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Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells

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SF2, an activity necessary for 5' splice site cleavage and lariat formation during pre-mRNA splicing *in vitro*, has been purified to near homogeneity from HeLa cells. The purest fraction contains only two related polypeptides of 33 kD. This fraction is sufficient to complement an S100 fraction, which contains the remaining splicing factors, to splice several pre-mRNAs. The optimal amount of SF2 required for efficient splicing depends on the pre-mRNA substrate. SF2 is distinct from the hnRNP A1 and U1 snRNP A polypeptides, which are similar in size. Endogenous hnRNA copurifies with SF2, but this activity does not appear to have an essential RNA component. SF2 appears to be necessary for the assembly or stabilization of the earliest specific prespliceosome complex, although in the absence of other components, it can bind RNA in a nonspecific manner. SF2 copurifies with an activity that promotes the annealing of complementary RNAs. Thus, SF2 may promote specific RNA–RNA interactions between snRNAs and pre-mRNA, between complementary snRNA regions, and/or involving intramolecular pre-mRNA helices. Other purified proteins with RNA annealing activity cannot substitute for SF2 in the splicing reaction.

Splicing of individual introns in eukaryotic pre-mRNAs takes place by a sequential two-step cleavage–ligation reaction, which has been reproduced *in vitro* (for reviews, see Green 1986; Padgett et al. 1986; Krainer and Maniatis 1988). In mammalian cell-free systems pre-mRNA splicing has been shown to require multiple nucleoprotein and protein factors (Krämer et al. 1984, 1987; Black et al. 1985; Krainer and Maniatis 1985; Krämer and Keller 1985; Berget and Robberson 1986; Black and Steitz 1986; Perkins et al. 1986; Krainer 1988; Winkelmann et al. 1989). These splicing factors include the major nucleoplasmic small nuclear ribonucleoprotein particles known as U snRNPs (Krämer et al. 1984; Krainer and Maniatis 1985; Black et al. 1985; Berget and Robberson 1986; Black and Steitz 1986; Krainer 1988; Winkelmann et al. 1989), which are composed of one or two U snRNAs and several common and unique polypeptides (for reviews, see Lührmann 1988; Reddy and Busch 1988). The snRNPs have been purified extensively in their active forms (Krainer 1988; Winkelmann et al. 1989). Additional protein factors are also required for splicing (Krainer and Maniatis 1985; Krämer and Keller 1985; Perkins et al. 1986; Krämer et al. 1987), but until now, none of these factors has been purified to homogeneity in an active form; thus, their identities and modes

of action are unknown. In addition, several HeLa cell polypeptides have been described and, in some cases, purified, that interact with RNA specifically and/or are present in spliceosomes and are thus potential splicing factors (Choi et al. 1986; Gerke and Steitz 1986; Sierakowska et al. 1986; Tazi et al. 1986; Kumar et al. 1987; Swanson and Dreyfuss 1988; García-Blanco et al. 1989; Zamore and Green 1989; Fu and Maniatis 1990).

The snRNA components of U1 and U2 snRNPs base-pair to complementary sequences within conserved splicing signals on pre-mRNA, and these interactions have been shown to play a fundamental role in splice site recognition (Zhuang and Weiner 1986, 1989; Parker et al. 1987; Seraphin et al. 1988; Siliciano and Guthrie 1988; Wu and Manley 1989). In addition, these and other snRNAs could potentially act as ribozymes, although it is not known at present whether RNA catalysis is involved in nuclear pre-mRNA splicing. Several of the protein factors mentioned above bind selectively to polypyrimidine-rich RNA and thus appear to be specific for pre-mRNA 3' splice sites (Choi et al. 1986; Gerke and Steitz 1986; Tazi et al. 1986; Kumar et al. 1987; Swanson and Dreyfuss 1988; García-Blanco et al. 1989; Zamore and Green 1989). In theory, one or more of these factors can be involved in 3' splice site recognition, as well as in interactions with the usually adjacent branch site. It remains to be determined which of these RNA

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and protein splicing factors contribute enzymatic activities that are important for splicing and which factors contain the active sites for the catalysis of the RNA cleavage–ligation reactions. These fundamental questions must be elucidated to provide a detailed understanding of the mechanism of pre-mRNA splicing and to deduce the origin of pre-mRNA introns and their possible evolutionary relations to self-splicing introns.

To identify the factors that are absolutely required for pre-mRNA splicing in vitro, we and others have employed complementation assays in which one or both cleavage–ligation reactions are restored upon addition of a purified or partially purified fraction (Krainer and Maniatis 1985; Krämer and Keller 1985; Perkins et al. 1986; Krämer et al. 1987; Krainer 1988; Winkelmann et al. 1989). Although this type of functional assay is tedious, we note that purification of factors on the basis of RNA binding or complex formation does not demonstrate a priori that such factors are essential for splicing. This is so even in cases in which RNA-binding proteins are involved in the formation of RNP complexes that behave as intermediates in the splicing pathway. A subset of such proteins could be involved in stabilizing the complexes so that they are detectable, but they do not necessarily play a direct role in splicing. In this study, we report the isolation and properties of an active protein splicing factor from HeLa cells. This factor, SF2, is shown to be an essential splicing factor required for cleavage at the 5' splice site and for lariat formation.

Results and discussion

Purification of SF2

Previous work demonstrated that the cytoplasmic S100 fraction obtained during the preparation of nuclear extracts (Dignam et al. 1983) contains active snRNPs and other splicing factors but is deficient in at least one protein activity, termed SF2, which is necessary for the first cleavage–ligation of pre-mRNA splicing (Krainer and Maniatis 1985). The reason for the selective retention of SF2 in the nuclear fraction in a hypotonic buffer is not known. It may be that the polypeptide(s) responsible for SF2 activity are tightly bound to the nuclear membrane

or to a subnuclear structure. Alternatively, SF2 could be part of large RNP complexes that cannot traverse the nuclear pores in an intact form. In fact, SF2 activity and other activities necessary for splicing cosediment with endogenous RNP particles and can be released by preincubation with ATP (Conway et al. 1989). To facilitate the purification of SF2 we developed a new extraction procedure, which maximizes the recovery of SF2, as judged by titration of the extracts against a constant amount of complementing S100 fraction (see Materials and methods). From this extract, SF2 has been purified by ammonium sulfate precipitation, Sephacryl S200-HR gel filtration chromatography, Mono Q anion exchange chromatography, and Phenyl-Superose hydrophobic interaction chromatography (Table 1; Figs. 1 and 2; Materials and methods).

The activities and polypeptide composition of the various chromatographic fractions are shown in Figures 1 and 2. The elution profile for the Phenyl-Superose column is shown in Figure 1. In the first active fraction, (Fig. 1A, fraction 9), only a doublet of 33 kD is detectable by Coomassie Blue (Fig. 1B) or silver staining (not shown). Complementing activity peaked in fraction 10 (Fig. 1A), which also corresponds to the 33-kD doublet peak (Fig. 1B). This fraction also contains several additional high-molecular-weight polypeptides. Complementing activity is also detectable in fractions 11–13 (Fig. 1A), although it did not correlate well with the abundance of the 33-kD doublet (Fig. 1B). A second peak of activity is centered in fraction 22 (Fig. 1A), which contains, among other minor bands, a polypeptide that comigrates precisely with the top band of the 33-kD doublet (Fig. 1B; data not shown). On the basis of these data, particularly the presence of complementing activity in fraction 9, we conclude that one or both of the 33-kD polypeptides possesses SF2 activity. At this stage we cannot rule out the existence of very minor polypeptides that are responsible for activity, although none were detected by silver staining. The reason for the distribution of SF2 activity into two well-separated peaks is not known.

The gel assay for splicing is not easily amenable to quantitation; thus, we cannot calculate precisely the overall purification of SF2. However, when SF2 is added in limiting amounts in the complementation assay, the

Table 1. *Purification of SF2*

Fraction	Volume (ml)	Protein (mg)[a]	Cumulative yield (%)[b]	Cumulative purification
I Crude extract	44	715	100	1
II Ammonium sulfate	4	80	40	4
III Sephacryl S200-HR	10	15	30	14
IV Mono Q	2	10	25	18
V Phenyl-Superose #9	1	0.03	5	1200

[a]Protein concentrations were determined by the method of Bradford, in the linear range of dye binding, using BSA as a standard (Bradford 1976).
[b]The approximate yield of SF2 activity was determined by the comparison of the extent of splicing in complementation assays with serial twofold dilutions of each fraction.
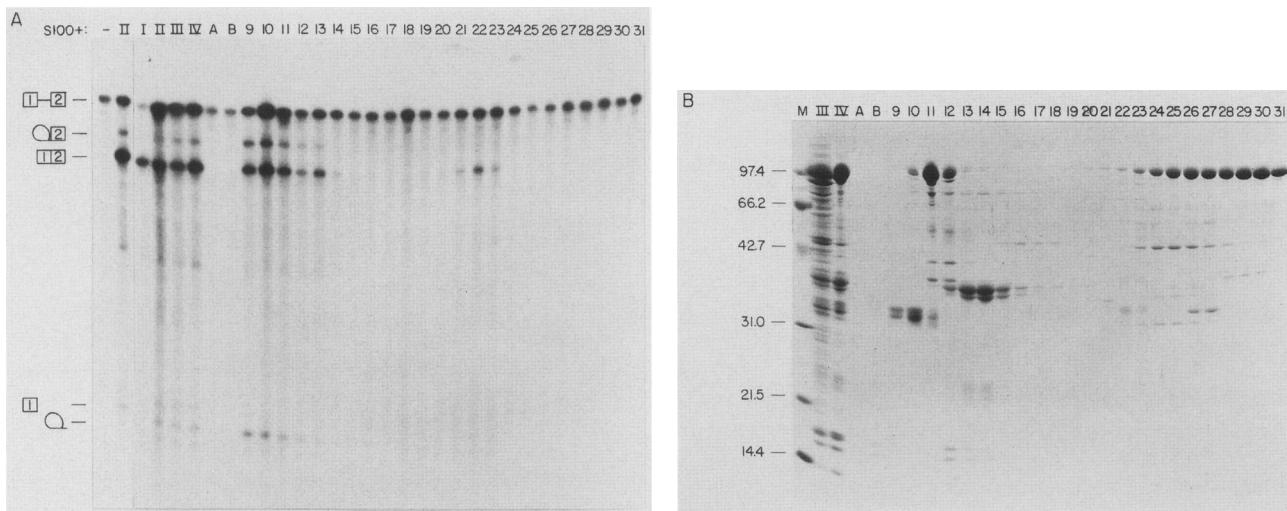
Figure 1. Purification of SF2. The Roman numerals denote pooled, active fractions from: crude extract (I); ammonium sulfate step (II); Sephacryl S200 HR step (III); Mono Q step (IV). The numbers 9–31 correspond to the Phenyl-Superose fractions; A and B are pooled flowthrough fractions 1–4 and 5–8, respectively. (*A*) *SF2 complementation assay.* Splicing reactions were carried out with 7.5 μl of the S100 extract supplemented with either 7.5 μl of the indicated Phenyl-Superose fractions or with 3.75 μl of I, 3 μl of II, 7.5 μl of III, or 1 μl of IV. The positions of the β-globin pre-mRNA, intermediates, and products of splicing are indicated at *left.* (*B*) *Polypeptide composition.* The proteins in the indicated fractions were analyzed by SDS-PAGE and Coomassie Blue staining. (M) Molecular weight markers; (III) 10 μl of fraction III; (IV) 2.5 μl of fraction IV; the remaining lanes contain 10 μl of the indicated fractions.

amount of spliced mRNA generated is roughly proportional to the concentration of SF2. Titration of the SF2-containing fractions against a constant amount of S100 can give an indication of the concentration of SF2 in each fraction. However, because the complementation assay involves addition of a crude S100 extract, these estimates are affected in an unpredictable manner by nonspecific protein–RNA and protein–protein interactions. Furthermore, the yield of spliced mRNA is affected by the presence of ribonucleases in the S100 extract and in the partially purified nuclear fractions. Often, when splicing is inefficient, the pre-mRNA is susceptible to degradation, suggesting that spliceosome formation protects the pre-mRNA from endogenous nucleases. With these caveats in mind, it is nevertheless useful to estimate the yield of SF2 during the various purification steps (Table 1; Fig. 2). There is roughly a 24,000-fold reduction in protein, when comparing the crude extract to the Phenyl-Superose fraction 9 (Table 1). Serial 2-fold dilutions of the complementing fractions indicate that ~5% of the initial SF2 is recovered in fraction 9, for an overall purification of 1200-fold. The overall yield of SF2 activity is ~20%, when the other active Phenyl-Superose fractions are considered (Fig. 1; data not shown).

A small amount of endogenous heterogeneous nuclear RNA (hnRNA) copurified with the SF2 polypeptides (see below), but SF2 activity does not appear to require an essential RNA component, as judged by its resistance to micrococcal nuclease treatment (Krainer and Maniatis 1985). Following inactivation of the nuclease by calcium chelation, purified SF2, like crude SF2, was fully active in the complementation assay (data not shown). In contrast, SF2 activity was susceptible to papain digestion. Following inactivation of the protease with a specific in-

hibitor, digested SF2 failed to complement the S100, whereas preinactivated papain had no effect (data not shown).

The similarity in the apparent molecular weights of the SF2 polypeptides and those reported for the hnRNP A1 (for review, see Dreyfuss et al. 1988) and for the U1 snRNP A polypeptides (for review, see Lührmann 1988) prompted us to compare them by SDS-PAGE analysis (Fig. 2, lanes 6–9). Both SF2 polypeptides are slightly smaller than hnRNP A1 and slightly larger than A, both derived from HeLa cells. The true molecular masses of A1 and A are 34,214 and 31,243 daltons, respectively, as derived from their cDNA sequences (Sillekens et al. 1987; Cobianchi et al. 1988). In addition, the SF2 polypeptides did not react in a Western blot analysis, using an anti-hnRNP antiserum that recognizes all the core hnRNP proteins (data not shown).

SF2 is a general splicing factor

The complementation assay employed for the purification of SF2 relied exclusively on a pre-mRNA substrate containing the first two exons and first intron of human β-globin. It was important to determine whether the requirement for SF2 is substrate dependent, and to this end, several different pre-mRNAs were spliced in vitro. An example of this is shown in Figure 3. A human immunodeficiency virus-1 (HIV-1) pre-mRNA, containing the coding exons of the *tat* gene and a truncated intron, was completely dependent on SF2 for splicing (lanes 5 and 6). In the S100 extract alone, no specific cleavage related to splicing was observed (lane 6). Unlike the β-globin pre-mRNA, which spliced equally well in the reconstituted system and in the nuclear extract (lanes 1
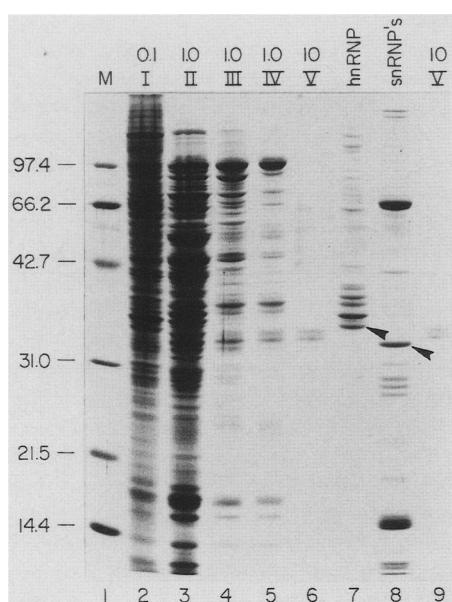
Figure 2. SDS-PAGE analysis of SF2 fractions and HeLa RNPs. (Lane *1*) Molecular weight markers (M); (lane *2*) crude extract, $0.1\times$ ($1\times = 2.4 \times 10^7$ cell equivalents); (lane *3*) ammonium sulfate active fraction, $1\times$; (lane *4*) Sephacryl S200 HR pooled active fraction, $1\times$; (lane *5*) Mono Q pooled active fractions, $1\times$; (lanes *6* and *9*) Phenyl-Superose fraction 9, $10\times$; (lane *7*) purified HeLa cell hnRNP particles (0.03 A_{260} units); (lane *8*) purified HeLa cell U snRNP particles (1.25 $\times 10^8$ cell equivalents). hnRNP A1 and U1 snRNP A polypeptides are indicated by arrowheads.

and 2), the HIV pre-mRNA was spliced much more efficiently in the reconstituted system (lanes 4 and 5). When nuclear extract was supplemented with additional purified SF2, there was no effect on the splicing efficiency of the β-globin pre-mRNA, whereas the HIV pre-mRNA was spliced as efficiently as with S100 and SF2 (data not shown). Therefore, higher amounts of SF2 are required for efficient splicing of the HIV pre-mRNA than for the β-globin pre-mRNA.

The inefficient splicing of the HIV pre-mRNA in nuclear extracts may reflect a general property of retroviral transcripts which, unlike most cellular pre-mRNAs, are spliced inefficiently to provide unspliced transcripts that are required for viral replication, packaging into virions, and expression of several proteins (for review, see Weiss et al. 1982). Retroviral *cis*-acting sequences appear to be responsible for inefficient splicing (Arrigo and Beemon 1988; Stoltzfus and Fogarty 1989; Katz and Skalka 1990). Our data suggest that in some cases, this type of negative *cis*-acting element may relate to the function of SF2.

In addition to these substrates, SF2 was found to be required for splicing both introns of rabbit β-globin pre-mRNA (data not shown) and several introns from a rat β-tropomyosin pre-mRNA (D. Helfman and A.R. Krainer, unpubl.). We were unable to demonstrate a requirement for SF2 to splice the adenovirus major late first intron, due to the instability of this pre-mRNA in the S100 extract in the absence of SF2 (data not shown).

This pre-mRNA was stable in the reconstituted system, but it was spliced less efficiently than with nuclear extract.

SF2 is required for spliceosome formation

The complementation assay employed for the purification of SF2 indicates that this activity is required for cleavage of pre-mRNA at the 5′ splice site and lariat formation. This reaction is normally preceded by the assembly of specific prespliceosome and spliceosome complexes, in which pre-mRNA interacts in a stable manner with multiple components of the splicing apparatus, such as the U snRNPs (for reviews, see Green 1986; Padgett et al. 1986; Krainer and Maniatis 1988). To determine the requirement for SF2 in this assembly pathway, RNP complexes were analyzed by a gel-retardation assay (Fig. 4). Under these electrophoretic conditions, three specific complexes are normally detected (Fig. 4, lane 2; Zillman et al. 1988). When pre-mRNA was incubated with the S100 extract under splicing conditions, no specific complexes were detected (lane 3). Incubation of pre-mRNA with purified SF2 also failed to generate specific complexes of discrete mobility (lane 4). The smear detected under these conditions is consistent with nonspecific RNA binding by SF2 (see below). When pre-mRNA was incubated with purified SF2 and S100, three discrete complexes were detected, which have the
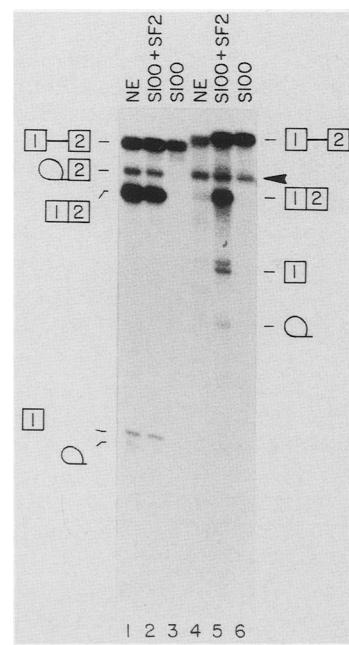

Figure 3. SF2-dependent splicing of two different pre-mRNAs. Human β-globin pre-mRNA (lanes *1–3*), or an HIV-1 pre-mRNA derived from the *tat* region (lanes *4–6*) were spliced with the indicated extracts and purified SF2, and the products were analyzed by urea–PAGE. The structures of the precursors, intermediates, and products of splicing are displayed on each side of the autoradiograph. The band indicated by the arrowhead above HIV mRNA is not a lariat intermediate but a truncated pre-mRNA arising from artifactual cleavage.
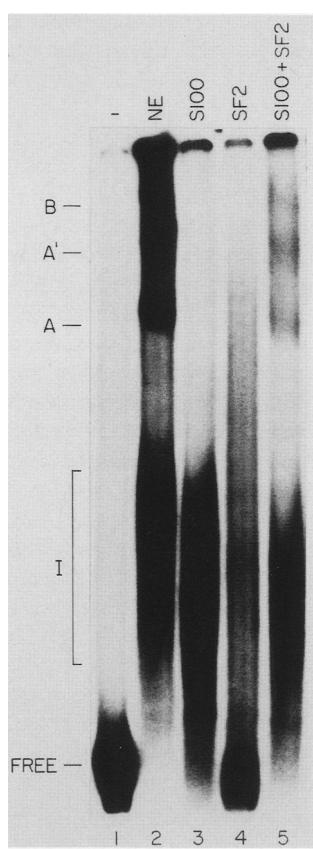
Figure 4. Spliceosome assembly. Human β-globin pre-mRNA was incubated for 45 min under splicing conditions, with the indicated fractions. The resulting complexes were analyzed by native gel electrophoresis, as described (Zillman et al. 1988). (Lane 1) Pre-mRNA alone; (lane 2) nuclear extract; (lane 3) S100 extract; (lane 4) purified SF2; (lane 5), S100 extract plus purified SF2. The electrophoretic positions of free RNA and of complexes I, A, A', and B (Zillman et al. 1988) are indicated.

same mobility as the A, A', and B complexes obtained with the nuclear extract (lane 5). We conclude that SF2 is required for the assembly or the stabilization of the earliest specific complex in the spliceosome assembly pathway, which is thought to be the A complex (Zillman et al. 1988). The intensity of the bands was much lower with the reconstituted system than with the crude nuclear extract (cf. lanes 2 and 5), although the extent of splicing was very similar in both cases (Fig. 3, lanes 1 and 2). This may simply reflect differences in the steady-state levels of the complexes formed in each reaction. Alternatively, the stability of these complexes may be different because of the influence of other specific and nonspecific components in each reaction. It is likely that the native gel assay conditions result in partial disassembly of the complexes.

Preincubation experiments with globin pre-mRNAs of different sizes were carried out to determine whether SF2 forms a stable, committed complex with pre-mRNA. Each pre-mRNA was preincubated with limiting amounts of SF2 in the presence or absence of ATP and magnesium, and then the second pre-mRNA and the

complementing S100 extract were added. The kinetics and extent of splicing of each substrate were the same (data not shown). Thus, SF2, itself, does not form a committed complex with pre-mRNA that persists through a rate-limiting step in splicing. In a reciprocal experiment, preincubations were done with the S100, and SF2 was added later with the second pre-mRNA. Again, no differences were detected in the splicing of the first and second substrate (data not shown), suggesting that no stable specific complexes are formed in the absence of SF2, in agreement with the native gel analysis. Further experiments are necessary to determine whether SF2 remains stably associated with the spliceosome after promoting the assembly of the initial specific complex and whether it is recycled to participate in a new round of splicing. The minimum amount of purified SF2 that is necessary to complement the S100 fraction represents a slight molar excess over the pre-mRNA concentration, suggesting that SF2 may act stoichiometrically, rather than catalytically. However, we do not know what proportion of the purified molecules is active, and furthermore, a fraction of these molecules may become trapped in nonproductive complexes upon addition to the crude S100 fraction.

RNA-binding properties of SF2

The effects of SF2 on splicing and splicing complex assembly may involve direct interactions between this factor and the pre-mRNA substrate, or SF2 may act without associating with the spliceosome, e.g., by modifying other factors. To determine whether SF2 binds RNA, several types of analyses were carried out. UV cross-linking demonstrated direct binding of purified SF2 to pre-mRNA and to mRNA (Fig. 5). When the cross-linked material is digested extensively with ribonuclease A, the protein–oligoribonucleotide adducts usually comigrate with, or migrate slightly behind, the untreated polypeptides on SDS-PAGE (Greenberg 1980; Economides and Pederson 1983; Choi and Dreyfuss 1984; García-Blanco et al. 1989). In the case of SF2, the mobility of the labeled adducts appears slightly slower than that of the untreated 33-kD doublet. At least one and, possibly, both polypeptides were cross-linked to pre-mRNA and to mRNA (Fig. 5, lanes 2 and 4). The poor resolution of the bands makes it difficult to determine to what extent each of the polypeptides is cross-linked. It is possible that only one polypeptide binds RNA, in which case the heterogeneity of the labeled adducts may be due to binding to different regions of the labeled RNA, which would give rise to heterogeneous RNase A digestion products. Thus, for instance, relatively nonspecific RNA binding and/or partial protection from RNase A could account for the observed pattern even if only the smaller of the two polypeptides binds RNA. A lighter band was also detected with an electrophoretic mobility expected for a protein dimer, and on longer exposures, labeled material with the mobility of trimers, tetramers, etc., was also seen. These forms could arise either by a low level of protein–pro-
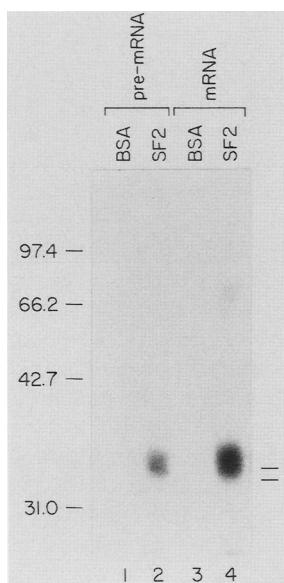
Figure 5. UV cross-linking. ^{32}P-labeled human β-globin pre-mRNA or mRNA *Bam*HI runoff transcripts were incubated with nuclease-free bovine serum albumin or with purified SF2 for 15 min and irradiated at 254 nm. After digestion with RNase A, the products were analyzed by SDS-PAGE and autoradiography. The electrophoretic mobilities of the untreated 33-kD polypeptides and of unlabeled molecular weight markers are indicated.

tein cross-linking or by protection from RNase A of small RNA fragments bound by two or more adjacent SF2 molecules.

These experiments gave the appearance of more efficient cross-linking to mRNA than to pre-mRNA (Fig. 5, cf. lanes 2 and 4). Both RNAs were labeled to the same specific activity with all four ribonucleoside triphosphates. However, the extent of RNase digestion could be different for each RNA, depending on, among other things, the distribution of pyrimidines in the vicinity of each cross-link. As a result, the molar amount of ^{32}P label in each adduct may differ, creating the appearance of different cross-linking efficiencies. Even if the cross-linking efficiencies do differ, this is not necessarily a reflection of different binding efficiencies, as some protein molecules may bind specific RNA sequences but they may fail to be cross-linked due to the specific bases and amino acid side chains present at or near the contact points. It is possible, although not directly addressed in these cross-linking experiments, that SF2 binding reflects the higher order structure of these RNAs or, perhaps, preferential binding to the spliced exon borders. The solution structure of naked human β-globin pre-mRNA truncated at the *Bam*HI site in exon 2 has been determined (Teare and Wollenzien 1989), and it includes helices between intron 1 and exon 2. Therefore, the solution structures of the exons in pre-mRNA and mRNA are substantially different.

No RNA binding was detected by Northwestern blotting, which could have been due to poor renaturation of

each polypeptide after SDS-PAGE (data not shown). Alternatively, both polypeptides might be necessary for RNA binding. We have not been able to separate the two polypeptides by nondenaturing techniques; thus, we cannot presently determine whether only one or both are responsible for the observed activities. We note, however, that preliminary amino acid analysis of the individual electrophoretically separated polypeptides suggests that they are related (data not shown). RNA footprinting experiments were carried out by partial modification of globin pre-mRNA with dimethylsulfate, followed by primer extension, and by partial digestion of end-labeled pre-mRNA with ribonucleases. No specific footprint was observed in the presence of SF2, suggesting that at least in the absence of other components, SF2 does not bind pre-mRNA in a sequence-specific manner (data not shown). However, because we do not know what fraction of the SF2 molecules is active in these experiments and because detection of a footprint requires binding to most or all of the RNA molecules, the possibility that SF2 binds RNA in a sequence-specific manner cannot be ruled out at present.

SF2 copurifies with an RNA annealing activity

We tested purified SF2 for possible RNA helicase and/or annealing activities (Fig. 6). Complementary ^{32}P-labeled RNAs were incubated with purified SF2 in splicing buffer, in the presence or absence of ATP. After incubation, digestion with ribonuclease T1 was carried out, and nuclease-resistant double-stranded RNAs were detected by urea–PAGE. In the absence of protein, complementary RNAs do not anneal in these buffer conditions at 30°C (Fig. 6, lanes 3 and 5), due to the higher order structures of the individual strands and to the slow rate of annealing at temperatures substantially below the melting temperature. Efficient annealing requires an initial incubation at high temperature to eliminate these intramolecular helices, followed by incubation at ~25° below the melting temperature. The theoretical melting temperature in these experiments is 86°C, or higher when magnesium is taken into account (for review, see Wahl et al. 1987). However, in the presence of SF2, rapid annealing can take place at 30°C, with or without ATP (Fig. 6, lanes 4 and 6) and only in the presence of magnesium (data not shown). When the complementary strands were preannealed, SF2 did not melt them, even when ATP was present (lanes 8 and 10). In summary, SF2 lowers the activation energy for intermolecular RNA duplex formation and thus behaves as a magnesium-dependent and ATP-independent RNA annealing activity. Under the conditions tested, SF2 lacks RNA helicase activity, as well as RNA-dependent ATPase activity (Fig. 6; data not shown).

To test further whether the RNA annealing activity is intrinsic to SF2 or whether it is a contaminating activity, we repeated the purification and assayed each fraction for both complementation of the S100 and for RNA annealing. Although other RNA annealing activities were present in the nuclear extract (Munroe 1988)
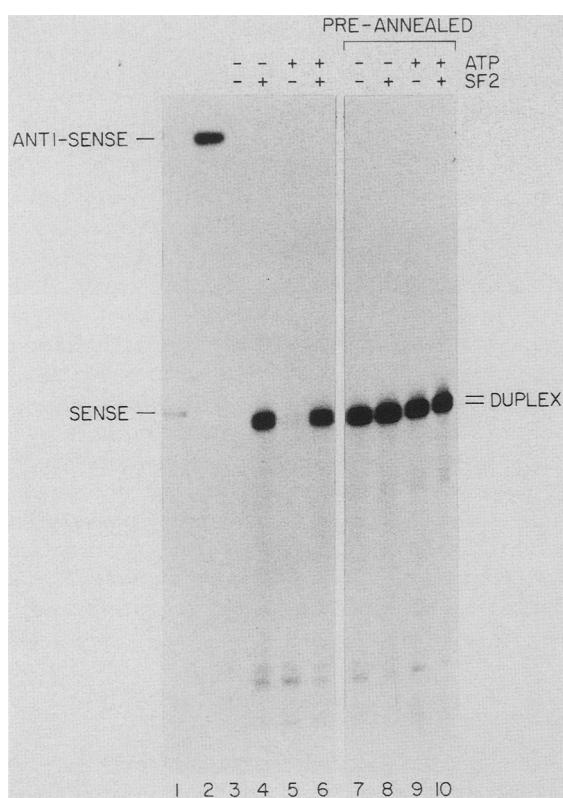
Figure 6. RNA annealing and helicase assays. Sense and anti-sense complementary β-globin RNAs were incubated as separate strands or as a preannealed duplex in the presence or absence of purified SF2 and ATP, as described in the text and diagram. After digestion with ribonuclease T1, the products were analyzed by urea–PAGE and autoradiography. The positions of the untreated sense and antisense RNAs and of the T1-resistant duplex are indicated on each side of the autoradiograph.

tions, and some, but not all, active fractions also contain discrete RNA species of tRNA size or larger. Thus, SF2 is not likely to have an essential RNA component, in agreement with its resistance to micrococcal nuclease (Krainer and Maniatis 1985; this study).

The 34-kD hnRNP A1 protein has been shown to have helix-destabilizing properties on double-stranded RNA. Likewise, proteolytic fragments of hnRNP A1, termed HDPs, or helix-destabilizing proteins, can lower the melting temperature of both double-stranded RNA and DNA (Cobianchi et al. 1988, and references therein). Under suitable conditions, this lowering of the melting temperature by hnRNP A1 can promote RNA annealing (S. Munroe, pers. comm.). We therefore tested purified A1 (kindly provided by S. Wilson) to see whether it could substitute for SF2 in the splicing complementation and RNA annealing assays. Concentrations of A1 that were active in RNA annealing did not substitute for SF2 in the complementation assay (data not shown). These observations suggest that if RNA annealing activity is integral to SF2 and necessary for splicing, it is not sufficient, implying that specific protein–protein or protein–RNA interactions are involved in mediating the role of SF2 in splicing.

Further experiments are necessary to elucidate the precise substrate specificity, if any, of the SF2 RNA annealing activity. However, we can envisage three types of potential complementary RNA targets upon which the SF2 RNA annealing activity could act to mediate its effects on splicing. First, at least two snRNA–pre-mRNA helices are known to form during splicing and to contribute to splice site selection; these involve base-pairing interactions between the 5′ terminus of U1 snRNA and pre-mRNA 5′ splice sites (Zhuang and Weiner 1986; Seraphin et al. 1988; Siliciano and Guthrie 1988) and between an internal region of U2 snRNA and pre-mRNA branch site sequences (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989). Second, U4 and U6 snRNAs coexist in a single snRNP particle held together by intermolecular base pairs (Bringmann et al. 1984; Hashimoto and Steitz 1984), and there is some evidence that the U4 subunit may be released from the spliceosome during the course of splicing, possibly to be recycled (Cheng and Abelson 1987; Konarska and Sharp 1987; Lossky et al. 1987; Lamond et al. 1988; Black and Pinto 1989; Blencowe et al. 1989). Third, pre-mRNA may be recognized as a proper splicing substrate only when it adopts a defined secondary and tertiary structure. Some of the putative intramolecular RNA helices may preferentially expose the splice sites and branch site.

and in several of the Phenyl-Superose fractions lacking SF2 (data not shown), one such activity copurified precisely with SF2 (Fig. 7A,B). Both activities peaked in fractions 24–28 and were reduced in fraction 26. Both activities correlate well with the 33-kD polypeptides in fractions 24 and 25 (Fig. 7C,D); however, it is difficult to detect these bands in fractions 27 and 28. It is not possible to assign activities to polypeptides in these fractions, but possible candidates are minor polypeptides of 55 (see below), 36, and 24 kD. Further experiments are necessary to determine whether these polypeptides are related to one another. A 32-kD polypeptide that peaks in fraction 23 is inactive (Fig. 7A–D) and may represent a degradation product of the 33-kD polypeptides. It is present in only some preparations (Fig. 7C,D, fractions 23–25 and lane C; Fig. 1).

Although the majority of the nuclear RNA in the crude extract was removed in the initial chromatographic steps, a small amount of UV_{260}-absorbing material copurified with SF2 and with the annealing activity. Therefore, the RNA content of the Phenyl-Superose fractions was analyzed by urea–PAGE (Fig. 7E). A smear corresponding to hnRNA is present in all the active frac-

Immunoprecipitation of SF2

To facilitate the characterization of native SF2 and to determine whether the 33-kD polypeptides are normally associated with other known or unknown polypeptides, polyclonal antisera were obtained by immunization of rabbits with purified SF2. After repeated injections, SF2 antisera were obtained and used for immunoprecipita-
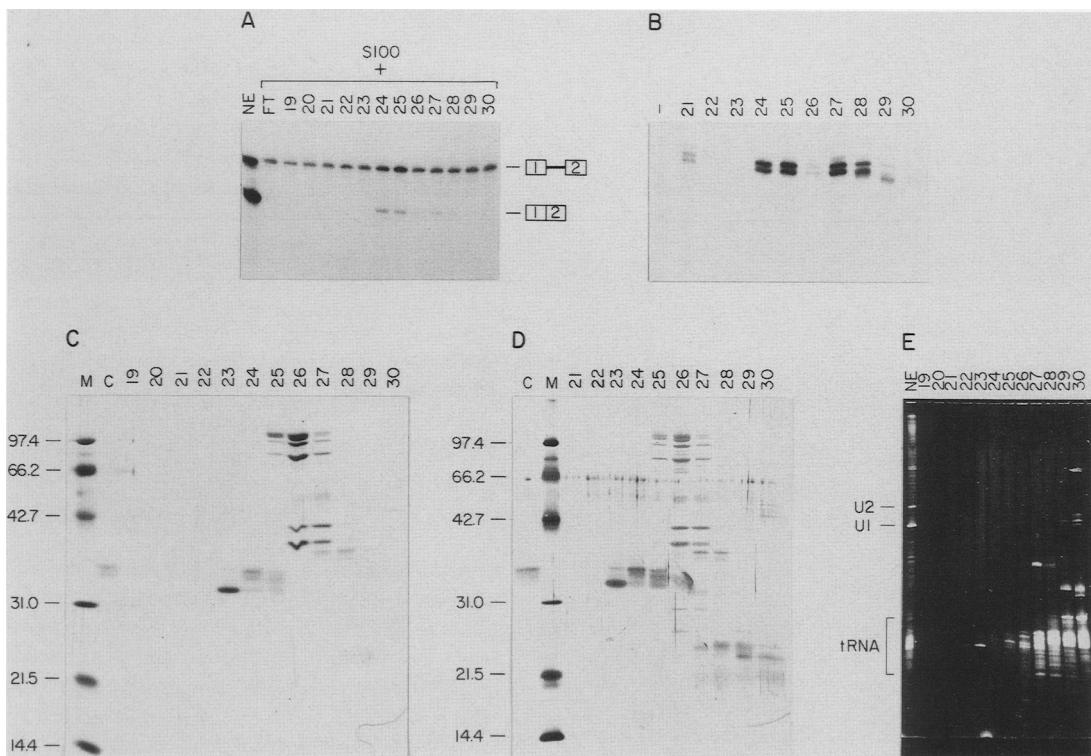
Figure 7. Copurification of SF2 and RNA annealing activities. Selected fractions from the Phenyl-Superose column were tested for complementing activity in splicing with the S100 extract (A) and for RNA annealing (B). The polypeptide composition of the same column fractions is shown in C (Coomassie Blue staining) and D (silver staining). (E) RNAs present in these fractions. The following amounts of each fraction were used: 7.5 μl (A), 15 μl (B), and 30 μl (C–E). The elution conditions for this column were slightly different from those in Fig. 1. Numbers at top of A–E are the fraction numbers. (FT) Flowthrough fraction; (NE) nuclear extract; (C) different purified preparation of SF2; (M) molecular weight markers with the indicated sizes. The amount of NE used in E represents 100 times fewer cell equivalents than the amount of each fraction. The positions of tRNAs and U1 and U2 snRNAs are indicated.

tions of ^{35}S-labeled HeLa cell nuclear extracts (Fig. 8). The SF2 antiserum immunoprecipitated a doublet of proteins of ~33 kD, as well as a polypeptide of 55 kD, which migrates more slowly than IgG heavy chain (lane 2). A 55-kD polypeptide could not be detected in the SF2 preparation employed for immunization. Its presence in the immunoprecipitates could be explained by contamination of the purified preparation with trace amounts of a highly antigenic protein. Alternatively, the 55-kD polypeptide may be a subunit of native SF2 that is associated with one or both 33-kD polypeptides in vivo. If this putative subunit is necessary for activity, it would have to associate with the purified 33-kD polypeptides upon incubation with the crude S100 fraction. An alternative explanation for the presence of the 55-kD band in the immunoprecipitates is that one or both 33-kD polypeptides may be specific proteolytic products of a putative 55-kD or larger SF2 precursor. Finally, the 55- and 33-kD proteins may share one or more epitopes. These possibilities can be explored by Western blotting analysis. Unfortunately, none of the SF2 antisera we have obtained so far are reactive in this type of analysis. We note that while optimizing the purification of SF2 we observed a 55-kD polypeptide that copurified precisely with highly purified 33-kD polypeptides (data not

shown). In the procedure that was optimized for SF2 activity (Figs. 1 and 7), a 55-kD polypeptide was no longer detected in the fractions with maximal activity and the largest amount of the 33-kD polypeptides. However, a small amount of a 55-kD polypeptide can be seen in adjacent fractions and may account for their activity in the complementation and annealing assays. Further work is necessary to determine the relation between the 33- and 55-kD polypeptides.

Relation of SF2 to other factors and activities

SF2 can be compared to other factors and activities on the basis of reported biochemical and physical properties. One distinguishing feature of SF2 is its apparent retention in the nuclear fraction until the salt concentration is raised in the extraction buffer (Krainer and Maniatis 1985). In our hands, the cytoplasmic S100 extract lacks a single component required for splicing, as assayed by complementation, but we believe that the ability to detect other components identified by different assays is likely to be variable. The relative concentration of various splicing factors is very different in nuclear and S100 extracts, but only SF2 is limiting for splicing in the latter extract. We have found, for ex-
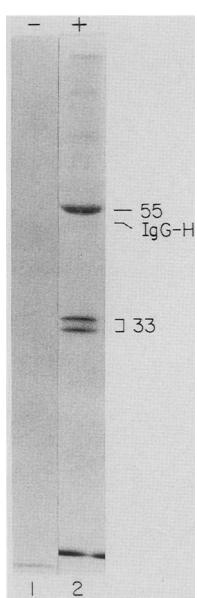
Figure 8. Immunoprecipitation of SF2. A nuclear extract prepared from HeLa cells labeled with [^{35}S]methionine and cysteine was immunoprecipitated with rabbit preimmune serum (lane *1*) or with antiserum from the same rabbit immunized with purified SF2 (lane *2*). The immunoprecipitates were recovered and analyzed by SDS-PAGE and fluorography. The sizes of the immunoprecipitated polypeptides (in kilodaltons) relative to unlabeled markers, as well as the position of immunoglobulin heavy chain, are indicated.

ample, that hnRNP C protein is also greatly reduced in S100 extracts (G.C. Conway and A.R. Krainer, unpubl.), yet we know that SF2 and C proteins are distinct. Thus, either C protein is not necessary for splicing or the small amount in the S100 extract is sufficient for activity (Choi et al. 1986; Sierakowska et al. 1986). We also note that there may be subtle differences in the composition of S100 extracts prepared in different laboratories. Therefore, the putative identity of other factors and SF2 should not be inferred solely on the basis of similar subcellular fractionation.

Splicing factors assayed by complementation   The SF2 polypeptides do not comigrate with any known snRNP polypeptide, and they do not copurify with snRNAs (Figs. 2 and 7E). This is in agreement with the observation that SF2 activity can be chromatographically separated from active snRNPs (Krainer 1988). Immunoprecipitation of the SF2 polypeptides under mild conditions does not bring down snRNP polypeptides, and conversely, immunoaffinity-purified snRNPs do not contain SF2 polypeptides (Figs. 2 and 8). Thus, the majority of SF2 and snRNPs do not appear to coexist in stable particles in the nuclear extract, and they may or may not contact each other upon spliceosome assembly.

In regard to non-snRNP factors that have been shown to be required for splicing by complementation, conclusive comparisons to SF2 are not always possible at this stage, because none of these factors have been identified

at the polypeptide level. On the basis of chromatographic properties, we believe that SF2 is present in fraction II described by Perkins et al. (1986), together with snRNPs and other protein factors. The factor described in the present study is different from a partially purified component, coincidentally designated *SF2* (Krämer and Keller 1985), which, unlike our factor, is enriched in the cytoplasmic fraction and is sensitive to the sulfhydryl reagent *N*-ethyl maleimide (NEM). Krämer and colleagues also described two partially purified components necessary for splicing, termed *SF1* and *SF3*, which, together with a fraction containing U2 snRNP, are minimally required to form a stable complex with pre-mRNA (Krämer et al. 1987; Krämer 1988). The combined activity of these two components is thought to correspond to a binding activity designated U2AF (Krämer 1988; Ruskin et al. 1988; see below). On the basis of the reported chromatographic and subcellular fractionation properties, it is possible that Krämer's *SF1* factor corresponds to SF4B described elsewhere (Krainer and Maniatis 1985); whereas her *SF3* factor is potentially analogous to the SF2 activity described in the present study (Krainer and Maniatis 1985; Krämer et al. 1987; Krämer 1988; this study). Other splicing activities designated SF3 and SF4A were shown previously to be distinct from SF2 (Krainer and Maniatis 1985). SCF, an activity necessary to complement presumptive in vivo assembled spliceosomes (Zeitlin et al. 1989) is thought to be related to *SF1* (Krämer et al. 1987) and distinct from SF2 (Krainer and Maniatis 1985; Zeitlin et al. 1989; this study).

hnRNP and intron-binding proteins   Several RNA-binding proteins may play a role in splicing, as inferred from their sequence-specific binding properties. Among these are the core hnRNP proteins, as well as several additional constituents of hnRNP particles (for review, see Dreyfuss et al. 1988; Kumar et al. 1987; Swanson and Dreyfuss 1988). Piñol-Roma et al. (1988) isolated multiple HeLa RNA-binding proteins present in hnRNP particles and characterized them by two-dimensional gel analysis. Because SF2 appears to be present in RNP particles in nuclear extracts (Conway et al. 1989; this study), it may be one of the known hnRNP proteins. However, the 33-kD polypeptides are smaller than all of the two dozen or so HeLa hnRNP polypeptides described by Piñol-Roma et al. (1988). Although they may represent proteolytic products of one of the larger hnRNP proteins, another possibility is that the SF2 polypeptides were not detected previously as hnRNA-binding proteins due to their low abundance or poor labeling with [^{35}S]methionine. The SF2 polypeptides migrate slightly ahead of the hnRNP core protein A1 (Fig. 2), which is the smallest of the known hnRNP proteins. However, degradation products of A1, ranging in size from 20 to 33 kD, have also been described (Cobianchi et al. 1988, and references therein). We used a polyclonal A1 antiserum for Western blot analysis and observed no cross-reactivity with the 33-kD proteins, even though this antiserum reacted strongly with all the core hnRNP polypeptides.

A family of RNA-binding proteins that bind preferentially to 3' splice sites and may exist in association with U5 snRNP has been described. These proteins have not been purified extensively, but they have been characterized by Northwestern blotting analysis and have reported molecular masses of 70 and 110 kD (Gerke and Steitz 1986; Tazi et al. 1986). None of their physical, biochemical, or chromatographic properties suggest a similarity to the SF2 polypeptides. The same can be said of a recently isolated RNA-binding protein of 62 kD, or pPTB, which cross-links at 3' splice sites and is present in spliceosomes (García-Blanco et al. 1989).

U2AF An activity termed U2AF was originally identified in crude nuclear extracts by a ribonuclease protection assay and was shown to protect the branch site region in combination with extracts containing U2 snRNP and to participate in splicing complex assembly (Ruskin et al. 1988). U2AF was reported to be absent or reduced in S100 extracts, and is sensitive to NEM (Ruskin et al. 1988). In contrast, SF2 activity is unaffected by treatment with NEM (data not shown). However, we note that the sensitivity of U2AF to NEM could have been due to inactivation of endogenous creatine kinase, which is necessary for ATP regeneration in splicing extracts (Krainer et al. 1984). This enzyme contains an essential reactive cysteine (Kumudavalli et al. 1970), and ATP is required for the binding of U2 snRNP to the branch site (Black et al. 1985). It is not known at present whether U2AF is an essential splicing factor, although Krämer has obtained two partially purified protein fractions necessary for splicing which, when combined, also have U2AF-like activity (Krämer 1988). U2AF was reported to bind in a sequence-specific manner to the 3' splice site (Ruskin et al. 1988). In contrast, we do not detect sequence specificity in the binding of SF2 to RNA (Fig. 5), but different RNA-binding assays were employed in each case. Two polypeptides of 65 and 35 kD were recently purified on the basis of a gel mobility shift assay and a UV cross-linking assay (Zamore and Green 1989), and may correspond to the U2AF activity described originally (Ruskin et al. 1988). The 65-kD component may be analogous to pPTB (García-Blanco et al. 1989). It is possible, although unknown at present, that the 35-kD component is the same as, or related to, the 33-kD SF2 polypeptides described in the present study. However, the 35-kD polypeptide does not cross-link to RNA (Zamore and Green 1989), in contrast to the 33-kD polypeptides (Fig. 5). In addition, we have found that SF2 mediates some of its effects at the 5' splice site (Krainer et al. 1990). In this respect, SF2 may be related to activities present in the nuclear extract, which enhance the binding of U1 snRNP to 5' splice sites (Mayeda et al. 1986; Zapp and Berget 1989).

SC-35 Recently, Fu and Maniatis obtained a monoclonal antibody against a protein, termed SC-35, that is stably associated with spliceosome complexes (Fu and Maniatis 1990). Immunodepletion of SC-35 from nuclear extracts results in loss of splicing activity and reduces the efficiency of complex formation, suggesting that this antigen or other nuclear proteins associated with it are necessary for splicing. Immunodepleted extracts could be complemented by micrococcal nuclease-treated nuclear extract but not by S100 extract, suggesting that SC-35 may be the same as SF2 or associated with it, for example, in an endogenous RNP complex (Conway et al. 1989). On Western blots, the αSC-35 antibody reacts with a 35-kD doublet in nuclear but not cytoplasmic extracts (Fu and Maniatis 1990). The similarity in size and other properties raises the possibility that this antigen and the 33-kD SF2 polypeptides may be the same or related. Experiments are in progress to address this possibility.

Conclusions

The complementation strategy employed here allowed us to isolate a factor that is essential for 5' splice site cleavage and lariat formation in vitro. It is always difficult to rule out completely the possibility of in vitro artifacts, and we do not know at present whether SF2 is required for splicing in vivo. However, we have recently found that SF2 mediates highly specific effects on splice site selection (Krainer et al. 1990). SF2 was found to be absolutely required for the splicing of several different introns. The S100 extract plus purified SF2 reconstituted system provides an alternative to nuclear extracts for in vitro splicing studies. We found that some pre-mRNAs spliced much more efficiently in this system than in nuclear extract. For example, splicing of an HIV-derived pre-mRNA was almost undetectable in nuclear extract and quite efficient in the reconstituted system; this was directly attributable to differences in the concentration of SF2. The opposite extract preference was observed with an adenovirus pre-mRNA, suggesting that the two in vitro systems probably differ in the relative concentrations of several splicing factors.

The precise mode of action of SF2 is unknown. Our current data indicate that SF2 has nonspecific RNA binding properties and the ability to promote annealing of complementary RNA strands. It does not form a stable complex with pre-mRNA in the absence of other factors, but the earliest requirement for SF2 appears to be for the assembly or stabilization of the pre-spliceosome complex. The associated RNA annealing activity suggests that SF2 may alter the higher order structure of the pre-mRNA substrate and/or the snRNAs. The 33-kD polypeptides represent minimal units of structure and complementing activity, and they retain RNA-binding and annealing functions. Whether these polypeptides must directly associate with other factors or subunits to promote splicing remains to be determined.

Materials and methods

Cell growth and sonicated nuclear extract preparation

HeLa S3 cells were grown in spinner flasks in Dulbecco's modified minimal essential medium (DMEM) supplemented with 5% calf serum. They were harvested at a density of 10^6/ml

by centrifugation at 2500 rpm ($1819g_{max}$) in a Sorvall H-6000A rotor for 10 min at 4°C. The cell pellet was resuspended in 50 % (vol/vol) glycerol and $1 \times$ DMEM (50 ml final volume per 3 liters of original culture) and stored at -70°C. Freezing the cells indefinitely in this manner did not adversely affect the splicing activity of the resulting extracts.

All of the extract preparation and SF2 purification procedures were performed at 0–4°C, unless otherwise noted. All buffers containing N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) were prepared from a 1 M stock that was adjusted to pH 8.0 at 25°C with NaOH. Dithiothreitol (DTT) was added fresh from a 1 M stock, and phenylmethylsulfonyl fluoride (PMSF) was added fresh from a 20 mg/ml stock in ethanol. Frozen HeLa cells (2.4×10^{10}; 400 ml suspension from a 24-liter culture) were thawed rapidly at 30°C and spun at 2500 rpm in the H-6000A rotor for 20 min. The cell pellet was resuspended in 250 ml of PBS plus 0.5 mM MgCl$_2$ and spun, as above, for 10 min. The supernatant was discarded and the packed cell volume noted. The cells were resuspended in an equal volume of buffer 1 [10 mM HEPES (pH 8.0), 10 mM KCl, 1.5 mM MgCl$_2$, and 0.5 mM DTT] and incubated for 10 min on ice. The cells were lysed in several batches in a 40-ml Dounce homogenizer with a B pestle (Kontes) and examined by phase-contrast microscopy for cell lysis. When >90% lysis was achieved, the nuclei were recovered by centrifugation at 2000 rpm ($1164g_{max}$) in the H-6000A rotor for 10 min. The cytoplasmic fraction was removed, and the nuclei were pelleted in a Sorvall SS34 rotor for 20 min at 16,500 rpm ($32,530g_{max}$). The supernatant was discarded and the packed nuclear volume noted. The nuclei were resuspended in an equal volume of buffer 2 [20 mM HEPES (pH 8.0), 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM PMSF] by 10 strokes of a B pestle in a 40-ml Dounce homogenizer. The nuclear suspension was kept in ice water and sonicated in four 10-sec bursts at 1-min intervals with a Branson sonifier with a ½-inch disruptor horn. The complete disruption of nuclei was verified by microscopy. The homogenate was rocked for 30 min and spun at 16,500 rpm in the SS34 rotor for 30 min to remove chromatin fragments and nucleoli. The supernatant was transferred to a clean tube, and the pellet was resuspended in an equal volume of buffer 2 containing only 0.21 M NaCl, spun at 16,500 rpm for 20 min, and pooled with the previous supernatant. The resulting extract was dialyzed overnight against 50 volumes of buffer 3 [20 mM HEPES (pH 8.0), 0.1 M KCl, 5% (vol/vol) glycerol, 0.2 mM EDTA, and 1 mM DTT] containing 0.5 mM PMSF. The dialysate was spun at 16,500 rpm in the SS34 rotor for 20 min to remove insoluble material. The dialysis step was omitted occasionally, but this often led to diminished solubility of SF2 in subsequent steps.

Purification of SF2

The sonicated nuclear extract was diluted with 1.5 volumes of buffer 4 [20 mM HEPES (pH 8.0), 0.2 mM EDTA, 1 mM DTT]. To the resulting solution, an equal volume of saturated (NH$_4$)$_2$SO$_4$ in buffer 4 was added slowly with gentle stirring. [Saturated (NH$_4$)$_2$SO$_4$ (at 0°C) was prepared by adding 69.7 g to each 100 ml of buffer 4]. The 50% saturated suspension was stirred for 1 hr and spun at 4000 rpm ($4657g_{max}$) in the H-6000A rotor for 30 min. The volume of the supernatant was measured, and the (NH$_4$)$_2$SO$_4$ concentration brought to 80% saturation (at 0°C) by the addition of 19.4 g of solid (NH$_4$)$_2$SO$_4$ per 100 ml of supernatant. The suspension was gently stirred for 1 hr and spun as above. The supernatant was discarded, and the 50–80% pellet was resuspended gently in buffer 3 in a final volume of 4 ml. This fraction contained the SF2 activity and only ~10% of the

total protein and nucleic acid. This first step also removed a great deal of particulate material, and essentially all detectable U snRNAs.

The (NH$_4$)$_2$SO$_4$ fraction was applied to a 200-ml Sephacryl S200-HR (Pharmacia-LKB) column (100 × 1.6 cm) pre-equilibrated in buffer 3, and eluted at 0.5 ml/min. Five-milliliter fractions were collected. The majority of SF2 activity eluted in the first two fractions after the void volume of this column, which has an exclusion limit for globular proteins of 200 kD. The highly reproducible behavior of SF2 on this column may reflect artifactual aggregation, the formation or maintenance of specific protein or nucleoprotein complexes, or a possible particulate nature. Because a small amount of hnRNA copurified with SF2 through subsequent steps and because SF2 can bind RNA, we favor the notion that, at this stage, SF2 is present as a ribonucleoprotein complex. The relative native molecular weight of SF2 later in the purification is ~30 kD, as determined by gel filtration on Superose 12.

The Sephacryl S200 HR peak fractions were pooled and applied to a 1-ml Mono Q column (Pharmacia-LKB) pre-equilibrated in buffer 3, at a flow rate of 0.5 ml/min; 0.5-ml fractions were collected. The column was washed with 5 ml of buffer 3 and subsequently eluted with 5 ml of buffer 3 containing 0.35 M KCl, followed by 5 ml of buffer 3 containing 1 M KCl (or a 20-ml gradient of 0.35–1 M KCl in buffer 3). SF2 bound very tightly to Mono Q and was recovered efficiently in the 0.35–1 M salt step. When a salt gradient was employed instead of a step gradient, SF2 activity trailed in a broad range of salt concentration, whereas most polypeptides eluted as sharp peaks (data not shown). This chromatographic behavior suggests that SF2 binds to Mono Q indirectly, via its interactions with high-molecular-weight RNA which, in turn, binds very tightly to anion exchangers.

The Mono Q fractions containing the protein peak were pooled (2 ml) and diluted with buffer 3 lacking KCl and containing 3.4 M (NH$_4$)$_2$SO$_4$ to a final concentration of 1.7 M (NH$_4$)$_2$SO$_4$ (2 M in the experiment shown in Fig. 7). This solution was applied to a 1-ml Phenyl-Superose column (Pharmacia-LKB) pre-equilibrated in buffer 3 lacking KCl and containing 1.7 M (or 2 M) (NH$_4$)$_2$SO$_4$, at a flow rate of 0.4 ml/min. The column was washed with 1 ml of the same buffer and eluted with a 25-ml gradient of this buffer, in which the (NH$_4$)$_2$SO$_4$ concentration decreased from 1.7 M (or 2 M) to 0, while 1-ml fractions were collected. SF2 activity emerged shortly after the gradient was started. The active fractions were pooled, dialyzed against buffer 3, and frozen in liquid nitrogen. Aliquots stored at -70°C remained active for several months.

Assays

The preparation of splicing extracts, pre-mRNA substrates, and the splicing reaction conditions, RNA extraction, and assays have been described (Krainer et al. 1984; Ruskin et al. 1984; Krainer and Maniatis 1985). The human β-globin pre-mRNA substrate is a BamHI runoff 497-nucleotide SP6 transcript containing a 158-nucleotide first exon, a 209-nucleotide second exon, and a 130-nucleotide intact first intron. The spliced mRNA is 367 nucleotides. The HIV-1 pre-mRNA substrate is a 525-nucleotide BamHI runoff SP6 transcript containing a 264-nucleotide first exon, a 108-nucleotide second exon, and a 153-nucleotide intron. The spliced mRNA is 372 nucleotides. The template was constructed by the subcloning of a 2.7-kb HindIII–BamHI fragment from the plasmid PBC12/RSV/t23 (Cullen 1986) into the corresponding sites in pSP64 (Melton et al. 1984). This fragment contains the two coding exons of the tat gene between the SalI (+5368) and BamHI (+8054) sites,

and a 2330-bp intron (Muesing et al. 1985). Internal intron sequences between an SspI site (+5738) and an RsaI site (+7916) were deleted to generate a 153-nucleotide truncated intron.

Nuclear and cytoplasmic S100 extracts (Dignam et al. 1983; Krainer et al. 1984) were dialyzed against buffer containing 5% instead of 20% glycerol. Micrococcal nuclease treatment and subsequent inactivation with ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) were as described previously (Krainer and Maniatis 1985). NEM (Fluka) treatments were performed on ice for 15 min in the presence of 10 mM NEM, followed by addition of DTT to 20 mM. As a control, NEM and DTT were premixed prior to the addition of SF2. Papain (Boehringer) treatments were done at 40 μg/ml for 15 min at 30°C, followed by inactivation with 10 μg/ml E-64 (Boehringer). As a control, the protease was inactivated with E-64 prior to the addition of SF2.

Complementation reactions were carried out for 2–4 hr with 7.5 μl of S100 extract and 1–7.5 μl of the appropriate protein fraction diluted to 7.5 μl with buffer 3 under otherwise standard splicing conditions (Krainer et al. 1984). Because splicing is highly sensitive to monovalent cation concentration (Krainer et al. 1984), small aliquots of the ammonium sulfate, Mono Q, and Phenyl-Superose fractions were dialyzed against buffer 3 prior to the assaying for SF2 activity. Protein concentrations were determined by the method of Bradford (1976), with BSA as a standard. Protein gels were run according to Laemmli (1970). Staining was with Coomassie Blue R-250 or by the ammoniacal silver method of Wray et al. (1981).

UV cross-linking Purified SF2 (6 μl, phenyl-Superose fraction 9) or 1.2 μg nuclease-free bovine serum albumin in 6 μl of buffer 3 was incubated with 10^5 cpm of globin pre-mRNA or mRNA, labeled with all four ^{32}P rNTPs to a sp. act. of 1.6×10^7 cpm/μg, in a 10-μl reaction containing 3.2 mM $MgCl_2$, for 15 min at 30°C. The samples were then spotted on parafilm and irradiated with 254-nm light at 2 mW/cm² for 10 min at room temperature. The samples were added to 10 μl of 2 mg/ml RNase A and incubated for 15 min at 30°C. They were then fractionated on a 12% SDS-polyacrylamide gel (Laemmli 1970). The gel was stained with Coomassie Blue R-250, destained, and autoradiographed.

RNA annealing The substrates in the annealing/helicase assays were a 212-nucleotide sense fragment of human β-globin transcribed from SP64-HβΔ6 (Krainer et al. 1984), truncated with NspI, and a 518-nucleotide antisense fragment transcribed from SP65-anti-Hβ, truncated with HindIII. This plasmid contains the HindIII–BamHI globin fragment from SP64-HβΔ6 subcloned into the corresponding sites in pSP65 (Melton et al. 1984). The transcripts were synthesized in the absence of a cap dinucleotide. The complementarity between the two RNAs is 204 bp, and T1-resistant fragments of 211 nucleotides are expected based on the sequence (Lawn et al. 1980). The 25-μl reactions contained 15 μl of buffer 3, including 3 μl of purified SF2 or hnRNP A1 protein (1–5 μg), 3.2 mM $MgCl_2$, 70 fmoles of each RNA and, in some cases, 0.5 mM ATP. The individual RNAs were either added separately to the reaction, or in some cases, they were first annealed by heating at 85°C for 3 min, followed by slow cooling to room temperature. Incubation in the presence of protein was for 30 min at 30°C, after which 0.1 unit of RNase T1 was added and allowed to digest the RNA for an additional 15 min at the same temperature. The RNAs were recovered by phenol extraction and ethanol precipitation and were analyzed on a 5% polyacrylamide/urea gel.

Spliceosome assembly Electrophoretic analysis of spliceosome complexes was carried out as described (Zillman et al.

1988), with BamHI runoff β-globin pre-mRNA and the appropriate extracts and fractions.

Immunoprecipitation Approximately 10 μg of purified SF2 (phenyl-Superose fraction 9) was used to immunize rabbits subcutaneously on days 1, 10, 22, and 35. Ten microliters of preimmune (day 0) or immune (day 43) sera were incubated with 10 μl of protein A–trisacryl beads (Pierce) in 0.5 ml of 20 mM HEPES (pH 8), 150 mM NaCl, and 0.05% Triton X-100 and washed four times with 1 ml of the same buffer. The immobilized IgG was incubated for 1 hr at 4°C with 60 μl of crude nuclear extract prepared from HeLa cells labeled with [^{35}S]methionine and cysteine. Labeling and extract preparation were as described (Conway et al. 1989). After four washes, as above, the immunoprecipitates were extracted by boiling in 50-μl of sample loading buffer and analyzed by SDS–PAGE and fluorography (Laemmli 1970).

RNP fractions snRNPs were purified by immunoaffinity chromatography as described (Krainer 1988). hnRNP was a kind gift from W. LeStourgeon.

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