The EMBO Journal vol.9 no.12 pp.4119-4126, 1990

Splicing factor SF4 is dispensable for the assembly of a functional splicing complex and participates in the subsequent steps of the splicing reaction

Ulrike Utans and Angela Krämer

Department of Cell Biology, Biocenter of the University of Basel, CH-4056 Basel, Switzerland

Communicated by W.Keller

The splicing of nuclear messenger RNA precursors (pre-mRNA) can be reconstituted *in vitro* with factors partially purified from HeLa cell nuclear extracts. Splicing complexes are assembled in the presence of the small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5 and U6 and the protein factors SF1, SF2, SF3 and U2AF. However, the complexes thus formed are inactive, i.e. they only contain unprocessed pre-mRNA. The intermediates and products of the splicing reaction are generated after addition of SF4. This splicing factor is a heat-labile protein which requires sulfhydryl groups for its activity. SF4 appears to participate, directly or indirectly, in the conversion of a functional but inactive splicing complex to the active spliceosome.

Key words: pre-mRNA splicing/RNA processing/snRNPs

Introduction

Within the last decade, *in vitro* systems have greatly contributed to our understanding of the process that removes introns from nuclear messenger RNA precursors (pre-mRNA) (for reviews see Green, 1986; Padgett *et al.*, 1986; Guthrie and Patterson, 1988). The splicing reaction is initiated by the association of multiple components with the pre-mRNA substrate, leading to the formation of splicing complexes or spliceosomes. Within these structures two distinct cleavage and ligation reactions take place. First, the pre-mRNA is cleaved at the 5′ splice site. At the same time the 5′ terminal guanosine of the intron is covalently attached via a 2′−5′ phosphodiester bond to an adenosine residue located close to the 3′ end of the intron. Thus, the intermediates of the splicing reaction, the cleaved exon 1 and the intron−exon 2 lariat, are formed. In the second step, cleavage occurs at the 3′ splice site concomitant with the ligation of the exons and the release of the intron in the lariat form.

A number of components involved in these reactions have been identified. Small nuclear ribonucleoprotein particles (snRNPs) interact with the pre-mRNA to assemble the spliceosome (for reviews see Guthrie and Patterson, 1988; Steitz *et al.*, 1988). U1 snRNP binds to conserved sequences at the 5′ splice site and U2 snRNP associates with the branch point sequence. Both of these interactions occur, at least in part, via RNA−RNA base pairing (Zhuang and Weiner, 1986; Parker *et al.*, 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989; Zapp and Berget, 1989; Heinrichs *et al.*, 1990). By binding of U4/U6 and U5 snRNPs, this pre-splicing complex is converted into the spliceosome (Konarska and Sharp, 1987; Cheng and Abelson, 1987; Lossky *et al.*, 1987; Lamond *et al.*, 1988). Although U4 and U6 RNAs have been found in the spliceosome their site of interaction within the complex has not yet been elucidated. Indirect evidence suggests that U5 snRNP binds to the canonical polypyrimidine tract that is located immediately upstream of the 3′ splice site (Chabot *et al.*, 1985). Concomitant with the appearance of splicing intermediates the spliceosome undergoes a conformational change which is reflected by a weakened U4/U6 interaction (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988; Blencowe *et al.*, 1989).

In addition to the snRNPs, protein factors have been detected that function in the splicing reaction. Several proteins have been described that are essential for pre-splicing complex assembly (Ruskin *et al.*, 1988; Krämer, 1988; Zamore and Green, 1989; Krainer *et al.*, 1990). Moreover, proteins have been identified that specifically bind to the pre-mRNA (Gerke and Steitz, 1986; Tazi *et al.*, 1986; Garcia-Blanco *et al.*, 1989) or are found within splicing complexes (Chang *et al.*, 1988; Pinto and Steitz, 1989; Fu and Maniatis, 1990; Garcia-Blanco *et al.*, 1990; Whittaker *et al.*, 1990). Members of the hnRNP class of proteins also interact with intron sequences in a specific manner (Swanson and Dreyfuss, 1988) and the class C proteins appear to be required for the assembly of spliceosomes (Choi *et al.*, 1986).

Less is known about the factors that function in the cleavage and ligation reactions that succeed spliceosome formation. In chromatographic fractions derived from HeLa cells at least two activities have been identified that are only required for cleavage at the 3′ splice site and exon ligation (Krainer and Maniatis, 1985; Perkins *et al.*, 1986). A similar activity has also been found after fractionation of whole cell extracts prepared from *Saccharomyces cerevisiae* (Cheng and Abelson, 1986). Recently two temperature-sensitive yeast mutants have been isolated that accumulate splicing intermediates and are thus defective in the second step of the splicing reaction (Vijayraghavan *et al.*, 1989; Vijayraghavan and Abelson, 1990).

Another temperature-sensitive yeast strain (*prp2*) is defective in the first cleavage and ligation reaction (Lustig *et al.*, 1986; Lin *et al.*, 1987). In heat-treated extracts derived from this mutant, inactive spliceosomes accumulate. The RNA can be spliced after addition of the wild type protein. In addition, a heat-stable protein appears to be required for the same step of the reaction (Lin *et al.*, 1987; Cheng and Abelson, 1987). For mammalian splicing systems, no equivalent factors have yet been isolated. However, evidence has been provided that such factors exist. Unspliced pre-mRNA in isolated spliceosomes can only be spliced after addition of appropriate extracts (Grabowski *et al.*, 1985; Abmayr *et al.*, 1988).

We have previously described the separation of splicing factors from HeLa cell nuclear extracts by chromatography on DEAE−Sepharose, heparin−Sepharose, and Mono Q

(Krämer *et al.*, 1987). Six fractions were obtained that have to be combined to splice a pre-mRNA substrate derived from the adenovirus 2 major late (AdML) transcription unit *in vitro*. Four of these fractions contain protein factors, termed SF1-SF4, and two fractions are enriched in U1 and U5, or in U2 and U4/U6 snRNPs. SF1 and SF3 activities, in combination with snRNPs, are required for pre-splicing complex assembly (Krämer, 1988). SF2 and SF4 act in subsequent steps of the reaction and are both required for cleavage at the 5′ splice site and lariat formation (Krämer *et al.*, 1987).

Here we have investigated the role of SF4 in more detail. In a reaction reconstituted with partially purified splicing components, splicing complexes are assembled in the absence of this factor. However, the pre-mRNA is not spliced. Upon addition of SF4 both cleavage and ligation reactions take place.

Results

For the experiments described below the following fractions were used as a source of splicing factors (see Krämer *et al.*, 1987; Krämer and Keller, 1990, for details). SF1 is supplied with the DS100 fraction. This fraction also contains U2AF (unpublished results) which functions early in spliceosome assembly (Ruskin *et al.*, 1988; Zamore and Green, 1989). In addition, small amounts of snRNPs are present in this fraction which can be eliminated by treatment with micrococcal nuclease. The source of SF2 is the HS100 fraction and SF3, U1/U5 and U2/U4/U6 snRNPs are individual pools derived from a Mono Q column. SF2 and SF3 fractions are free of snRNPs.

Figure 1 shows some characteristics of SF4 activity. In a splicing reaction complemented with factors SF1−SF4, U2AF and both snRNP-containing fractions, the AdML pre-mRNA is efficiently spliced (lane 1). When SF4 is missing from such a reaction, splicing is almost completely abolished (lane 2). The small amount of residual splicing activity is caused by the presence of SF4 in one of the complementing fractions and was not observed in other experiments (see Figures 4 and 5). This confirms our previous result that SF4 is required for 5′ cleavage and lariat formation (Krämer *et al.*, 1987). When SF4 is treated with micrococcal nuclease prior to its addition to the complementation reaction, full splicing activity is retained (lane 3). This suggests that SF4 is a protein that is not associated with an essential RNA moiety. Preincubation of the SF4 fraction with *N*-ethylmaleimide abolishes splicing (lane 5), implying that sulfhydryl groups are required for SF4 activity. The reactions displayed in lanes 7−12 show that SF4 activity is stable after a 10 min preincubation at 45°C, but incubation at higher temperatures leads to a loss of its activity in the splicing reaction. Taken together these results demonstrate that SF4 is a heat-labile protein which is essential for the first cleavage and ligation reaction.

We have shown previously that SF1 and SF3 in combination with snRNPs assemble the pre-mRNA into a pre-splicing complex. SF4 fractions do not contribute any activity that is required for this step (Krämer, 1988). SF4 could therefore function later in spliceosome assembly or, alternatively, an inactive splicing complex may be formed in its absence and it could directly participate in the cleavage and ligation reactions. This distinction is based on the fact that, in the presence of low concentrations of EDTA, splicing complexes containing only unprocessed pre-mRNA can be obtained in extracts from HeLa cells (Abmayr *et al.*, 1988) or from *S.cerevisiae* (Cheng and Abelson, 1987). Furthermore, a block in splicing is observed after spliceosome assembly but before 5′ cleavage and lariat formation in heat-treated extracts prepared from the temperature sensitive yeast mutant *prp2* (Lin *et al.*, 1987). The inhibition can be overcome by addition of appropriate extracts or functional PRP2 protein, respectively.

To distinguish between a role for SF4 in spliceosome assembly or in the subsequent cleavage and ligation reactions, complex formation was examined in the presence or in the absence of SF4 in the standard assay containing SF1, SF2, SF3, U2AF and snRNPs. In initial experiments a low level of spliceosome formation was observed in the absence of SF4 and the addition of SF4-containing fractions had varying effects (not shown). Further experiments revealed that the variability was caused by different amounts of snRNPs that were present in SF4-containing fractions.

SF4 is separated from the bulk of the snRNPs by chromatography on a Mono Q column (Krämer *et al.*, 1987). The activity reproducibly elutes between the main peaks of U1/U5 and U2/U4/U6 snRNPs (Figure 2A and B). The residual snRNPs that are present in SF4-containing fractions tend to cofractionate with SF4 activity on a variety of columns, such as Blue-Sepharose or spermine−agarose (unpublished results). When spliceosome formation was monitored with consecutive column fractions in which SF4 activity was partially separated from snRNPs, it became apparent that the residual particles present in the SF4 fractions contributed to spliceosome formation, but not SF4 activity itself. As an example, Figure 2C shows the splicing complexes generated with consecutive Mono Q fractions obtained by elution with a salt gradient. The assay was

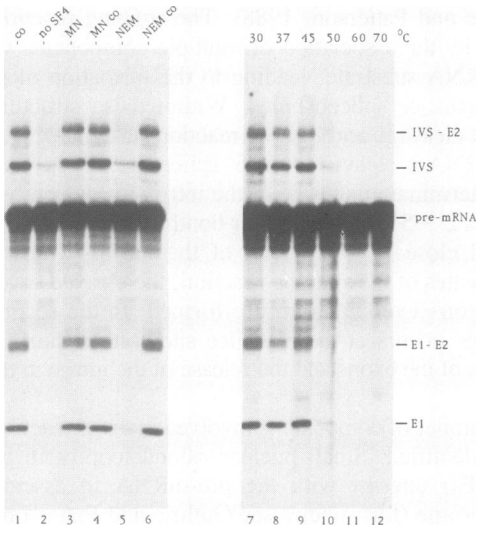

Fig. 1. Characterization of SF4 activity. Standard splicing reactions were performed for 2 h in the presence (lane 1) or in the absence of SF4 (lane 2). In the reactions shown in lanes 3−6, the SF4 fraction was pretreated with (lane 3) or without (lane 4) micrococcal nuclease; or with *N*-ethylmaleimide (lane 5) or *N*-ethylmaleimide and dithiothreitol (lane 6). Lanes 7−12 display reactions that contain SF4 which was incubated for 15 min at the indicated temperature before it was added to the remaining splicing factors. See Materials and methods for details.

performed in the presence of SF1, SF2, SF3 and U2AF. The SF1 fraction was treated with micrococcal nuclease to destroy residual snRNPs. With these fractions neither a pre-splicing complex (complex A) nor a spliceosome (complex B) is formed due to the lack of U1 and U2 snRNPs. Upon addition of the U2/U4/U6 fraction (0.4 μl/reaction) complex A is efficiently made, but complex B does not form. It should be noted that the U2/U4/U6 fraction also contains some U1 and U5 snRNPs. The concentration of U1 in this fraction is sufficient to assemble complex A. In fact, the fraction can be diluted up to 100-fold before U1 snRNP becomes limiting and pre-splicing complex formation is abolished (unpublished results). Complex B is detected only when fractions enriched in U1 and U5 snRNPs are included in the reaction. The addition of Mono Q fractions 76 and 78, which contain high concentrations of U2 and U4/U6 snRNPs, does not result in the appearance of complex B. These results indicate that a minimal amount of U1/U5 fraction has to be included in the *in vitro* reaction to support spliceosome assembly. In addition, the observation that SF4 activity is not detected in these fractions suggests that this factor functions subsequent to the assembly of the splicing complex.

To analyze the effect of the snRNPs in spliceosome formation in more detail and to test the possibility that SF4 is dispensable for this step a standard assay was performed

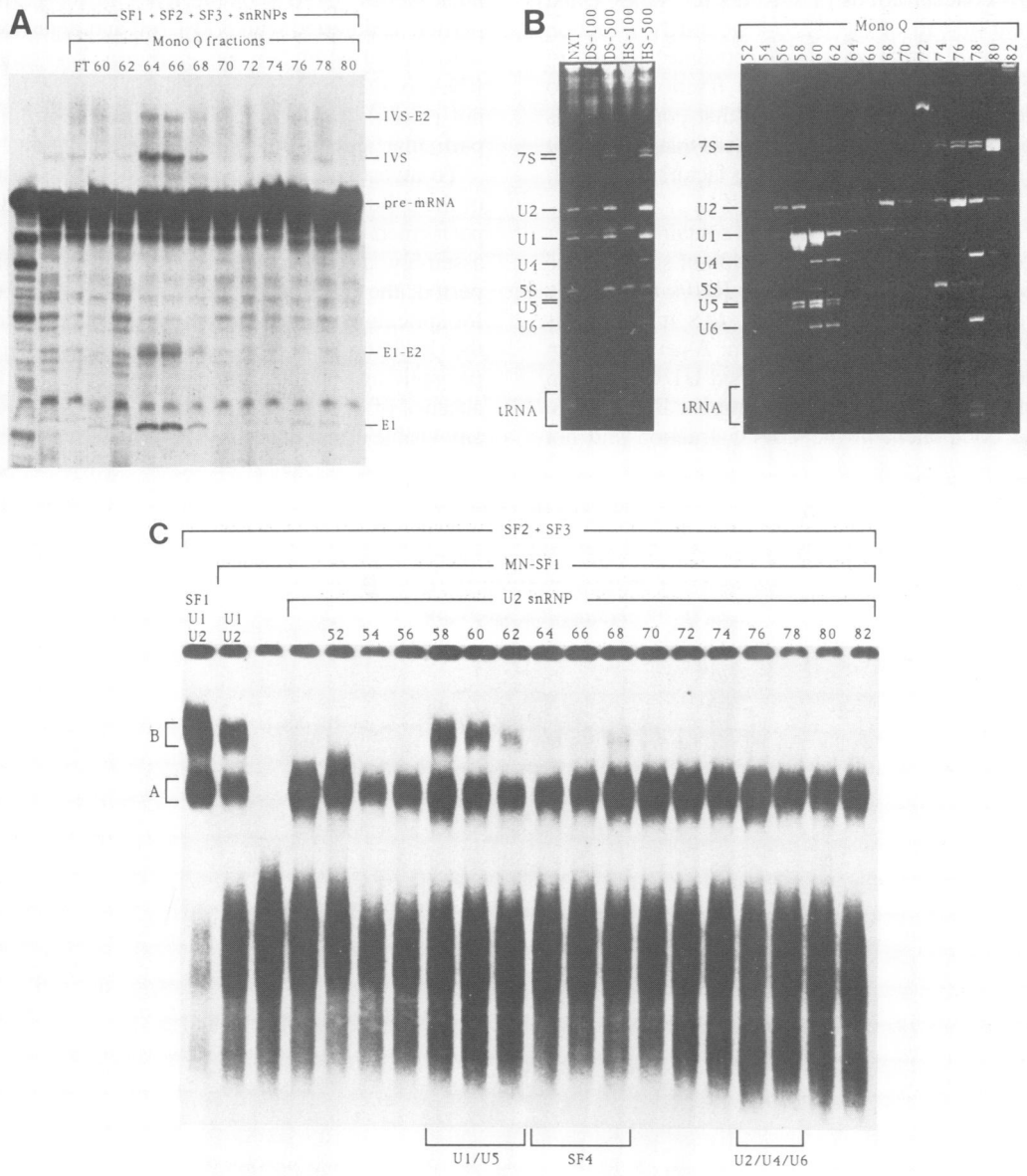

Fig. 2. Analysis of SF4 activity, snRNPs and splicing complex formation in Mono Q fractions. (**A**) Standard splicing reactions containing SF1, SF2, SF3, and snRNP fractions were performed in the presence of 3.5 μl of the Mono Q fractions indicated above each lane. The products of the reaction are indicated on the right. IVS-E2: intron−exon 2 lariat; IVS: intron lariat; pre-mRNA: unprocessed RNA; E1-E2: spliced RNA; E1: cleaved exon 1. (**B**) RNA from 20 μl of the fractions indicated above the figure was size-fractionated in a denaturing 10% polyacrylamide gel. Lanes: NXT, nuclear extract; DS-100, DS-500, HS-100, and HS-500, DEAE− or heparin−Sepharose flow-through and bound material, respectively. The RNAs are indicated on the left of both panels. (**C**) Splicing reactions (10 μl) were reconstituted as indicated above the panel with 0.6 μl of SF2 (derived from a Mono Q column), 1 μl of SF3, 1.2 μl of SF1 or 1.5 μl of micrococcal nuclease-treated SF1 (MN-SF1), 1.8 μl of a U1/U5 snRNP-containing fraction (U1), and 0.4 μl of a U2/U4/U6 snRNP-enriched fraction (U2 snRNP). Mono Q fractions 52−82 (1.8 μl each) were added to a mixture of SF2, SF3, MN-SF1 and U2 snRNP where indicated. The positions of complexes A and B are indicated on the left. Fractions enriched in U1/U5 snRNPs, SF4 activity, or U2/U4/U6 snRNPs are marked below the figure.

in the absence of SF4 and the concentration of the snRNPs was titrated. The snRNP fractions used are derived from a Mono Q column and lack any detectable SF4 activity (not shown). In a reaction that contains SF1, SF2, SF3 and U2AF, only complex A is formed (Figure 3A, lane 2). The snRNPs necessary for its appearance are supplied by the SF1 fraction (Krämer, 1988). Splicing complexes are beginning to form when 0.5 μl of each of the snRNP fractions are added. A further increase in the concentration of U1/U5 snRNPs in the reaction up to 4 μl results in a marked enhancement of the assembly of complex B (lanes 3–6). With 4 μl of the U1/U5 fraction the level of spliceosome formation observed is similar to that found in the presence of SF4 and low concentrations of snRNPs (cf. lanes 1 and 6). The addition of 1 μl of the U2/U4/U6 snRNP fraction also results in a stimulation of spliceosome assembly; however, higher concentrations of this fraction have no additional effect. These results indicate that complex B is formed in the absence of SF4 provided that sufficient quantities of snRNPs are added to splicing factors SF1, SF2, SF3, and U2AF.

Apart from snRNPs the U1/U5 fraction contains a number of proteins. To ascertain that the stimulation of spliceosome assembly is caused by the addition of snRNPs and not by some as yet unidentified protein factor, this fraction was subjected to micrococal nuclease digestion. As evident from Figure 3B, the micrococcal nuclease-treated U1/U5 fraction does not function in the formation of complex B. Thus, an essential RNA component in the U1/U5 fraction and not merely protein is essential at this step, supporting our assumption that higher concentrations of snRNPs are required for spliceosome assembly than for the formation of a pre-splicing complex.

Chabot *et al.* (1985) have shown that in nuclear extracts, U5 RNA, in contrast to most other snRNAs, is rather inaccessible to digestion with micrococcal nuclease. Although this characteristic should help to determine whether it is U1 or U5 snRNP that is responsible for the stimulation of spliceosome formation, we found that the partially purified U5 particle is readily digested by the nuclease (not shown). Whereas only ~50% of U5 RNA in nuclear extract is degraded at a concentration of 10 000 U micrococcal nuclease/ml, >90% of the RNA in the partially purified particle is digested with 500 U/ml and digestion is complete with a ten-fold higher concentration. The sensitivity of U1 RNA to micrococcal nuclease was similar. It was therefore not possible to distinguish between the requirement for a particular snRNP in spliceosome formation.

To investigate the function of SF4 in the following steps of the splicing pathway, the factor was titrated in a reaction performed under conditions optimal for spliceosome assembly, as established above. At the end of the incubation period, the reactions were divided. One half was analyzed for splicing complex formation, the other half was used for the detection of splicing products. As shown in Figure 4, pre-splicing complexes and spliceosomes are formed in the absence of SF4 and the addition of the factor does not have any visible effect (lanes 1–4). When the RNA isolated from

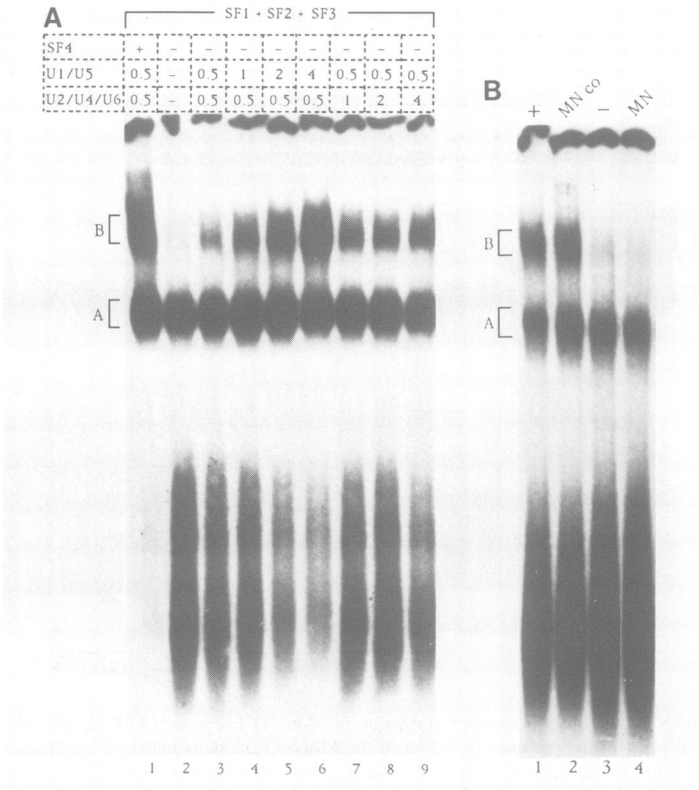

Fig. 3. (**A**) Titration of snRNPs. Splicing reactions were performed in a total volume of 25 μl in the presence of 3 μl of SF1, 2 μl of SF2, 3 μl of SF3, and 3.5 μl of SF4 (where indicated). U1/U5 and U2/U4/U6 snRNP-enriched fractions were added at the concentrations indicated above the figure. Ten microliters of the reaction were used for the experiment shown. (**B**) Micrococcal nuclease sensitivity of spliceosome formation. Splicing reactions were performed as in (A) in the absence of SF4. The U1/U5 fraction (4 μl) that was added to the reaction was either untreated (lane 1) or preincubated in the absence (lane 2) or in the presence (lane 4) of micrococcal nuclease. U1/U5 was omitted from the reaction shown in lane 3. The positions of complexes A and B are indicated on the left of the figure.

these reactions is analyzed it is apparent that splicing does not occur in the absence of SF4 (lane 5). Increasing the SF4 concentration in the reaction results in the appearance of splicing products (lanes 6–8). From this result we conclude that SF4 functions subsequent to the formation of complex B.

Lamond *et al.* (1987) and Konarska (1989) have previously reported the existence of three splicing-related complexes in the mammalian splicing system. Complex α (or A) corresponds to the pre-splicing complex and contains unspliced pre-mRNA as well as as U2 RNA. Complex β (or B) also contains unprocessed RNA and, in addition U2, U4, U5 and U6 RNAs. Complex γ (or C) is characterized by the presence of splicing intermediates as well as U2, U5 and U6 RNAs and thus represents the active spliceosome. In the system used here, only two complexes specific for intron-containing RNA substrates can be separated unambiguously. Complex B appears to be a mixture of splicing complexes B and C. We obtained a similar separation to Konarska (1989) in reactions performed with nuclear extract and AdML pre-mRNA in the absence of polyvinylalcohol and omitting the heparin treatment prior to electrophoresis in Tris–borate buffered gels (not shown). In the presence of heparin, however, only two complexes (A and B) are usually detected and Frendewey *et al.* (1987) have shown that complex B contains splicing intermediates, i.e. it represents (at least in part) active spliceosomes. Splicing in the presence of fractionated components requires the inclusion of polyvinylalcohol in the assay which markedly stimulates splicing activity (Krainer *et al.*, 1984). These reactions have to be treated with heparin prior to gel electrophoresis to avoid retention of complexed RNA at the top of the gel and to obtain a separation of specific complexes. Under these conditions a structure similar to complex C has not been observed consistently. It is therefore difficult to assess, whether both complexes B and C or only complex B is formed in the absence of SF4.

We have however analyzed whether the splicing complex assembled in the absence of SF4 is functional, i.e. whether the pre-mRNA within complex B can be chased into spliced products after the addition of the factor. If this were the case, we would expect that the lag phase observed during the regular splicing reaction (Hernandez and Keller, 1983) disappears or is considerably reduced. Reactions were incubated in the absence of SF4 for 60 min to allow splicing complexes to form. At this time SF4 was added and incubation was continued for increasing periods up to 120 min. In a control experiment performed in parallel, the kinetics of the splicing reaction in the presence of SF4 were analyzed. As shown in Figure 5, in the control reactions spliced products are generated within 60 min (panel A,

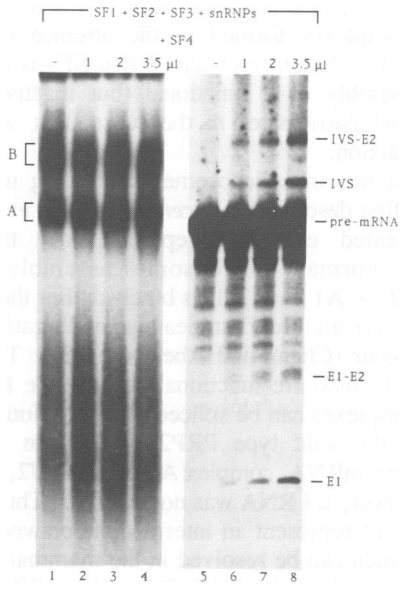

Fig. 4. Titration of SF4 activity in splicing complex assembly and splicing. Standard splicing reactions (25 µl) were performed in the presence of increasing concentrations of SF4 (as indicated above the figure). After 2 h at 30°C, 10 µl of the reactions were analyzed for complex assembly (lanes 1–4) and 15 µl were analyzed for splicing products (lanes 5–8). Splicing complexes A and B are indicated on the left, splicing products are shown on the right.

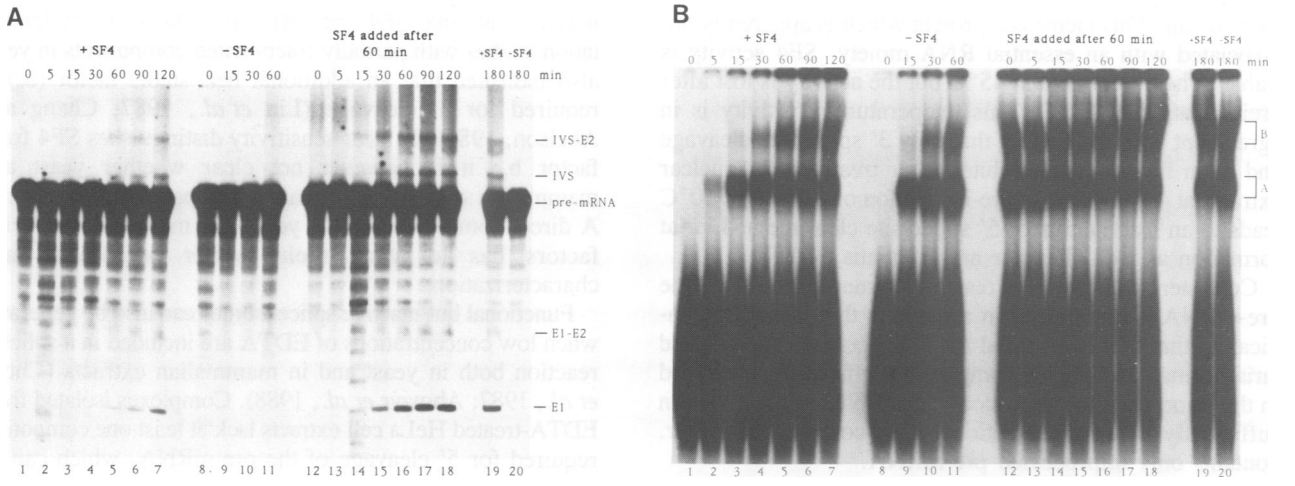

Fig. 5. Chasing of pre-assembled substrate RNA into splicing products. Splicing reactions (25 µl) contained 4 µl SF1 (devoid of U2AF; see Materials and methods), 1 µl SF2, 2.5 µl SF3, 1 µl U2AF (purified as described in Materials and methods), 2 µl U1/U5 and 0.5 µl U2/U4/U6 snRNPs. SF4 (2.5 µl) was added to the reactions before the start of the reaction (lanes 1–7, 19) or after a 60-min incubation at 30°C (lanes 12–18). Reactions shown in lanes 8–11 and 20 were performed in the absence of SF4. The length of incubation at 30°C is indicated on top of each panel. (**A**) 15 µl of the reactions were processed for the analysis of splicing products. (**B**) The remaining 10 µl were used for the analysis of splicing complexes. The positions of the reaction products and of complexes A and B are indicated on the right side of the respective panel.

lanes 1–7) concomitant with the assembly of complex B (panel B). When SF4 is omitted from the reaction complex B forms with similar kinetics and, as expected, the pre-mRNA is not spliced (lanes 8–11 and 20). The addition of SF4 to such a reaction results in splicing of the pre-mRNA within 15 min and reaction products accumulate upon further incubation (panel A, lanes 12–18). The reduction of the lag phase in this experiment from 30–60 min to ~5–15 min strongly implies that the complexes formed in the absence of SF4 are functional. This observation makes it unlikely that pre-mRNA which was not sequestered into a specific complex is spliced upon addition of SF4. It is also apparent from Figure 5A that splicing proceeds most efficiently when the pre-mRNA is assembled into a splicing complex prior to the addition of SF4. The reason for this observation is not known. One possible explanation could be that a component present in the SF4 fraction interferes with spliceosome formation.

Close inspection of the splicing complexes assembled in those reactions that generated spliced RNA reveals a faint smear above complex B (cf. lanes 12 and 18, and lanes 19 and 20 in panel B) which could possibly represent complex C. This may indicate that only complex B but not complex C is formed in the absence of SF4. At present, this question cannot be answered conclusively because of the limitations of the electrophoretic separation methods available.

Discussion

To elucidate the function of components that splice nuclear pre-mRNA, we have used a reconstituted system which contains factors partially purified from HeLa cell nuclear extracts. We have previously shown that SF1 and SF3 in combination with snRNPs participate in pre-splicing complex formation (Krämer, 1988). More recently it has become evident that a third factor, U2AF, is required for this step as well (Zamore and Green, 1989; our unpublished data). The remaining two factors, SF2 and SF4, are dispensable for pre-splicing complex formation and thus act at some later step in the splicing pathway.

In this report we have analyzed the function of SF4 in more detail. This factor is a protein which is apparently not associated with an essential RNA moiety. SF4 activity is stable to heat treatment at 45°C but the activity is lost after preincubation at 50°C. This temperature-sensitivity is in agreement with the finding that only 3′ splice site cleavage and exon ligation are inhibited after treatment of nuclear extracts at 45°C whereas pre-incubation of extracts at 50°C leads to an inactivation of 5′ splice site cleavage and lariat formation as well (Krainer and Maniatis, 1985).

Consistent with previous results (Krämer *et al.*, 1987) the pre-mRNA is not spliced in a reaction that lacks SF4, indicating that SF4 is essential for 5′ splice site cleavage and lariat formation. Splicing complex B is efficiently assembled in the same reaction, provided that snRNPs are included in sufficiently high concentrations. This complex, however, contains only unprocessed pre-mRNA.

As mentioned above, the originally defined splicing complex or spliceosome (Grabowski *et al.*, 1985; Frendewey and Keller, 1985) can be separated into two distinct structures with suitable methods (Lamond *et al.*, 1987; Konarska, 1989). For technical reasons this separation was not achieved with splicing reactions containing fractionated components (see Results). It therefore remains unclear whether only complex B or an inactive complex C is formed in the absence of SF4. In the experiment shown in Figure 5 we do, however, observe a faint smear above complex B in reactions that contained spliced RNA, which may correspond to a low amount of complex C. Although a better resolution of the splicing complexes is required, this result would imply that, in the absence of SF4, complex B is formed whereas its conversion to the active splicing complex C is impaired. We have shown that substrate RNA which is preassembled into complex B is spliced with accelerated kinetics after addition of SF4 compared with a reaction that contained SF4 throughout the entire incubation time. This result suggests that the complexes formed in the absence of SF4 are functional. We therefore conclude that SF4 is dispensable for the assembly of a functional (but inactive) splicing complex and participates in the subsequent steps of the splicing reaction.

The existence of spliceosomes containing unprocessed RNA was first described in *S.cerevisiae* (Lin *et al.*, 1987). In heat-treated extracts prepared from the mutant *prp2* the normal spliceosome assembly pathway (B → A2-2 → A1 → A2-1) is blocked after the formation of complex A1 and the first cleavage and ligation reaction does not occur (Cheng and Abelson, 1987). The splicing complexes formed are functional because the RNA in the isolated complexes can be spliced after addition of extracts containing the wild type PRP2 protein. In addition to unspliced pre-mRNA, complex A1 contains U2, U5 and U6 RNA; however, U4 RNA was not detected. Thus, complex A1 appears to represent an intermediate between complex B and C which can be resolved in the mammalian splicing system (Lamond *et al.*, 1987; Konarska, 1989).

SF4 acts at a similar step of the splicing reaction as PRP2, in that a functional splicing complex is generated in its absence and the pre-mRNA remains unprocessed. It is thus possible that SF4 represents the mammalian counterpart of the yeast protein. In the most purified SF4 preparation a polypeptide of ~110 kd cofractionates with the activity (unpublished data), whereas PRP2 has an estimated molecular weight of 100 kd (Last and Woolford, 1986). This may indicate that SF4 and PRP2 are related. Complementation studies with partially fractionated components in yeast also indicated that an additional heat-stable factor (b_n) is required for 5′ cleavage (Lin *et al.*, 1987; Cheng and Abelson, 1987). Its heat-sensitivity distinguishes SF4 from factor b_n; it is however not clear whether yeast and mammalian splicing factors can be compared by this criteria. A direct comparison of the yeast and mammalian splicing factors has to await their further purification and characterization.

Functional but inactive spliceosomes can also be generated when low concentrations of EDTA are included in a splicing reaction both in yeast and in mammalian extracts (Cheng *et al.*, 1987; Abmayr *et al.*, 1988). Complexes isolated from EDTA-treated HeLa cell extracts lack at least one component required for 5′ cleavage of the pre-mRNA, which can be supplied either by a micrococcal nuclease-treated nuclear extract or by a S-100 fraction (Abmayr *et al.*, 1988). This factor is resistant to temperatures of at least 50°C. Considering that the first cleavage and ligation reaction can be inactivated by preincubation of a nuclear extract for 10 min at 50°C but not at 45°C (Krainer and Maniatis, 1985), it

appears that in addition to the factor identified by Abmayr *et al.* (1988) another component(s) participates in this reaction. Based on the observation that SF4 activity is abolished at 50°C we conclude that this splicing factor is different from the factor mentioned above.

Although we have shown that SF4 is dispensable for the assembly of a functional splicing complex, it remains unclear how this factor participates in the splicing reaction. Several possible functions can be envisioned. First, from the data available it appears that SF4 can interact with the splicing complex that is formed in its absence. It could then function directly in the subsequent steps of the reaction. Alternatively SF4 may become assembled into the spliceosome allowing other factors to associate which in turn may allow splicing to proceed. Second, the reactive sites in the pre-mRNA have to be aligned prior to cleavage and ligation. Although the snRNPs are thought to be the major determinants of this alignment, SF4 could aid in conformational changes that take place within the spliceosome. The PRP2 protein in yeast which functions in a similar step to SF4 is one component necessary for the conversion of complex A1 (the functional but inactive splicing complex) to complex A2-2 (the active spliceosome). Although both complexes sediment with 40S in glycerol gradients (Lin *et al.*, 1987) they can be separated by gel electrophoresis (Cheng and Abelson, 1987), suggesting a conformational change within the splicing complex. Third, ATP hydrolysis is necessary for the formation of the active splicing complex and for both cleavage and ligation reactions (Lin *et al.*, 1987; Cheng and Abelson, 1987; Abmayr *et al.*, 1988; Sawa *et al.*, 1988; Vijayraghavan and Abelson, 1990) implying the action of ATPases. The association of an ATPase activity with SF4 can probably be ruled out since no ATP-hydrolyzing activity could be detected in SF4-containing fractions (unpublished observation).

At this point we cannot address the question of whether SF4 is only needed for the conversion of a functional into an active splicing complex or whether it is directly required for 5′ cleavage and lariat formation and/or 3′ cleavage and exon ligation. Two factors have been described that function in the second step of the reaction (Krainer and Maniatis, 1985; Perkins *et al.*, 1986; Vijayraghavan *et al.*, 1989; Vijayraghavan and Abelson, 1990). These proteins are most likely distinct from SF4, since 5′ cleavage occurs in their absence. In addition, a factor essential for 3′ cleavage cofractionates with SF3 activity on a Mono Q column but not with SF4 (unpublished results).

During the characterization of SF4 activity we observed that a factor limiting for spliceosome formation in the reconstituted system is enriched in the U1/U5 fraction. Micrococcal nuclease treatment of this fraction confirmed that the limiting component is a snRNP and not a protein factor. As all spliceosomal snRNPs are present in this fraction it is not possible to determine which snRNP is present in limiting amounts. However, a few points should be considered. First, it has been shown that U4, U5 and U6 snRNPs bind to the pre-mRNA as a preformed complex and multi-snRNP assemblies have been detected in splicing extracts (Konarska and Sharp, 1987, 1988; Cheng and Abelson, 1987; Black and Pinto, 1989). Chromatography of snRNPs on Mono Q columns results in the separation of different snRNP assemblies. U4, U5 and U6 snRNPs appear to exist in one complex in the early eluting fractions (58–62) whereas snRNPs eluting at high salt (fraction 78) occur as smaller independent species (S.Bienroth and W.Keller, personal communication). It could thus be that only those snRNPs present in larger assemblies are active in spliceosome formation.

Second, chromatography of snRNPs may lead to a loss of proteins specifically associated with one type of particle (Bach *et al.*, 1989). Interestingly, one of the U5-specific proteins becomes incorporated into the spliceosome (Lossky *et al.*, 1987; Pinto and Steitz, 1989; Anderson *et al.*, 1989; Whittaker *et al.*, 1990; Garcia-Blanco *et al.*, 1990). If U5 snRNP present in the U1/U5 fraction was partially depleted of this protein this could explain why spliceosome formation occurs efficiently only with relatively high concentrations of the U1/U5 fraction. Similarly, U1 snRNP lacking one or more of its specific proteins can be obtained by Mono Q chromatography, although under conditions that are slightly different from the ones used here (Bach *et al.*, 1990), and it has been shown that the U1 specific C-protein plays a role in the binding of U1 snRNP to the 5′ splice site (Heinrichs *et al.*, 1990).

Third, U1 and U2 snRNP interact with the pre-mRNA to form the pre-splicing complex but they also function in the subsequent conversion of this complex to the spliceosome (for review see Guthrie and Patterson, 1988; Steitz *et al.*, 1988). Although it has been shown that different portions of these snRNPs are required at specific steps of spliceosome assembly (Frendewey *et al.*, 1987; Zillmann *et al.*, 1988; Lamond *et al.*, 1989), it is unknown whether the concentration of U1 and/or U2 snRNP also effects spliceosome formation. To distinguish between these possibilities it will be necessary to separate the individual snRNP species and to test them directly in the reconstituted system.

In conclusion, we have characterized a splicing factor that acts subsequent to the formation of a functional spliceosome. Two proteins have been identified in yeast which act at a similar step in the splicing pathway and it is possible that SF4 represents the mammalian analogue of one of these factors.

Materials and methods

Fractionation of splicing activities

Splicing activities have been partially purified from HeLa cell nuclear extracts (Dignam *et al.*, 1983). The details of the fractionation procedure, including chromatography on DEAE–Sepharose, heparin–Sepharose and Mono Q columns, have been described (Krämer *et al.*, 1987; Krämer and Keller, 1990). In the experiments presented here, the DEAE–Sepharose flow-through fraction (DS100) was used as a source of SF1 and U2AF, and the heparin–Sepharose flow-through (HS100) as a source of SF2. SF3 and SF4 activities were pools of active Mono Q fractions. SnRNP fractions enriched in either U1 and U5 or in U2, U4 and U6 snRNPs were also derived from Mono Q columns.

For the experiment shown in Figure 5, a DS100 fraction (SF1) was used that was free of U2AF (unpublished results). This factor was purified over DEAE–Sepharose, heparin–Sepharose, Poly U–Sepharose (Zamore and Green, 1989) and Mono Q where it elutes at ~0.2 M KCl (Ruskin *et al.*, 1988; unpublished results). The SF4 activity used in this experiment was further purified from Mono Q fractions by chromatography on Blue-Sepharose and spermine–agarose, the details of which will be published elsewhere.

Preparation of [α-^{32}P]UTP labeled pre-mRNA

The pre-mRNA used as a substrate for splicing is a derivative of the AdML transcription unit. It is synthesized *in vitro* from plasmic pSP62Δil as described (Frendewey and Keller, 1985). The transcript contains 102 nucleotides of exon 1, 113 nucleotides of intron sequences and 38 nucleotides of exon 2.

In vitro splicing reactions

A standard 25 μl splicing reaction contained 0.4 mM ATP, 10 mM creatine-phosphate, 2.5 mM $MgCl_2$, 50 mM KCl, 10% (v/v) glycerol, 10 mM HEPES–KOH (pH 7.9), 0.05 mM EDTA, 0.25 mM dithiothreitol, 1.25 μg tRNA, 2.6% (v/v) polyvinyl alcohol (Sigma, type 2) and 20 000 Cerenkov c.p.m. of uniformly labeled pre-mRNA (5.5 fmol). Unless indicated otherwise, the following amounts of column fractions were included in the assays: 2.5 μl SF1, 1.5 μl SF2, 2.5 μl SF3, 3.5 μl SF4, 2 μl U1/U5 snRNPs and 0.5 μl U2/U4/U6 snRNPs. Reactions were performed for 2 h at 30°C and analyzed for splicing complexes in native 4% polyacrylamide gels or for splicing products in denaturing 12% polyacryalmide gels (Krämer and Keller, 1985; Krämer, 1988). When a single reaction was used for the analysis of both, 50 000 Cerenkov c.p.m. were included in a 25 μl reaction, 10 μl of which were used for complex formation, and 15 μl for the analysis of spliced RNA.

Micrococcal nuclease digestion and NEM treatment of SF4-containing fractions were performed as described (Krämer and Keller, 1985; Krämer *et al.*, 1987). Heat treatment of SF4 activity was done for 10 min at the temperature indicated in the figure legend.

Acknowledgements

We are grateful to Walter Keller for his support throughout this work, for stimulating discussions and the revision of the manuscript. We thank Barbara Widmer for cell culture and extract preparation and Graeme Bilbe for comments on the manuscript. This work was supported by the Kantons of Basel and by the Schweizerischer Nationalfonds.

References

Abmayr,S.M., Reed,R. and Maniatis,T. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7216–7220.

Anderson,G.J., Bach,M., Lührmann,R. and Beggs,J.D. (1989) *Nature*, **342**, 819–821.

Bach,M., Winkelmann,G. and Lührmann,R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6038–6042.

Bach,M., Krol,A. and Lührmann,R. (1990) *Nucleic Acids Res.*, **18**, 449–457.

Black,D.L. and Pinto,A.L. (1989) *Mol. Cell. Biol.*, **9**, 3350–3359.

Blencowe,B.J., Sproat,B.S., Ryder,U., Barabino,S. and Lamond,A.I. (1989) *Cell*, **59**, 531–539.

Chabot,B., Black,D.L., LeMaster,D.M. and Steitz,J.A. (1985) *Science*, **230**, 1344–1349.

Chang,T.H., Clark,M.W., Lustig,A.J., Cusick,M.E. and Abelson,J. (1988) *Mol. Cell. Biol.*, **8**, 2379–2393.

Cheng,S.-C. and Abelson,J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2387–2391.

Cheng,S.-C. and Abelson,J. (1987) *Genes Dev.*, **1**, 1014–1027.

Choi,Y.D. Grabowski,P.J., Sharp,P.A. and Dreyfuss,G. (1986) *Science*, **231**, 1534–1539.

Dignam,J.D., Lebovitz,R.M .and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.

Frendewey,D. and Keller,W. (1985) *Cell*, **42**, 355–367.

Frendewey,D., Krämer,A. and Keller,W. (1987) *Cold Spring Harbor Symp. Quant. Biol.*, **52**, 287–298.

Fu,X.-D. and Maniatis,T. (1990) *Nature*, **343**, 437–441.

Garcia-Blanco,M.A., Jamison,S.F. and Sharp,P.A. (1989) *Genes Dev.*, **3**, 1874–1886.

Garcia-Blanco,M.A., Anderson,G.J., Beggs,J. and Sharp,P.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3082–3086.

Gerke,V. and Steitz,J.A. (1986) *Cell*, **47**, 973–984.

Grabowski,P.J., Seiler,S.R. and Sharp,P.A. (1985) *Cell*, **42**, 345–353.

Green,M.R. (1986) *Annu. Rev. Genet.*, **20**, 671–708.

Guthrie,C. and Patterson,B. (1988) *Annu. Rev. Genet.*, **22**, 387–419.

Heinrichs,V., Bach,M., Winkelmann,G. and Lührmann,R. (1990) *Science*, **247**, 69–72.

Hernandez,N. and Keller,W. (1983) *Cell*, **35**, 89–99.

Konarska,M.M. (1989) *Methods Enzymol.*, **180**, 442–453.

Konarska,M.M. and Sharp,P.A. (1987) *Cell*, **49**, 763–774.

Konarska,M.M. and Sharp,P.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5459–5462.

Krainer,A.R. and Maniatis,T. (1985) *Cell*, **42**, 725–736.

Krainer,A.R., Maniatis,T., Ruskin,B. and Green,M.R. (1984) *Cell*, **36**, 993–1005.

Krainer,A.R., Conway,G.C. and Kozak,D. (1990) *Genes Dev.*, **4**, 1158–1171.

Krämer,A. (1988) *Genes Dev.*, **2**, 1155–1167.

Krämer,A. and Keller,W. (1985) *EMBO J.*, **4**, 3571–3581.

Krämer,A. and Keller,W. (1990) *Methods Enzymol.*, **181**, 3–19.

Krämer,A., Frick,M. and Keller,W. (1987) *J. Biol. Chem.*, **262**, 17630–17640.

Lamond,A.I., Konarska,M.M. and Sharp,P.A. (1987) *Genes Dev.*, **1**, 532–543.

Lamond,A.I., Konarska,M.M., Grabowski,P.J. and Sharp,P.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 411–415.

Lamond,A., Sproat,B., Ryder,U. and Hamm,J. (1989) *Cell*, **58**, 383–390.

Last,R.L. and woolford,J.L.,Jr (1986) *J. Cell Biol.*, **103**, 2103–2112.

Lin,R.-J., Lustig,A.J. and Abelson,J. (1987) *Genes Dev.*, **1**, 7–18.

Lossky,M., Anderson,G.L., Jackson,S.P. and Beggs,J. (1987) *Cell*, **51**, 1019–1026.

Lustig,A.J., Lin,R.-J. and Abelson,J. (1986) *Cell*, **47**, 953–963.

Padgett,R.A., Grabowski,P.J., Konarska,M.M., Seiler,S.R. and Sharp,P.A. (1986) *Annu. Rev. Biochem.*, **55**, 1119–1150.

Parker,R., Siliciano,P. and Guthrie,C. (1987) *Cell*, **49**, 229–239.

Perkins,K.K., Furneaux,H.M. and Hurwitz,J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 887–891.

Pikielny,C.W., Rymond,B.C. and Rosbash,M. (1986) *Nature*, **324**, 341–345.

Pinto,A.L. and Steitz,J.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8742–8746.

Ruskin,B., Zamore,P.D .and Green,M.R. (1988) *Cell*, **52**, 207–219.

Sawa,H., Ohno,M., Sakamoto,H. and Shimura,Y. (1988) *Nucleic Acids Res.*, **16**, 3157–3164.

Steitz,J.A., Black,D.L., Gerke,V., Parker,K.A., Krämer,A., Frendewey,D. and Keller,W. (1988) In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer-Verlag, Heidelberg, pp. 115–154.

Swanson,M.S. and Dreyfuss,G. (1988) *Mol. Cell. Biol.*, **8**, 2237–2241.

Tazi,J., Alibert,C., Temsamani,J., Reveillaud,I., Cathala,G., Brunel,C. and Jeanteur,P. (1986) *Cell*, **47**, 755–766.

Vijayraghavan,U., Company,M. and Abelson,J. (1989) *Genes Dev.*, **3**, 1206–1216.

Vijayraghavan,U. and Abelson,J. (1990) *Mol. Cell. Biol.*, **10**, 324–332.

Whittaker,E., Lossky,M. and Beggs,J.D. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2216–2219.

Wu,J.L. and Manley,J.L. (1989) *Genes Dev.*, **3**, 1553–1561.

Zamore,P.D. and Green,M.R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9243–9247.

Zapp,M.L. and Berget,S.M. (1989) *Nucleic Acids Res.*, **17**, 2655–2674.

Zhuang,Y. and Weiner,A.M. (1986) *Cell*, **46**, 827–835.

Zhuang,Y. and Weiner,A.M. (1989) *Genes Dev.*, **3**, 1545–1552.

Zillmann,M., Zapp,M. and Berget,S.M. (1988) *Mol. Cell. Biol.*, **8**, 814–821.

Received on June 11, 1990; revised on August 20, 1990