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# Purification of a protein required for the splicing of pre-mRNA and its separation from the lariat debranching enzyme

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**We have used a complementation assay to test for activities required for the splicing of pre-mRNA *in vitro*. During the hypotonic lysis of HeLa cells, two components are released from the nuclei that specifically stimulate splicing in an extract prepared from washed nuclei. The two activities separate during chromatography on DEAE-Sepharose. One of these activities [splicing factor (SF)2] co-purified through several steps with the lariat debranching enzyme and with a nuclease which degrades the linear portion of lariat RNAs. These enzymes could, however, be separated from SF2 by chromatography on heparin-Sepharose. SF2 fractionates as a single protein with an apparent mol. wt. of 50 000. SF2 is resistant to mild heat treatment and to treatment with micrococcal nuclease, but it is inactivated by N-ethylmaleimide, suggesting that it is a protein which is not associated with an essential RNA component. When SF2 is absent in a complementation assay, the generation of both intermediates and final products of the splicing reaction is completely abolished. Thus, SF2 functions in an early step of the splicing process.**

*Key words:* fractionation/lariat debranching enzyme/messenger RNA splicing/splicing factors

## Introduction

The development of *in vitro* systems that mimic the *in vivo* pathway of pre-mRNA splicing (Goldenberg and Raskas, 1981; Kole and Weissman, 1982; Padgett *et al.*, 1983a; Hernandez and Keller, 1983; Krainer *et al.*, 1984) has added to our understanding of the events leading to the precise excision of the introns that are present in most protein coding eukaryotic pre-mRNAs (for reviews, see Breathnach and Chambon, 1981; Keller, 1984; Padgett *et al.*, 1985). The process by which two exons are accurately aligned and subsequently ligated is not a simple one-step reaction; it can be separated into the events that occur during the characteristic lag period of 15-30 min observed in nuclear extracts of HeLa cells (Hernandez and Keller, 1983; Krainer *et al.*, 1984; Hardy *et al.*, 1984) and the actual splicing reaction which takes place in two distinct phases. Synthetic pre-mRNA added to a HeLa cell nuclear extract is assembled into a structure that sediments at 50-60S in sucrose or glycerol gradients (Frendewey and Keller, 1985; Grabowski *et al.*, 1985). The formation of this complex seems to be a prerequisite for splicing, since reaction products are observed only in its presence. A similar structure has previously been identified in a yeast *in vitro* splicing system (Brody and Abelson, 1985).

In the first step of the actual splicing reaction, which coincides with the appearance of the splicing complex, the pre-mRNA is cleaved at the 5′ splice site (Ruskin *et al.*, 1984; Padgett *et al.*, 1984), giving rise to two reaction intermediates, the first exon and the intron-exon 2 lariat RNA. The latter structure is formed by joining of the 5′-terminal G residue of the intron to an A residue near the 3′ splice site *via* a 2′,5′-phosphodiester bond (Ruskin *et al.*, 1984; Padgett *et al.*, 1984; Konarska *et al.*, 1985). The next step involves cleavage at the 3′ splice site and ligation of the two exons, leading to the release of the intron which remains in the lariat configuration. All of these products are associated with the 50-60S splicing complex (Frendewey and Keller, 1985; Grabowski *et al.*, 1985). Reaction products corresponding to those found *in vitro* have also been detected *in vivo* (Domdey *et al.*, 1984; Rodriguez *et al.*, 1984; Zeitlin and Efstratiadis, 1984), supporting the conclusion that they represent genuine intermediates and products of the splicing pathway.

A requirement for distinct components that generate intermediates and final splicing products, respectively, is indicated by the observation that the two steps can be uncoupled. If a nuclear extract is subjected to mild heat treatment prior to splicing, only reaction intermediates are observed (Krainer and Maniatis, 1985). Thus, heat-stable components act early in the splicing process, whereas heat-labile activities are required to complete the reaction. Likewise, a nuclear extract can be fractionated into separate components, a subset of which are sufficient for the generation of intermediates (Krainer and Maniatis, 1985; Krämer and Keller, in preparation).

In addition, uncoupling of the two steps of the splicing reaction can be achieved by use of mutant splicing substrates, indicating that distinct sequence elements in the intron are recognized at different times during the splicing reaction. A 5′ splice site, an RNA branch point and a polypyrimidine stretch at the 3′ splice site are the minimal requirements for splicing complex formation and generation of intermediates, whereas the AG dinucleotide defining the 3′ end of the intron is only essential for 3′ cleavage and ligation of the exons (Reed and Maniatis, 1985; Frendewey and Keller, 1985; Ruskin and Green, 1985b).

Two of the components required during the lag period have been identified. Based upon the complementarity between sequences in the intron (Breathnach *et al.*, 1978; Mount, 1982; Ruskin *et al.*, 1984; Keller and Noon, 1984) and in U1 and U2 snRNP, these particles have been proposed to participate in splicing (Lerner *et al.*, 1980; Rogers and Wall, 1981; Keller and Noon, 1984, 1985; Oshima *et al.*, 1981). Experimental evidence from several laboratories supports such a model and provides a mechanism which could explain the high degree of accuracy observed in splicing both *in vivo* and *in vitro*. Recognition of the 5′ splice site is achieved by binding of U1 snRNP, thereby protecting this region from RNase digestion (Mount *et al.*, 1983; Black *et al.*, 1985). The interaction most probably occurs through base pairing between the 5′ splice site and the 5′-terminal sequences of U1 snRNA in the intact RNP particle (Krämer *et al.*, 1984; Black *et al.*, 1985; Krainer and Maniatis, 1985). Similar experiments have shown the interaction of U2 snRNP with the intron near the 3′ splice site (Black *et al.*, 1985; Krainer and Maniatis, 1985; B. Ruskin and M. Green, personal communication), again leading to a protection from RNase digestion. The

region of interaction includes the nucleotide of subsequent branch formation (Ruskin *et al.*, 1984; Keller and Noon, 1984; Padgett *et al.*, 1984; Konarska *et al.*, 1985). Thus, U2 snRNP acts in the selection of the 3′ splice site (Black *et al.*, 1985). Furthermore, the presence of U1 and U2 snRNPs in the large splicing complexes (Grabowski *et al.*, 1985) and the inhibition of complex formation in their absence (Frendewey and Keller, 1985) is consistent with the binding of these particles to the pre-mRNA.

In addition to components that mediate splicing of pre-mRNA molecules, HeLa cell extracts contain enzymatic activities that specifically act on lariat RNA species (Ruskin and Green, 1985a). These include a debranching enzyme which cleaves the 2′,5′-phosphodiester bond formed at the branch point, giving rise to a linear excised intron, and a nucleolytic activity which degrades the linear portion of the lariat RNA. Since corresponding RNA products have also been found *in vivo* (Domdey *et al.*, 1984; Rodriguez *et al.*, 1984; Zeitlin and Efstratiadis, 1984), an involvement of these enzymes in the degradation of introns after their removal from the pre-mRNA seems plausible. Moreover, Ruskin and Green (1985a) have suggested that the physiological role of the debranching enzyme might be to function in the actual splicing reaction by catalyzing isoenergetic transesterifications (Cech *et al.*, 1981) that were proposed as one possible mechanism for pre-mRNA splicing (Padgett *et al.*, 1984).

To gain insight into the splicing mechanism, we have begun to fractionate HeLa cell extracts with the final goal of reconstituting pre-mRNA splicing with purified components. Here we describe the partial purification and characterization of a protein factor which appears to play an essential role early in the splicing process. During initial chromatographic steps this factor co-purifies with the lariat debranching enzyme and a nuclease which degrades the linear portion of lariat molecules. Upon further fractionation these activities can be separated.

## Results

### *Assay system*

For the experiments described here nuclear and cytoplasmic extracts prepared according to Dignam *et al.* (1983) were used. The model substrate for *in vitro* splicing was derived from the adenovirus 2 major late (AdML) transcription unit. RNA was synthesized from plasmid pSP62Δi1, which was linearized at the *Sca*I site within the second exon (see Frendewey and Keller, 1985, for details). Transcription from the phage SP6 promoter with SP6 RNA polymerase (Melton *et al.*, 1984; Georgiev *et al.*, 1984) results in an RNA with a chimeric first exon (E1) of 102 nucleotides (containing SP6 sequences and the entire AdML leader 1), an intron (IVS1) of 113 nucleotides and a second exon (E2) of 38 nucleotides (corresponding to about half of the AdML leader 2). The RNA products generated during the splicing reaction were identified by the criteria described by Frendewey and Keller (1985; see also below).

### *Components essential for in vitro splicing leak out of the nuclei during extract preparation*

In their splicing system Hernandez and Keller (1983) observed an activity in the cytoplasmic fraction that stimulated splicing in a nuclear extract. The cytoplasmic extract by itself was inactive in splicing. This result suggested that components involved in the *in vitro* splicing reaction leak out of the nuclei during preparation of the extracts. To pursue this observation, crude nuclei were prepared (Dignam *et al.*, 1983). The nuclei were divided into aliquots and either extracted immediately or washed with hypotonic buffer before extraction. The extracts were then tested

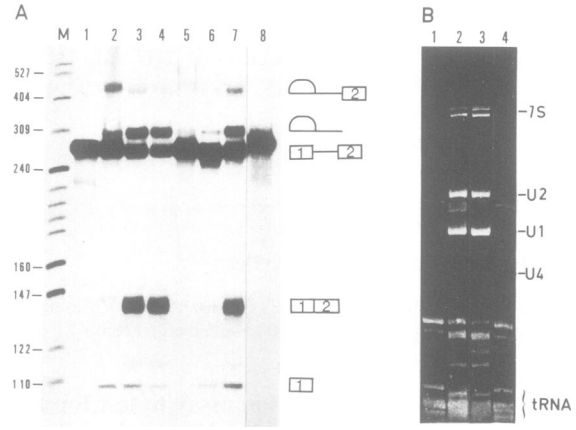


**Fig. 1.** Components required for efficient *in vitro* splicing leak into the cytoplasmic fraction during extract preparation. **(A)** Standard splicing reactions were performed for 30 min **(lanes 2** and **5)** or 90 min **(lanes 1, 3, 4, 6, 7** and **8)**. The reactions contained: no extract **(lane 1)**, 15 μl of untreated nuclear extract **(lanes 2** and **3)**, 15 μl of untreated nuclear extract plus 5 μl of cytoplasmic extract **(lane 4)**, 15 μl of washed nuclear extract **(lanes 5** and **6)**, 15 μl of washed nuclear extract plus 5 μl of cytoplasmic extract **(lane 7)** or 5 μl of cytoplasmic extract **(lane 8)**. Reaction products were fractionated on a 12% acrylamide gel and visualized by autoradiography. **Lane M:** [$^{32}$P]DNA markers from a *Hpa*II digest of pBR322 DNA. The sizes of the fragments are indicated on the left. Pre-mRNA and reaction products are identified by schematic drawings. Exons are shown as open boxes, the intron as a line. **(B)** The gel shown in **(A)** was stained with ethidium bromide. **Lane 1:** carrier tRNA. **Lane 2:** 15 μl of untreated nuclear extract. **Lane 3:** 15 μl of washed nuclear extract. **Lane 4:** 5 μl of the cytoplasmic fraction. The RNA species are indicated at the right.

for splicing activity in the absence or presence of the cytoplasmic fraction prepared from the same cells (Figure 1A). The nuclear extract prepared without the hypotonic wash step (untreated extract) splices the pre-mRNA efficiently. Splicing intermediates (E1 and IVS1-E2) as well as a small amount of final reaction products (E1-E2 and IVS1) are visible after 30 min of incubation (lane 2). After 90 min the spliced product and the excised IVS1 lariat have accumulated (lane 3). In contrast, the extract prepared from washed nuclei has lost almost all of its splicing activity. No products are apparent in a 30 min reaction (lane 5); at 90 min a low level of splicing is observed (lane 6). However, when this extract is complemented with the cytoplasmic fraction, which itself is inactive (lane 8), splicing activity is greatly increased (lane 7) and is now as efficient as in the extract prepared from untreated nuclei. Addition of the cytoplasmic fraction has no effect on the splicing efficiency of the untreated nuclear extract (lane 4), suggesting that all components required for splicing are present in sufficient quantity. These results confirm that components necessary for *in vitro* splicing are present in the cytoplasmic fraction and that these activities leak out of the nuclei during preparation of the extracts.

To test the possibility that the loss of splicing activity observed in the washed nuclear extract is due to leakage of snRNPs into the cytoplasmic fraction, RNA prepared from the different extracts was analyzed by polyacrylamide gel electrophoresis (Figure 1B). Extracts from untreated and washed nuclei contain comparable amounts of U1 and U2 snRNPs which are essential for *in vitro* splicing (Padgett *et al.*, 1983b; Krämer *et al.*, 1984; Black *et al.*, 1985; Krainer and Maniatis, 1985). In the cytoplasmic fraction no snRNPs can be detected, making it unlikely that the stimulatory effect is due to U1 or U2 snRNP (see also below).

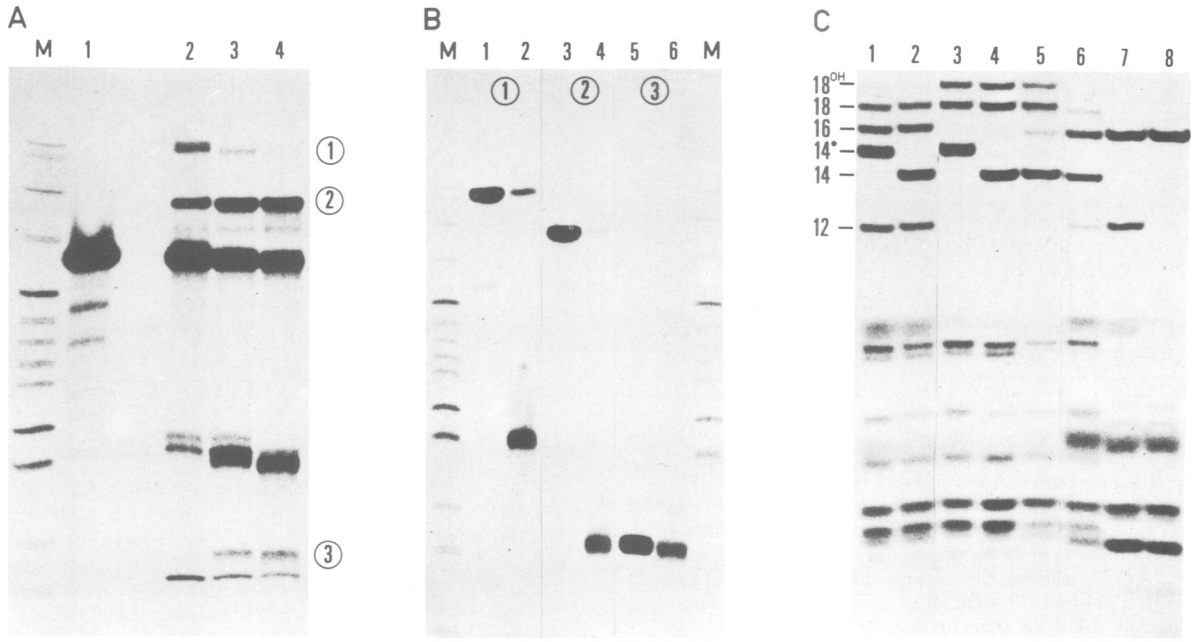


**Fig. 2.** Analysis of the IVS1 lariat, which is debranched in a splicing reaction, by a debranching assay and RNase T1 digestion. **(A)** Splicing was performed in the absence of extract **(lane 1)** or in the presence of 15 μl of nuclear extract for 30 min **(lane 2)**, 60 min **(lane 3)** or 120 min **(lane 4)**. The circled numbers indicate the IVS1-E2 lariat (1), the IVS1 lariat (2) and the debranched IVS1 (3). **(B)** The IVS1-E2 lariat, the IVS1 lariat and the linearized lariat were eluted from the gel shown in **(A)**, as indicated by the circled numbers. Debranching reactions were performed for 15 min in the absence **(lanes 1, 3** and **5)** or the presence **(lanes 2, 4** and **6)** of 5 μl of cytoplasmic extract. **(C)** Products from splicing and debranching reactions were eluted from 12% acrylamide gels and digested with RNase T1 as described in Materials and methods. **Lane 1:** IVS1-E2 lariat. **Lane 2:** IVS1-E2 debranched. **Lane 3:** IVS1 lariat. **Lane 4:** IVS1 lariat debranched in a debranching reaction. **Lane 5:** IVS1 lariat debranched during a standard splicing reaction. **Lane 6:** pre-mRNA. **Lane 7:** spliced product. **Lane 8:** exon 1. Numbers at the left indicate the size of the RNase T1 digestion products. See text for a description of oligonucleotides $18^{OH}$ and 14*. Reaction products were fractionated on a 20% acrylamide/8 M urea gel.

*Intron lariats are debranched during the splicing reaction*

Among the products from a standard splicing reaction we observed an RNA species of ~115 nucleotides that first appears between 30 and 60 min and remains stable for at least 2 h (Figure 2A). The fact that this RNA species (113-nucleotide RNA) is a late product of the *in vitro* splicing reaction led us to suspect that it arises by debranching of the intron lariat, which is 113 nucleotides long. To compare the 113-nucleotide RNA with the linear form of IVS1, the lariat debranching assay described by Ruskin and Green (1985a) was used. In the cytoplasmic extract deproteinized IVS1-E2 or IVS1 lariats are converted to products of ~151 and 113 nucleotides, the sizes expected for the linearized lariat molecules (Figure 2B, lanes 2 and 4). The 113-nucleotide RNA is not susceptible to the debranching activity (lanes 5 and 6) and does not change its electrophoretic mobility in gels of different acrylamide concentrations (see below), indicating that it is a linear molecule. Comparison of the electrophoretic mobilities of the 113-nucleotide RNA with that of the debranched IVS1 reveals that both RNA species are of the same size (lanes 4 and 5).

For further analysis, lariat and debranched RNA species were digested with RNase T1, followed by electrophoresis in a 20% sequencing gel (Figure 2C). All RNase T1 fragments characteristic of splicing products of the AdML pre-mRNA are generated (Padgett *et al.*, 1984). These are, for the IVS1-E2 lariat (lane 1), a fragment of 18 nucleotides derived from the 3′ end of IVS1, oligonucleotide 14* which contains the additional G residue derived from the 5′ end of IVS1 linked to an A residue at the branch point, and the 12-nucleotide digestion product indicative of E2 sequences. The IVS1 lariat (lane 3) differs from the IVS1-E2 lariat in the reduced electrophoretic mobility of the 18-nucleotide RNase T1 fragment. This is due to the presence of a 3′ hydroxyl group (instead of a 3′ phosphate) generated by cleavage of the IVS1-E2 intermediate at the 3′ splice site. The oligonucleotides of 16 and 18 nucleotides in the digest of IVS1-E2 and IVS1, respectively, probably represent partial RNase T1 digestion products. The debranched counterparts of IVS1-E2 (lane 2) and IVS1 (lane 4) contain the 14-nucleotide fragment present in the pre-mRNA (lane 6). RNase T1 digestion of the 113-nucleotide RNA (lane 5) yields fragments $18^{OH}$ and 14 characteristic of the debranched intron. The band of 16 nucleotides (indicative of exon 1 sequences) within this digest is possibly derived from E1 sequences that had been trapped within the 113-nucleotide RNA which migrates close to exon 1 in a 12% gel. For comparison lanes 6–8 of Figure 2C show RNase T1 digestion products derived from the pre-mRNA, the spliced product and E1, respectively. In summary, the presence in the 113-nucleotide RNA of the 3′-terminal hydroxyl group of IVS1, the absence of a modified oligonucleotide containing the branch point and the appearance of this RNA at late times during the splicing reaction suggest that a small amount of the IVS1 lariat is converted to its debranched linear counterpart in the course of the splicing reaction.

*Fractionation of splicing and debranching activities*

Ruskin and Green (1985a) suggested that an enzyme catalyzing the debranching reaction might also be involved in putative transesterifications leading to the formation of lariats. Since the debranching activity is found in both nuclear and cytoplasmic extracts (Ruskin and Green, 1985a) probably it is partially lost from the nuclei during extract preparation, as shown above for components involved in pre-mRNA splicing. To test the hypothesis that the debranching enzyme is associated with an activity required for splicing, fractions obtained during the purification of a splicing factor from cytoplasmic extracts were tested for

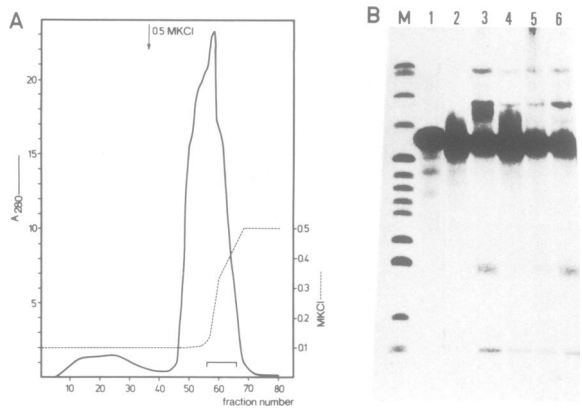


**Fig. 3.** DEAE-Sepharose chromatography of the cytoplasmic fraction. **(A)** Elution profile of proteins derived from a cytoplasmic extract. The horizontal bar indicates the fractions containing stimulatory and debranching activities. **(B)** Standard splicing reactions were performed for 60 min in the absence of extract **(lane 1)** or in the presence of 15 μl of washed nuclear extract **(lanes 2–6)**. In addition, the reactions contained 5 μl of cytoplasmic fraction **(lane 3)**, 5 μl of DE-FT **(lane 4)**, 5 μl of DE-500 **(lane 5)** or 5 μl of DE-FT plus 5 μl of DE-500 **(lane 6)**.

splicing as well as for debranching activity.

As a first purification step, a cytoplasmic extract (Dignam *et al.*, 1983) was fractionated on DEAE-Sepharose (Figure 3A). The pooled flow-through (DE-FT) and individual step fractions (DE-500) were tested for stimulatory activity in the presence of washed nuclear extract (Figure 3B). Complementation of the extract with either DE-FT or DE-500 fractions of the descending part of the protein peak (see Figure 3A) results in a slight stimulation of the splicing activity which is obtained with washed extract alone (Figure 3B, compare lanes 4 and 5 with lane 2). The splicing efficiency cannot be increased by adding larger amounts of these fractions. However, complementation of the extract with both DE-FT and DE-500 results in a further stimulation of splicing activity (lane 6), comparable with that observed with the cytoplasmic fraction (lane 3). These results indicate that the cytoplasmic extract contains at least two components required for optimal splicing efficiency and that these components can be separated by DEAE-Sepharose chromatography. The activity which elutes from the column at 500 mM KCl most probably corresponds to the stimulatory activity previously observed by Hernandez and Keller (1983) in an equivalent fraction.

When the DEAE-Sepharose fractions were tested for debranching activity, only the DE-500 fractions eluting in the late part of the protein peak efficiently converted the IVS1-E2 lariat in the debranched product (Figure 3A and Figure 4C, lane 3). Thus, the fractions which contain splicing activity are also active in debranching.

Components corresponding to the cytoplasmic DE-FT and DE-500 activities which are required for splicing are also obtained after chromatography of a nuclear extract (Krämer and Keller, in preparation). From a DEAE-Sepharose column eluted with a salt gradient, the activity equivalent to the cytoplasmic DE-500 activity is eluted between 0.2 and 0.26 M KCl. According to its order of elution from DEAE-Sepharose this activity has been designated splicing factor (SF) 2. For reasons of simplicity we shall refer to the cytoplasmic DE-500 activity as SF2 in the following sections. A lariat debranching assay on the gradient fractions revealed that the debranching activity co-elutes with SF2 between 0.19 and 0.28 M KCl (data not shown).

In an attempt to devise a complementation system where the

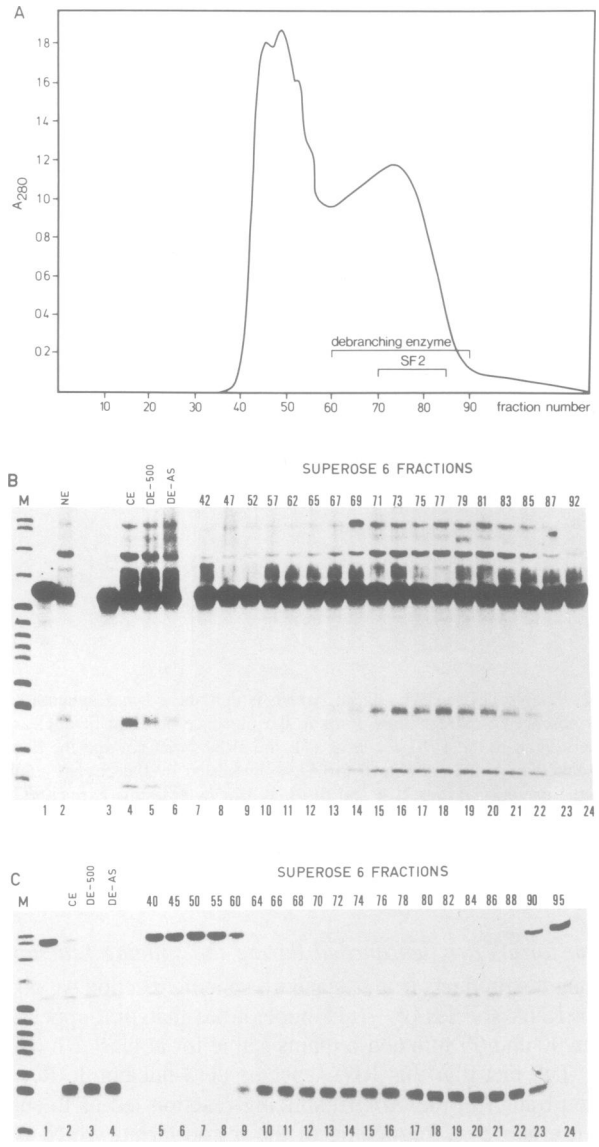


**Fig. 4.** Superose 6 gel filtration of SF2 activity and debranching enzyme. **(A)** Elution profile of proteins upon fractionation on a Superose 6 column. The horizontal bars indicate SF2 and debranching activity, respectively. **(B)** Standard splicing reactions contained no extract **(lane 1)**, 15 μl of nuclear extract **(lane 2)**, 15 μl of washed nuclear extract plus 2 μl of DE-FT **(lanes 3–24)** and 5 μl of the fractions indicated at the top of each lane. Incubation was for 50 min. **(C)** Standard debranching reactions contained buffer B **(lane 1)** or 5 μl of the fractions indicated above each lane. IVS1-E2 lariat was used as the substrate. Incubation was for 15 min.

stimulatory effect of SF2 is optimal, the washed nuclear extract was titrated with increasing amounts of DE-FT (derived from a nuclear extract) in the presence of the DE-500 fraction (data not shown). As a result, all subsequent column fractions were tested for splicing activity in the presence of 15 μl of washed nuclear extract and 2 μl of DE-FT.

Active DE-500 fractions were pooled and concentrated by ammonium sulfate precipitation. This material was applied to a Superose 6 gel filtration column and fractions were tested for splicing and debranching activity. As shown in Figure 4B, the activity that stimulates splicing elutes in fractions 71–85. The debranching activity shows a rather broad elution profile, being most prominent in fractions 64–88 (Figure 4C). The IVS1-E2 lariat used as a substrate is almost completely converted to the

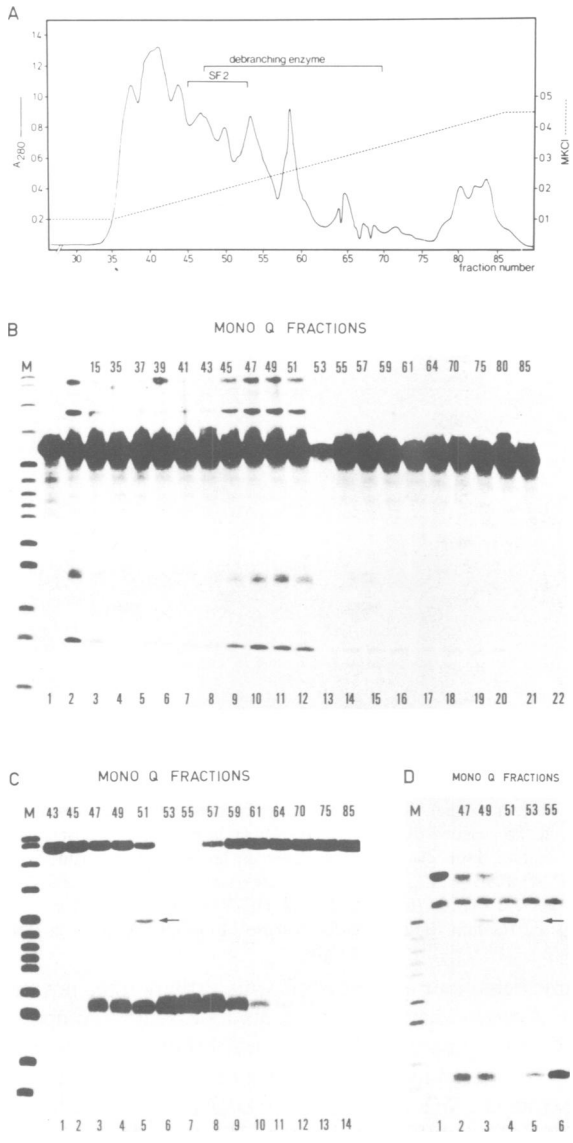


**Fig. 5.** First Mono Q chromatography. **(A)** Profile of proteins eluted from a Mono Q column with a linear salt gradient. The protein concentration in the flow-through fractions (not shown) was $<0.2$ $A_{280}$ units/ml. **(B)** Standard splicing reactions contained buffer B **(lane 1)** or 15 $\mu$l of washed nuclear extract and 2 $\mu$l of DE-FT plus 5 $\mu$l of buffer B **(lane 2)** or 5 $\mu$l of the fractions indicated above each lane **(lanes 3–22)**. Incubation was for 60 min. **(C)** IVS1-E2 lariat was subjected to debranching in the presence of 5 $\mu$l of the Mono Q fractions indicated above each lane. The arrow indicates the 240-nucleotide RNA (see text). **(D)** IVS1 lariat was debranched in the presence of buffer B **(lane 1)** or Mono Q fractions as shown on the top of each lane. The arrow indicates the 240-nucleotide RNA.

debranched form. Some pre-mRNA is present in this lariat preparation, due to trapping of the precursor in the excised band. (This has been observed whenever the lariats were eluted from 12% acrylamide gels, where they migrate slower than the pre-mRNA.)

Superose 6 fractions active in splicing were pooled and further fractionated on a Mono Q column by gradient elution (Figure 5A). Splicing activity is present in fractions 45–51 eluting between 0.16 and 0.21 M KCl (Figure 5B). When the Mono Q fractions are tested for debranching with the IVS1-E2 lariat as the substrate, activity is observed in fractions 47–64 (Figure 5C) with a peak of activity in fractions 53–55. From these results it is evident that fractions which quantitatively linearize the substrate lariat do not stimulate splicing in the complementation assay. In fraction 51 an RNA species migrating slightly faster than the pre-mRNA with an electrophoretic mobility of ~240 nucleotides is apparent (arrow in Figure 5C). When the IVS1 lariat is used as a substrate in this assay, the same product is observed (Figure 5D). The conversion of the IVS1 lariat to the 240-nucleotide RNA seems to be more efficient than the conversion of the IVS1-E2 lariat. This RNA species was identified as a circular molecule containing IVS1 sequences and will be described in detail below.

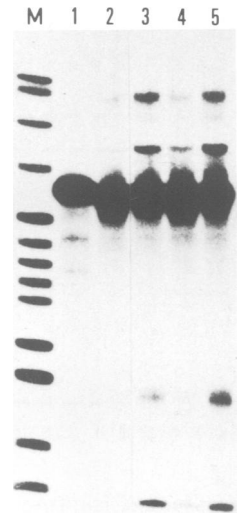


**Fig. 6.** SF2 activity in heparin-Sepharose and Mono QII fractions. Standard splicing reactions were performed for 60 min in the absence of extract **(lane 1)** or in the presence of 15 $\mu$l of washed nuclear extract and 2 $\mu$l of DE-FT **(lanes 2–5)**. In addition, the reactions contained 5 $\mu$l of HS-FT **(lane 3)**, 5 $\mu$l of HS-500 **(lane 4)** or 5 $\mu$l of the Mono QII-500 fraction **(lane 5)**.

To purify further SF2, Mono Q fractions enriched in this activity were fractionated on a heparin-Sepharose column. The column washed (HS-FT) and bound proteins were eluted with a 0.5 M KCl step (HS-500). The HS-FT fractions were immediately concentrated on a second Mono Q column by elution with 0.5 M KCl (MQII-500). Aliquots of the HS-500 and MQII-500 fractions were dialyzed and tested for splicing activity. As shown in Figure 6, SF2 did not bind to heparin-Sepharose, but was recovered in the HS-FT (lane 3). After concentration of this fraction by Mono Q chromatography, an increase in stimulation by SF2 is observed in the complementation assay (lane 5). No stimulation of splicing is found when the HS-500 fraction is tested in the presence of washed extract and DE-FT (lane 4).

Aliquots of the MQII-500 and HS-500 fractions were sedimented through glycerol gradients. As expected, only components in the glycerol gradient of the MQII-500 fraction caused a stimulation of splicing in the complementation assay (Figure 7A). The activity sediments in fractions 9–16. According to size markers run in a parallel gradient, SF2 has an apparent mol. wt. of 50 000. The same native mol. wt. was determined when SF2 was subjected to gel filtration on a Superose 12-FPLC column (results not shown). In a denaturing acrylamide gel of the glycerol gradient proteins a prominent band of ~50 000 is detected in the SF2-containing fractions (results not shown). Whether or not this protein represents SF2 has to await further purification.

Debranching activity in the glycerol gradient fractions was tested in the presence of the IVS1 lariat. Activity was found only in the gradient of the HS-500 fraction. It is present in fractions

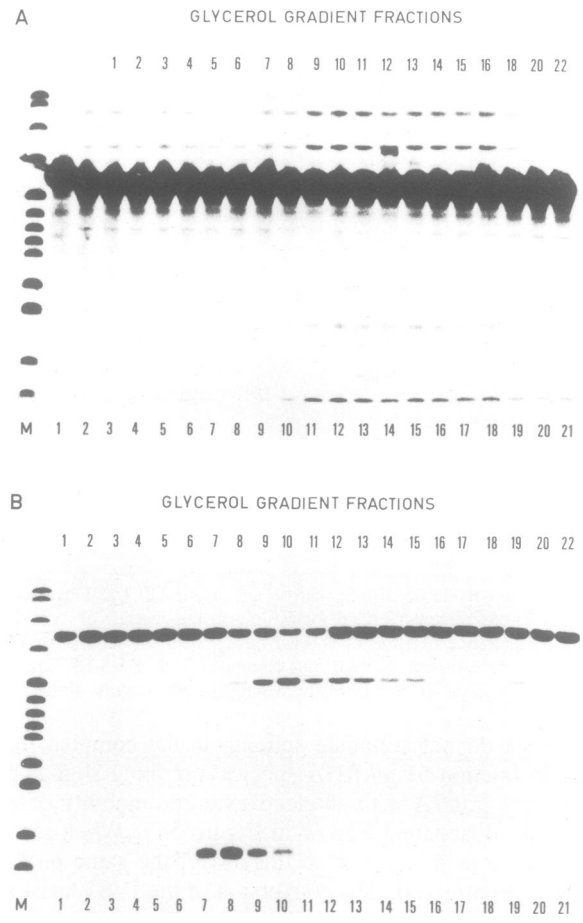


**Fig. 7.** Sedimentation of SF2 and lariat debranching activities, in glycerol gradients. **(A)** Glycerol gradient sedimentation of SF2 activity (Mono QII-500 fraction). Standard splicing reactions were performed for 60 min in the absence of extract **(lane 1)** or in the presence of 15 μl of washed nuclear extract plus 2 μl of DE-FT **(lanes 2–21)**. Reactions shown in **lanes 3–21** contained, in addition, 5 μl of the glycerol gradient fractions indicated on top of each lane. **(B)** Glycerol gradient sedimentation of debranching activity (HS-500 fraction). IVS1 lariat was debranched in a standard reaction in the presence of 5 μl of the glycerol gradient fractions indicated on top of each lane.

7–10 with some trailing up to fraction 15 (Figure 7B). Comparison of the sedimentation profile with size markers suggests an approximate mol. wt. of 30 000 for the debranching activity. In addition to the linearization of the IVS1 lariat, fractions 9–13 converted the lariat to the 240-nucleotide RNA which was previously observed when fractions of the first Mono Q column were tested for debranching activity (Figure 5C). The activity involved in this conversion sediments with a mol. wt. of ~40 000.

These results reveal that SF2 is a distinct activity that can be separated from the debranching enzyme by heparin-Sepharose chromatography. It behaves as a single component during anion exchange and affinity chromatography as well as during gel filtration and sedimentation through glycerol gradients. Therefore, we conclude that SF2 represents a single activity required for splicing *in vitro*.

*Characterization of SF2*

As mentioned above, SF2 activity can also be purified from a nuclear extract. Upon gradient elution from DEAE-Sepharose it is separated from three other fractions which are all required for *in vitro* splicing (Krämer and Keller, in preparation). During gel filtration and Mono Q chromatography nuclear SF2 shows

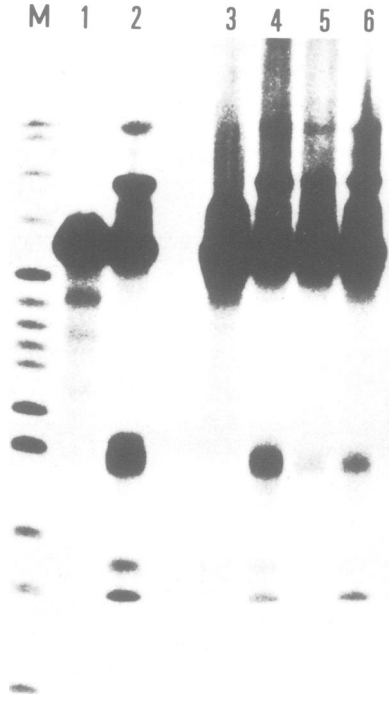


**Fig. 8.** Splicing in a system reconstituted with SF2 and fractions obtained from a nuclear extract. Standard splicing reactions were performed for 60 min in the absence of extract **(lane 1)**, or in the presence of 15 μl of nuclear extract **(lane 2)**. Reactions shown in **lanes 3–6** contained 5 μl of SF0, 10 μl of SF1 and 5 μl of SF3 (see text) plus 5 μl of DE-500 derived from a nuclear extract **(lane 4)**, 5 μl of DE-500 derived from the cytoplasmic fraction **(lane 5)** or 5 μl of the Mono QI-500 fraction **(lane 6)**.

the same behaviour as the cytoplasmic activity (data not shown). Figure 8 shows that SF2 represents an essential component of the splicing apparatus. When, in a reconstitution assay, SF0 (the activity recovered in the DE-FT from either nuclear or cytoplasmic extracts), SF1 (eluting from DEAE-Sepharose at 0.12–0.18 M KCl) and SF3 (eluting at 0.25–0.35 M KCl) are tested for splicing activity, neither intermediates nor final reaction products are generated (Figure 8, lane 3). Splicing activity is only recovered when these fractions are tested in the presence of SF2. This component can either be derived from the nuclear (lane 4) or from the cytoplasmic extract (lanes 5 and 6). Addition of the different SF2 fractions yields varying amounts of reaction products. This can be attributed to differences in the concentration of SF2 activity in the fractions tested. Similar reconstitution assays were performed with the glycerol gradient fractions containing SF2. Although this component was considerably diluted during sedimentation, all products of the splicing reaction are generated, however, at a lower level (not shown). From these results we infer that SF2 is one of the integral activities involved in pre-mRNA splicing. When it is missing from a system reconstituted with other components required for splicing, neither intermediates nor final reaction products are generated. This observation suggests that SF2 functions at an early stage of the splicing pathway.

Hernandez and Keller (1983) showed that splicing only occurs within a narrow temperature range. Treatment of nuclear extract at 46°C for 15 min totally inactivated the production of spliced RNA. Since the primer extension assay employed in those studies could only detect the final spliced RNA but not the intermediates and the IVS1 lariat, the experiment was repeated with $^{32}$P-labeled pre-mRNA, followed by direct gel analysis of the reaction

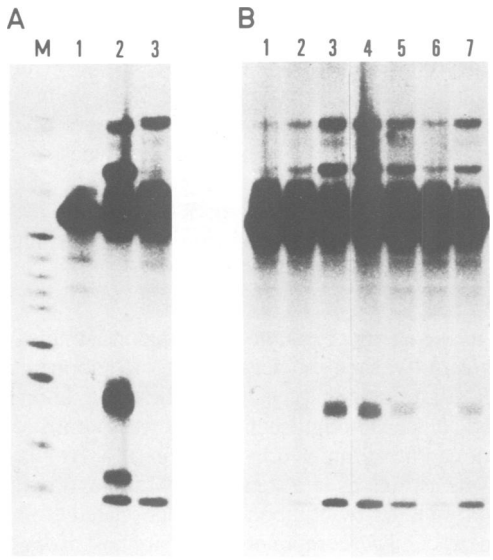


**Fig. 9.** Heat inactivation of the second step of the splicing reaction and characterization of SF2 activity. **(A)** Standard splicing reactions were performed for 60 min in the absence of extract **(lane 1)**, in the presence of 15 μl of nuclear extract **(lane 2)**, or in the presence of 15 μl of nuclear extract which had been pre-incubated at 46°C for 15 min **(lane 3)**. **(B)** Standard splicing reactions were performed for 60 min in the presence of 15 μl of washed nuclear extract. The reactions shown in **lanes 2–7** also contained 2 μl of DE-FT. SF2 activity was supplied by addition of 5 μl of HS-FT, which was either untreated **(lane 3)**, incubated at 46°C for 15 min **(lane 4)**, pre-treated with micrococcal nuclease **(lane 5)** or with NEM **(lane 6)**. In the reaction shown in **lane 7** NEM was inactivated with DTT prior to addition of SF2, DE-FT and extract. See Materials and methods for details.

products. As shown in Figure 9A (lane 3) treatment of the nuclear extract at 46°C for 15 min still allows the production of the splicing intermediates (IVS1-E2 and E1). However, neither the spliced product nor the excised IVS1 lariat are made. Thus, the first step of the splicing reaction is unaffected by the elevated temperature, indicating that heat-resistant components are responsible for the formation of the splicing intermediates. In contrast, a component(s) that can be inactivated by heat is required for the subsequent steps in the pre-mRNA pathway (see also Krainer and Maniatis, 1985). If SF2 activity was required at an early step, we would expect it to be resistant to mild heat treatment. Thus, partially purified SF2 (HS-FT fraction) was incubated at 46°C for 15 min and then tested in combination with washed nuclear extract and DE-FT. As shown in Figure 9B (lane 4), no loss of splicing activity is evident in this reaction, indicating that SF2 is resistant to mild heat treatment and is, therefore, a likely candidate for an activity required early during the splicing reaction.

U1 and U2 snRNPs are essential for *in vitro* splicing (Padgett *et al.*, 1983b; Krämer *et al.*, 1984; Black *et al.*, 1985; Krainer and Maniatis, 1985). Recent experiments suggest that these components associate with the pre-mRNA in the initial events of the splicing reaction (Frendewey and Keller, 1985; Grabowski *et al.*, 1985; Black *et al.*, 1985; B. Ruskin and M. Green, personal communication). Although neither U1 nor U2 snRNAs could be detected in the partially purified SF2 fractions by ethidium bromide staining of acrylamide gels, SF2 was treated with micrococcal nuclease to examine the possibility that an RNA species contributes to its activity. Figure 9B (lane 5) shows that SF2 activity is unaffected by micrococcal nuclease treatment, demonstrating that it is not associated with an active RNA moie-

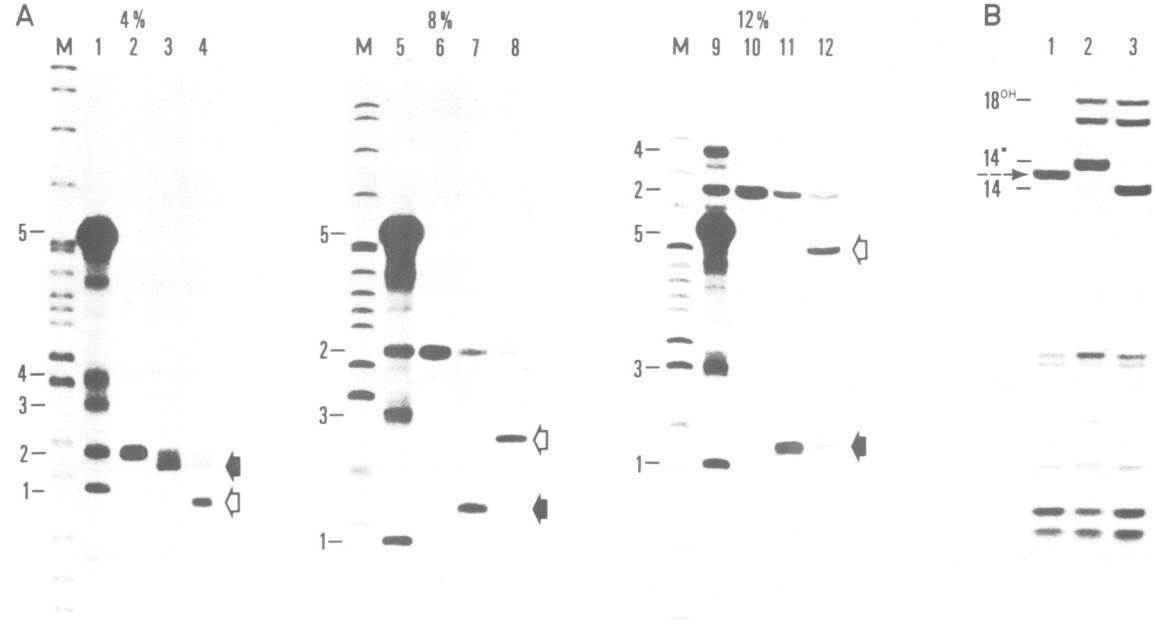


**Fig. 10.** Characterization of the 240-nucleotide RNA by gel analysis and RNase T1 digestion. **(A)** Splicing reactions were performed in the presence of nuclear extract for 30 min **(lanes 1, 5** and **9)**. IVS1 lariat was used as the substrate in debranching reactions in the presence of buffer B **(lanes 2, 6** and **10)** or in the presence of debranching enzyme (glycerol gradient fraction 8, see Figure 7B) **(lanes 3, 7** and **11)**, or nuclease (glycerol gradient fraction 10, see Figure 7B) **(lanes 4, 8** and **12)**. Incubation was for 15 min. Reaction products were separated in 4%, 8% or 12% acrylamide/8 M urea gels, respectively, as indicated above each panel. Numbers at the left side of each panel indicate exon 1 (1), IVS1 lariat (2), spliced product (3), IVS1-E2 lariat (4) and pre-mRNA (5). The closed arrows at the right side of each panel indicate the debranched IVS1, the open arrows the 240-nucleotide RNA. **(B)** The 240-nucleotide RNA **(lane 1)**, as well as the lariat **(lane 2)** and debranched IVS1 RNAs **(lane 3)** were eluted from a gel and digested with RNase T1 as described in Materials and methods. Reaction products were fractionated on a 20% acrylamide/8 M urea gel. Numbers on the left indicate the size of RNase T1 digestion products. For a description of oligonucleotides $18^{OH}$ and 14* see text. The arrow indicates the RNase T1 fragment specific for the 240-nucleotide RNA.

ty. (The slightly reduced splicing efficiency observed in this reaction is due to the presence of 0.8 mM EGTA.) Under the same experimental conditions splicing in a nuclear extract is totally abolished (data not shown; see Krainer and Maniatis, 1985).

The requirement for active sulfhydryl groups for SF2 activity was examined by treatment of the partially purified component with N-ethylmaleimide (NEM). Unreacted NEM was inactivated with an excess of dithiothreitol (DTT) before washed nuclear extract and DE-FT were added. Figure 9B (lane 6) shows that SF2 activity was abolished by NEM, indicating that sulfhydryl groups are essential for SF2 function in the splicing reaction. When NEM was inactivated by DTT before the addition of extract, DE-FT and HS-FT in a control reaction, no effect on splicing was observed (lane 7).

*Characterization of the 240-nucleotide RNA*

During the purification of SF2 we obtained fractions which are enriched in an activity that converts deproteinized lariat RNAs to a discrete product with an electrophoretic mobility of 240 nucleotides in a 12% acrylamide gel. Taking into account that treatment of both IVS1-E2 and IVS1 lariats with this activity yields the same smaller RNA species, it seemed that the 240-nucleotide RNA still contained the circular component of the IVS1 lariat. Its faster electrophoretic mobility as compared with the IVS1-E2 or IVS1 lariats is probably caused by a nucleolytic degradation of the linear sequences present in the lariat molecules. A similar product was observed upon incubation of a globin pre-mRNA in a HeLa cell nuclear extract (Ruskin *et al.*, 1984; Ruskin and Green, 1985a).

One feature of RNAs containing circular or branched components is their aberrant electrophoretic mobility in gels of different acrylamide concentrations (Grabowski *et al.*, 1984; Ruskin *et al.*, 1984). This property was used to confirm the circular nature of the 240-nucleotide RNA. In a 4% acrylamide gel the RNA produced by the nucleolytic activity migrates with 100 nucleotides (lane 4). Its mobility is disproportionately slower in 8% or 12% acrylamide gels (lanes 8 and 12). A similar behaviour is observed for the IVS1 lariat, whereas the linear form of IVS1 migrates as expected from its length (lanes 3, 7 and 11). From these results we conclude that the 240-nucleotide RNA is indeed a circular molecule. In addition to the 240-nucleotide RNA and a small amount of debranched IVS1 (due to some debranching activity in the fraction used) a very faint band migrating with 95–97 nucleotides in the 4% and 8% polyacrylamide gels, respectively, is visible in the original autoradiographs. The electrophoretic mobility of this debranched form of the 240-nucleotide RNA suggests that ~15–20 nucleotides are missing from the 3′ sequences of the IVS1 lariat.

To confirm the removal of sequences from the linear portion of the lariat RNA, gel-purified 240-nucleotide RNA was treated with RNase T1. This RNA (derived from the IVS1 lariat) lacks oligonucleotides $18^{OH}$ and 14* both of which are characteristic for the 3′ end of the excised IVS1 (Padgett *et al.*, 1984). This indicates that the nuclease has partially degraded the linear 3′ portion of the intron lariat. A new RNase T1 product migrating between fragments 14 and 14* is generated. Although we have not characterized this oligonucleotide in more detail, it could represent a 13-nucleotide RNase T1 fragment with the G residue derived from the 5′ end of the intron attached to the A residue at the branch point. In addition, the new fragment could terminate with a 3′ hydroxyl instead of a phosphate group, decreasing its electrophoretic mobility even further (as shown for fragments 18 and $18^{OH}$; see Figure 2 and Padgett *et al.*, 1984). If this is true, then the 240-nucleotide RNA can be defined as a circular molecule with a short linear portion of four nucleotides beyond the branch point.

Thus, along with the fractionation of SF2, we have partially purified the lariat debranching enzyme and a nucleolytic activity which removes linear sequences from both types of lariat molecules. This activity probably corresponds to the nuclease previously observed in nuclear extracts (Ruskin *et al.*, 1984; Ruskin and Green, 1985a) and is different from the debranching enzyme.

## Discussion

We have used a complementation system consisting of an extract prepared from 'washed' nuclei to test for components involved in pre-mRNA splicing that leak out of the nuclei upon hypotonic lysis of the cells. Although such an extract is almost completely inactive, it can be stimulated by addition of a cytoplasmic fraction to levels as high as in a standard splicing extract. Likewise, it can be stimulated by components fractionated from the cytoplasmic extract. The components that are lost from the nuclei during extract preparation are present in the untreated nuclear extract in excess, since addition of the cytoplasmic fraction in this case does not result in a further increase in splicing efficiency. The fact that either SF2 or DE-FT stimulate splicing in the washed extract to a small extent shows that this extract contains both components, however, in limiting quantities. Only when SF2 and DE-FT are added in combination, can optimal splicing be restored. In the absence of added SF2 or DE-FT the generation of all splicing products is decreased, indicating that components required for the first step of the splicing reaction are limiting. It is unlikely that U1 and U2 snRNPs, which have been shown to be required at an early stage of pre-mRNA splicing (Black *et al.*, 1985; Frendewey and Keller, 1985), are responsible for the observed stimulation, since the washed nuclear extract contains substantial quantities of these particles. Therefore, we assume that other components essential for the first splicing events are present in the cytoplasmic fraction.

The use of the complementation assay has helped in the purification of a component (SF2) which is necessary for *in vitro* splicing. SF2 is a protein, as demonstrated by its sensitivity to NEM. It behaves as a single component during all of the purification steps employed here. SF2 is not associated with an essential RNA moiety, since its activity cannot be inhibited by treatment with micrococcal nuclease, which totally abolishes splicing in a nuclear extract (data not shown; see Krainer and Maniatis, 1985). Therefore, SF2 is distinct from U1 or U2 snRNP. If SF2 is missing from a system reconstituted with components fractionated from a nuclear extract (Krämer and Keller, in preparation), neither intermediates nor final splicing products are formed, suggesting a function of SF2 at an early stage in pre-mRNA splicing. This idea is supported by two observations. First, the production of splicing intermediates is resistant to mild heat treatment and SF2 is stable under the same conditions; second, the production of intermediates is increased when additional SF2 is combined with a heat-treated extract. Only upon addition of a different fraction does 3′ cleavage and exon ligation occur (Krämer and Keller, in preparation).

Although it is clear that SF2 represents an essential factor of the *in vitro* splicing apparatus, the mechanism of its action is not known. Because of its apparent requirement early in the reaction, it may function in the assembly of the pre-mRNA into a complex competent for splicing. For example, it has been proposed that protein factors may be binding to intron sequences near the 3′

splice site in conjunction with U2 snRNPs (Black *et al.*, 1985; Ruskin and Green, 1985c). Alternatively, SF2 could be involved directly or indirectly in the catalysis of the cleavage-ligation reaction that leads to the formation of the IVS1-E2 lariat. The finding that SF2 is required early in the splicing pathway, however, does not exclude a function in a later step as well.

At this point we are unable to correlate SF2 activity with splicing components identified in HeLa cell extracts by other laboratories. On the basis of the criteria used by Krainer and Maniatis (1985) to define splicing components (the sensitivity to micrococcal nuclease or heat treatment and the distribution of components between nuclear and cytoplasmic fractions), SF2 seems to be different from the activities they described. Although these authors also report the fractionation of splicing activities, a direct comparison cannot be made due to different chromatographic procedures employed. Furneaux *et al.* (1985) recently reported the separation of a nuclear extract into two fractions which, when combined, are active in splicing. Since this system is still rather impure, it is not possible to make predictions about the presence of SF2 in either of the two fractions.

Ruskin and Green (1985a) have proposed that the lariat debranching enzyme may be involved in pre-mRNA splicing. We have demonstrated that SF2 can be chromatographically separated from this enzyme, but we cannot rule out a role for this activity in the splicing process. Although the debranching enzyme leaks into the cytoplasmic fraction during cell lysis, the extract prepared from washed nuclei still contains considerable amounts of this activity (not shown). Likewise, a splicing system reconstituted with components fractionated from a nuclear extract is not completely devoid of the debranching enzyme. However, since we have never observed a stimulation of splicing in our reconstitution system upon addition of the purified debranching enzyme, we consider it unlikely that this enzyme is required in the splicing of pre-mRNA.

In contrast to the results of Ruskin *et al.* (1984), who did not observe any debranching in a splicing reaction containing unfractionated nuclear extract, we detect the linear IVS1 species as a late product in our *in vitro* system. From their observations Ruskin and Green (1985a) argued that the branch point in the IVS1 lariat is protected from debranching by a component(s) binding to this region. A protection of the pre-mRNA in the 3′ portion of the intron including the branch point has recently been shown (Black *et al.*, 1985; Ruskin and Green, 1985c). It is unclear, however, whether this component(s) stays associated with the intron after lariat formation occurs. The presence of the excised IVS1 lariat in large splicing complexes (Frendewey and Keller, 1985; Grabowski *et al.*, 1985) indicates an association of this RNA species with components of the nuclear extract. However, when comparing the distribution of the IVS1 lariat in sucrose or glycerol gradients with the migration of the other reaction products, it is apparent that part of the IVS1 lariat sediments with a lower S-value (Frendewey and Keller, 1985; Grabowski *et al.*, 1985). It is possible that the slower sedimenting IVS1 lariats represent those molecules that are released from the splicing complex. The released IVS1 lariats could then become debranched in the nuclear extract. Debranched intron RNA has never been detected in the splicing complexes, indicating that these reaction products are no longer associated with components that bind to the intron lariat.

During the purification of the splicing factor we enriched an activity that converts the deproteinized lariat RNAs to a distinct new species which migrates with an electrophoretic mobility of 240 nucleotides in a 12% acrylamide gel. We demonstrated that this RNA retains the circular component of the IVS1 lariat and ~4 nucleotides of the linear portion of the excised intron beyond the branch point, suggesting that it is derived by the action of a nuclease. Although we have not characterized this activity further, we assume that it acts in a 3′ to 5′ exonucleolytic fashion, since the IVS1 lariat is converted to the new RNA species considerably faster than the larger IVS1-E2 lariat. In addition, we have failed to detect any RNA species corresponding in size to a fragment expected after an endonucleolytic cleavage. We did not observe any degradation of the pre-mRNA or other linear RNAs by the purified nuclease under conditions where >50% of the IVS1 lariat is converted (not shown). This observation raises the possibility that the nucleolytic activity acts specifically on lariat-containing structures.

An intron lariat which is missing ~10 nucleotides from its 3′-terminal sequences is generated during the splicing reaction from a globin pre-mRNA upon prolonged incubation in a HeLa cell nuclear extract (Ruskin and Green, 1985a). Since the branch point in the globin intron is located 37 nucleotides upstream of the 3′ splice site (Ruskin *et al.*, 1984), a considerable portion of the linear part of IVS1 is left intact in this molecule, suggesting a protection from nuclease attack in the presence of other nuclear components (Ruskin and Green, 1985a). Our finding that the intron sequences are degraded almost up to the branch point can be explained by the use of deproteinized lariat RNA and a partially purified nuclease. Thus, this activity might be useful to monitor the protection of intron sequences by nuclear components in partially purified systems.

Products similar to the 240-nucleotide RNA and linear intron RNAs have also been detected *in vivo* (Domdey *et al.*, 1984; Rodriguez *et al.*, 1984; Zeitlin and Efstratiadis, 1984). Therefore, the nucleolytic activities described here might be responsible for the degradation of intron lariats which would otherwise accumulate in the cell nucleus.

In conclusion, we have isolated a protein factor that participates in the early events of the *in vitro* splicing reaction. SF2 represents one of a number of proteins that are required in addition to snRNPs for the splicing of pre-mRNAs. The use of systems which examine binding to the pre-mRNA substrate or splicing complex formation, as well as the development of different complementation assays, should make it possible to purify all the components involved in the splicing reaction and to determine the mechanism of their action.

## Materials and methods

*Preparation of extracts*

HeLa cells were grown as described (Hernandez and Keller, 1983). Nuclear and cytoplasmic extracts were prepared according to Dignam *et al.* (1983). Extracts from washed nuclei were prepared as follows: the crude nuclear pellets obtained after low-speed centrifugation (see Dignam *et al.*, 1983) were resuspended in two original cell volumes of 10 mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT and homogenized and centrifuged as before. The nuclear pellet obtained was designated 'washed nuclei'.

*Fractionation*

All steps were carried out at 4°C. Buffers used for DEAE-Sepharose and Superose 6 chromatography contained 0.5 mM phenylmethylsulfonyl fluoride (PMSF).

*DEAE-Sepharose chromatography.* DEAE-Sepharose (Pharmacia, 15 × 2.5 cm) was equilibrated with buffer A [10% (v/v) glycerol; 20 mM Hepes-KOH, pH 7.9; 3 mM $MgCl_2$; 0.1 mM EDTA; 0.5 mM DTT] + 100 mM KCl. Cytoplasmic extract (120 ml, 5.3 mg protein/ml, corresponding to $3.2 \times 10^{10}$ cell equivalents) was loaded at 1.5 column volumes/h. The column was washed with 100 ml buffer A + 100 mM KCl. Bound proteins were step-eluted with buffer A + 500 mM KCl. Fractions of 5 ml were collected for flow-through and wash fractions; during elution the fraction size was 3 ml. Protein-containing flow-through fractions were pooled and frozen (DE-FT). The step fractions (DE-500) were

dialyzed individually against three changes of buffer B [same as buffer A but 20% (v/v) glycerol] + 100 mM KCl. The fractions were tested for splicing and debranching activity and stored at −80°C.

DEAE-Sepharose chromatography of a nuclear extract was carried out as above, except that proteins were eluted with a linear salt gradient of 100−500 mM KCl (Krämer and Keller, in preparation).

*Ammonium sulfate precipitation.* DE-500 fractions active in splicing (containing 150 mg of protein) were pooled and precipitated by addition of 0.44 g $AmSO_4$/ml. The pH was adjusted to 7.5 with 1 N NaOH. After stirring for 60 min the precipitate was collected by centrifugation in a SW 60 rotor (Beckmann) for 30 min at 30 000 r.p.m. The pellets were dissolved in one-tenth the original volume of buffer B + 100 mM KCl. This DE-AS fraction was stored at −80°C. An aliquot was dialyzed against buffer B + 100 mM KCl and tested for splicing and debranching activity.

*Superose 6 gel filtration.* A Superose 6 column (Pharmacia, 55 × 1.5 cm), equilibrated with buffer C (same as buffer A but 50 mM Tris-HCl, pH = 7.9) + 100 mM KCl, was loaded with 1 ml of the DE-AS fraction (50 mg of protein). This corresponds to about half of the material obtained after concentration of the DE-500 fraction. The column was run at 15 ml/h and 1 ml fractions were collected. Splicing and debranching assays were performed and the fractions were frozen at −80°C (Sup 6 fraction).

*Mono Q chromatography.* A Mono Q column (FPLC-system, Pharmacia, 1 ml) was equilibrated with buffer C + 100 mM KCl. Superose 6 fractions active in splicing (21 mg of protein) were loaded and the column was run at 60 ml/h resulting in a pressure of 3.5 MPa. Unbound proteins were removed with a 10 ml wash of buffer C + 100 mM KCl, followed by a 30 ml gradient of buffer C + 100 to 500 mM KCl to elute bound proteins. Fractions of 0.5 ml were collected during elution. Protein-containing fractions were dialyzed against three changes of buffer D (same as B but 50 mM Tris-HCl, pH 7.9) + 20 mM KCl (Mono QI fraction).

*Heparin-Sepharose chromatography.* Heparin-Sepharose (Pharmacia, 1 ml) was equilibrated with buffer C + 20 mM KCl. Mono QI fractions active in splicing were pooled (2.24 mg of protein) and loaded onto the column at 2.5 column volumes/h. After washing with four column volumes of buffer C + 20 mM KCl (HS-FT), bound proteins were eluted with the same buffer containing 500 mM KCl (HS-500). Fractions of 0.5 ml were collected.

*Second Mono Q chromatography.* The HS-FT fractions were pooled (1.28 mg of protein) and loaded immediately onto a second Mono Q column (see above) which was equilibrated with buffer E [same as buffer C but 5% (v/v) glycerol] + 20 mM KCl. The column was run at 60 ml/h. After a four column volume wash with buffer E + 20 mM KCl, proteins were eluted with buffer E + 500 mM KCl. Fractions of 0.4 ml were collected (Mono QII fraction).

*Glycerol gradients*

Gradients of 4 ml containing 15−30% (v/v) glycerol, 50 mM Tris-HCl, pH 7.9, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 0.5 mM DTT were loaded with 100 μl of the Mono QII fraction (130 μg of protein) or the HS-500 fraction (140 μg of protein), respectively. The gradients were run at 4°C in a SW 60 rotor (Beckmann) for 21.5 h at 55 000 r.p.m. Fractions of 190 μl were collected from the top of the gradients. The fractions were tested immediately for splicing and debranching activity and stored at −80°C.

*Preparation of [$^{32}P$]pre-mRNA*

*In vitro* transcription of the SP6 template was performed in a cap-primed reaction (Contreras *et al.*, 1982; Georgiev *et al.*, 1984) as described by Melton *et al.* (1984). RNA was synthesized from 1 μg of plasmid pSP62Δi1 (Frendewey and Keller, 1985) which was linearized at a *Sca*I site within the second exon. Reaction conditions were as follows: 50 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 500 μM each of ATP and CTP, 100 μM GTP, 20 μM UTP, 2 mM G(5′)ppp(5′)G (P-L Biochemicals), 10 μCi of [α-$^{32}$P]UTP (Amersham Buchler), 5 units SP6 polymerase (Renner, Dannstadt, FRG) and 32 units RNasin (Promega Biotec). Transcripts were purified and stored as described by Frendewey and Keller (1985).

*In vitro splicing*

The standard splicing reaction contained, in a volume of 50 μl, 0.4 mM ATP, 10 mM creatine phosphate, 1.5 mM $MgCl_2$, 10% (v/v) glycerol, 10 mM Hepes-KOH, pH 7.9 (or 25 mM Tris-HCl, pH 7.9, depending on the fractions tested), 50 mM KCl, 0.05 mM EDTA, 0.25 mM DTT, 0.2 mg/ml tRNA, 16 units RNasin and the amount of extract or column fractions indicated in the figure legends. In the reconstitution experiment shown in Figure 8 the $MgCl_2$ concentration was 2.5 mM [$^{32}$P]Pre-mRNA substrate was used at 10 000 Cerenkov c.p.m./reaction (~0.1 nM). Reactions were performed for 60 min at 30°C, unless stated otherwise. Proteinase K treatment, phenol extraction and precipitation of the RNA were as described (Hernandez and Keller, 1983). Reaction products were fractionated on 12% acrylamide/8 M urea gels. [$^{32}$P]RNA was visualized by autoradiography.

Heat treatment of nuclear extract or column fractions was done at 46°C for 15 min. Micrococcal nuclease digestion was performed for 5 min at room temperature in a 7 μl reaction containing 5 μl of HS-FT (in buffer C + 20 mM KCl), 22.5 units of micrococcal nuclease (Serva) and 1 mM $CaCl_2$. The enzyme was inhibited with 5 mM EGTA before the remaining constituents of the complete splicing reaction were added (see above). NEM treatment was performed by incubation of SF2 (HS-FT fraction) in the presence of 5 mM NEM for 5 min on ice. Unreacted NEM was inactivated with 10 mM DTT before addition of the remaining constituents of the splicing reaction (see above). In a control reaction NEM was inactivated with DTT prior to addition of SF2.

*Debranching assay*

For the production of the substrate lariats the splicing reaction was scaled up, the reaction products were separated on a 12% acrylamide gel, and the lariats were eluted from gel slices as described by Frendewey and Keller (1985). Debranching reactions contained, in a total volume of 25 μl, 10% (v/v) glycerol, 10 mM Hepes-KOH, pH 7.9 (or 25 mM Tris-HCl, pH 7.9, depending on the fractions tested), 50 mM KCl, 0.6 mM $MgCl_2$, 0.05 mM EDTA, 0.25 mM DTT, and 5 μl of extract or column fractions. As a substrate either the IVS1-E2 or the IVS1 lariat was used at a concentration of 20−30 pM (~500 Cerenkov c.p.m./reaction). Incubation was for 15 min at 30°C. Reactions were stopped by proteinase K treatment and products were extracted as described (Hernandez and Keller, 1983). Reaction products were electrophoresed as above.

*RNase T1 digestion*

Gel-purified RNA was digested for 15 min at 37°C with 20 U of RNase T1 (Boehringer Mannheim) in 4 μl of 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA containing 20 μg of carrier tRNA. RNase T1 digestion products were fractionated on a 20% acrylamide/8 M urea sequencing gel.

*Protein determination*

The protein concentration of extracts and pooled fractions was determined according to Bradford (1976).

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**Note added in proof**

We would like to point out that although the splicing factors we have described coincidentally have the same designations as those employed by Krainer and Maniatis (1985), this does not necessarily imply physical or functional identity.