
SF*1, SF*2 (50 kD), SF*3, SF*4 (Krämer and Keller, 1985; Krämer et al., 1987)*
Ia, Ib (Furneaux et al., 1985; Perkins et al., 1986)
[Matrix complementation assays]
SCF (This paper)
[RNA-binding assays]
IBP (100-kD intron binding protein; Tazi et al., 1986)‡
3SSBC (70 kD 3′ splice site binding component; Gerke and Steitz, 1986)‡
U2AF (U2 snRNP auxiliary factor; Ruskin et al., 1988)

Factors Present Exclusively in Nuclear Extract

SF2, SF*3, SF*4, U2AF

Factors Present in Both the Nuclear Extract and the S100 Fraction

SF3, SF4A, SF4B, SF*1, SF*2, SCF, "IBP"§

Heat Sensitivity

45°, 10 min: resistant: SF2, SF4A, SF4B, SF*2, Ia (53%), Ib (38%), SCF
sensitive: SF3 (also U2AF; conditions not reported)
55°, 10 min: resistant: Ia (39%), SCF
sensitive: Ib

N-Ethylmaleimide Sensitivity

Relatively Resistant (reduction, but not elimination, of activity): Ia, SCF
Sensitive: Ib, SF*2, U2AF

Reactions

SF1 + SF2 + SF3 = no splicing
[SF1 + SF2 + SF3] + SF4B = first step [SF4B is a 1st step factor]
[SF1 + SF2 + SF3 + SF4B] + SF4A = both splicing steps [SF4A is a 2nd step factor]
Heated extract ($SF3^-$) = first step [SF3 is a second step factor]
Ia + Ib + II = both steps
Ib + II = first step [Ia is a second step factor]
SF*1 + SF*2 + SF*3 + SF*4 + QU1 + QU2 = both steps [uncoupling of steps not observed by omission of any of the factors]
Matrix + SCF = both steps

Chromatographic Correlations

1. SF*2 binds to DEAE-Sepharose; SF*1 is in the flowthrough (application of extract in 0.1 M KCl, 3 mM Mg^{2+}). Under identical conditions, SCF is in the flowthrough. Ia and Ib are in the flowthrough of a DEAE-cellulose column (application in 0.2 M KCl without Mg^{2+}); they are fractionated by Bio-Rex 70 column chromatography.
2. U2AF binds to Mono Q, and is eluted with 0.3 M KCl. The behavior of SF*3 is the same. Further fractionation of SF*3 on Mono S indicates that it consists of at least two components.

Conclusions

1. SF*3 and SF*4 are subsets of SF2; U2AF is a subset of SF*3.
2. SCF corresponds to SF*1.

(For details see Discussion.)

* To avoid confusion from the coincidental use of the same designations for different activities by the two groups, we refer to one set of factors as SF*1–SF*4 (instead of SF1–SF4).
‡ IBP and 3SSBC are not necessarily identical as generally suggested. In 15 mM Mg^{2+}, 3SSBC dissociates from snRNPs and does not bind to DEAE-Sepharose. The behavior of IBP is the opposite.
§ The 70-kD IBP activity described by Ruskin et al. (1988) was purified according to Tazi et al. (1986), but assayed according to Gerke and Steitz (1986).

from the adenovirus-2 E3 transcription unit has indicated that they begin appearing within 1–2 and 3–4 min, respectively, after the synthesis of the precursor (Sittler et al., 1986).

Within their limitations all of these measurements, including ours, indicate reaction times of the same order of magnitude. Thus, we suggest that the results obtained with the advantageous "open" matrix system, which is amenable to in vitro manipulation, can be extrapolated reliably as a close approximation to the behavior of in vivo native spliceosomes.

The complementable structures on the matrix might represent a graded series of complexes with various degrees of assembly. Alternatively, if rapid cotranscriptional assembly of presplicing complexes (Beyer and Osheim, 1988) is a general phenomenon, most of our complementable structures might be missing only SCF. The same might apply to the structures remaining after high-salt extraction. Salt resistance of spliceosome structure has also been observed with the in vitro splicing system (see Grabowski et al., 1985). Since matrix complementation can be attained with extracts devoid of activities with an RNA component, the salt-resistant complexes certainly contain all of the snRNPs participating in splicing. Such complexes, partially stripped of some protein component(s), are not rearranged and can be fully restored by complementation. In fact, as discussed below, most of the known non-snRNP (protein) splicing factors (Table I) should also be present on the salt-washed complementable complexes. We note that in vitro–assembled complementable complexes, derived by interruption of an SP6 splicing reaction ~20 min after its initiation (after spliceosome assembly, but before the appearance of products), require for function not only SCF but also other activities present in the DEAE–Sepharose-bound fraction (Parent, A., R. Wilson, S. Zeitlin, and A. Efstratiadis, manuscript in preparation).

The chromatographic behavior of SCF, which is present in the flowthrough fraction after DEAE–Sepharose column chromatography, suggests that SCF corresponds to a component of the previously described (unbound) factor SF*1 (Krämer et al., 1987; see Table I). As SF*1, SCF is present in both the nuclear extract and the S100 fraction. Further purification of SF*1 has indicated that the fraction contains at least two activities, one of them acting at the level of spliceosome assembly (Krämer, 1988). It is unlikely that the latter activity is related to SCF.

SCF is clearly unrelated to SF2 (Krainer and Maniatis, 1985), SF*3 and SF*4 (Krämer et al., 1987), and U2AF (Ruskin et al., 1988), all of which are absent from the S100 fraction, and to the DEAE–Sepharose-bound 50-kD polypeptide SF*2 (Krämer and Keller, 1985; Krämer et al., 1987). We believe that the heat-sensitive SF3 component (Krainer and Maniatis, 1985), which has been implicated in the second splicing step, is also unrelated to the relatively heat-resistant SCF. Moreover, we exclude the possibility that SCF could be related to the HnRNP C protein (proposed to be a splicing factor; Choi et al., 1986). After ultracentrifugation, the HnRNP C protein is found exclusively in the pellet (Ruskin et al., 1988), whereas SCF is in the supernatant. A relationship between SCF and the fraction Ia described by Perkins et al. (1986), which is involved in the second splicing step, cannot be excluded because of similar heat- and *N*-eth-

ylmaleimide-sensitivities (Table I). However, a chromatographic correlation is currently missing. A basis for correlating SCF with the remaining first (SF4B) and second step (SF4A) activities described by Krainer and Maniatis (1985) is not available.

Our current information is not sufficient to determine whether SCF is one or more splicing activities. It is possible that SCF is composed of at least two activities, SCFl and SCF2, involved in the first and second splicing steps, respectively. Resolution of this issue could facilitate the eventual interpretation of the uncoupling of the two splicing steps upon extract dilution. One model is that the dissociation constant of the SCF2/matrix complex is higher than that of the SCFl/matrix complex (SCF2 has less affinity for the complementable spliceosome than SCFl). Neither SCFl nor SCF2 could be a limited extract component (see Results). A more complicated model based on the same principle is that SCF2 can bind to the spliceosome only via SCFl (in the form of an SCFl/SCF2 complex), but the affinity of SCFl for the spliceosome is higher than its affinity for SCF2. If dilution promotes dissociation of the SCFl/SCF2 complex, SCFl can still bind to the complementable spliceosome propagating the first splicing step. If, on the other hand, SCF is a unique factor, it might act as a multimeric protein (for example, a dimer). In this model, concerted propagation of both splicing steps depends on the presence of the dimer. However, one subunit could suffice to promote the first step, either directly or by providing an auxiliary function for another component already present on the complementable spliceosome. The postulated dimer need not be a preexisting entity (dissociating into subunits upon dilution), but it might be formed after independent binding of two monomers to proximal sites of the complementable spliceosome. For the moment we cannot discriminate between these models, which certainly do not exhaust all possibilities. However, the dilution phenomenon we described suggests that binding of SCF is an absolute prerequisite for the occurrence of at least the second splicing step. If all of the putative second step factors were present on the complementable spliceosome, uncoupling of the two steps after the occurrence of the first would not have been observed.

The function of SCF at the molecular level is unknown. As previously pointed out (see Cech and Bass, 1986), the splicing reactions themselves might be catalyzed by RNA. In that case, instead of an enzymatic activity, SCF or its potential components might be the last assembly activities to become associated with the spliceosome, triggering some conformational change.

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