Heterogeneous Nuclear Ribonucleoproteins: Role in RNA Splicing

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Splicing in vitro of a messenger RNA (mRNA) precursor (pre-mRNA) is inhibited by a monoclonal antibody to the C proteins (anti-C) of the heterogeneous nuclear RNA (hnRNA)–ribonucleoprotein (hnRNP) particles. This antibody, 4F4, inhibits an early step of the reaction: cleavage at the 3′ end of the upstream exon and the formation of the intron lariat. In contrast, boiled 4F4, or a different monoclonal antibody (designated 2B12) to the C proteins, or antibodies to other hnRNP proteins (120 and 68 kilodaltons) and nonimmune mouse antibodies have no inhibitory effect. The 4F4 antibody does not prevent the adenosine triphosphate–dependent formation of a 60*S* splicing complex (spliceosome). Furthermore, the 60*S* splicing complex contains C proteins, and it can be immunoprecipitated with 4F4. Depletion of C proteins from the splicing extract by immunoadsorption with either of the two monoclonal antibodies to the C proteins (4F4 or 2B12) results in the loss of splicing activity, whereas mock-depletion with nonimmune mouse antibodies has no effect. A 60*S* splicing complex does not form in a C protein–depleted nuclear extract. These results indicate an essential role for proteins of the hnRNP complex in the splicing of mRNA precursors.

MOST PROTEIN-CODING GENES IN HIGHER EUKARYOTES contain intervening, noncoding sequences (introns). The precise splicing of precursor nuclear RNA's to remove the intervening sequences and yield mature messenger RNA's (mRNA's) is central to eukaryotic gene expression (*1*). Distinct canonical sequences define the 5′ and 3′ boundaries of introns and their junctions with the flanking coding sequences (exons) (*2*). The intron-exon junctions are common to and therefore potentially interchangeable between junctions in the same or different precursor mRNA's (pre-mRNA's) (*2, 3*). Because the splicing of pre-mRNA involves endonucleolytic cleavage and ligation of intron-exon junctions, it follows that the splicing machinery must be capable of aligning and holding together the intermediates of pre-mRNA splicing during the splicing process in order to prevent undesirable formation of alternate splicing and of chimeric mRNA's.

Biochemical and microscopic evidence demonstrates that mRNA precursors exist in the nucleus in association with specific protein complexes to form ribonucleoprotein (RNP) particles, termed heterogeneous nuclear RNP (hnRNP) complexes or hnRNP particles (*4–11*). It was therefore considered likely that these structures are important for the biogenesis of mRNA, and that the conversion of pre-mRNA to mRNA takes place in these particles. Large hnRNP complexes have been isolated (*11*) with the use of monoclonal antibodies to some of the major hnRNP proteins (*9, 10*), and some of the major components and structural features of the hnRNP complex were characterized (*11, 12*). The major structural element of the hnRNP complex is a monomer particle that sediments in sucrose gradients at about 30*S* (at 100 m*M* NaCl) (*4–8*). The hnRNP complex is composed of 10 to 15 major [^{35}S]methionine-labeled proteins as determined by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). These include the A1, A2, B1, B2, C1, and C2 proteins (32 to 43 kD) and doublets at 68 and 120 kD (*11*). The 30*S* monoparticle normally accommodates about 500 ± 100 nucleotides (nt) of hnRNA. Large hnRNP complexes are composed mostly of multiples of 30*S* particles that are connected by highly nuclease-sensitive stretches of hnRNA. At least 75 percent of the hnRNA is associated with 30*S* particles, and most, if not all, of this RNA sequence is accessible to nuclease. Nuclease digestion experiments also revealed that after cleavage of the hnRNA within a 30*S* particle the hnRNA fragments and the proteins remain associated in a single complex; therefore these experiments suggested that the hnRNP complex can serve as an "operating table" for hnRNA processing (*12*). The proteins of the hnRNP complex may, therefore, be important in both the packaging of the hnRNA and in pre-mRNA splicing by functioning in the crucial substrate presentation and in the positioning of splicing intermediates.

With the development of in vitro cell-free systems which faithfully splice mRNA precursors (*13*) and with the availability of specific antibodies to proteins of the hnRNP complexes (*9, 10*), it has become possible to address the long-standing issue of the involvement of the hnRNP proteins and of hnRNP particles in the splicing of pre-mRNA. We report here that a monoclonal antibody to the hnRNP C proteins (C1 and C2, 41 and 43 kD, respectively), two of the major proteins of the hnRNP complex in vertebrate cells, inhibits the splicing of pre-mRNA in vitro. Furthermore, the C proteins are part of the splicing complexes, and depletion of the C proteins from splicing extracts abolishes splicing activity and the capacity to form a 60*S* splicing complex.

A monoclonal antibody to the hnRNP C proteins inhibits the splicing of pre-mRNA in vitro. A transcript corresponding to pre-mRNA (454 nt) (Fig. 1) was prepared by transcription with the *Salmonella* phage SP6 polymerase (*14*) and used as a substrate for splicing in vitro (*15–17*). The transcript contains a 5′-m^{7}GpppG cap structure added in vitro by the SP6 polymerase (*17*). Incubation of this ^{32}P-labeled pre-mRNA in the cell-free nuclear extract (*18*) for 30 minutes at 30°C under conditions optimal for splicing (*15, 16*)

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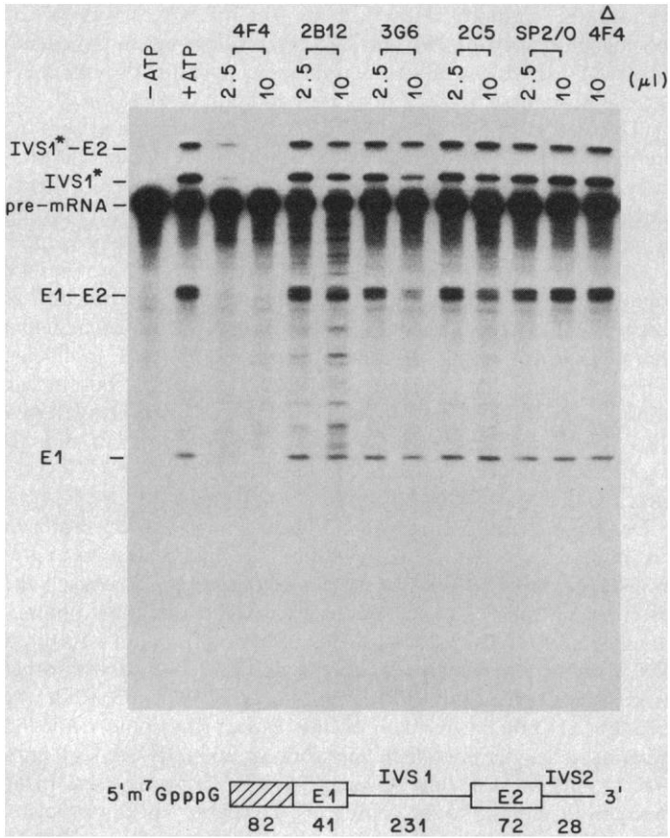

Fig. 1. The effect of monoclonal antibodies to hnRNP proteins on pre-mRNA splicing in vitro. (Lanes −ATP and +ATP) Control splicing reaction without or with ATP, respectively. Monoclonal antibodies (2.5 or 10 μl) added to each splicing reaction were as follows. (Lanes 4F4 and 2B12) Monoclonal antibodies to the C proteins of hnRNP (*9, 10*). (Lanes 3G6) Monoclonal antibody to the 120-kD protein of hnRNP (*9*). (Lanes 2C5) Monoclonal antibody to the 68-kD proteins of hnRNP (*20*). (Lanes SP2/0) Nonimmune mouse antibodies (*9, 10*). (Lane Δ4F4) Boiled 4F4. The structure of the precursor RNA is shown below, and the size of each sequence is shown in nucleotides. The pre-mRNA transcript (454 nt) contains the L1 exon (41 nt preceded by 82 nt of the sequence of the pSP62 plasmid downstream from the SP6 promoter, E1), a deleted form of the first intervening sequence (231 nt, IVS1), the L2 exon (72 nt, E2), and 28 nt of part of the second intervening sequence of adenovirus 2 (Ad-2) major late transcription unit. Splicing products were assigned as described (*16, 19*). IVS1* denotes the lariat form of IVS1. Precursor RNA was prepared by in vitro transcription with SP6 polymerase (*14*) from Bgl I linearized DNA template of pRSP-1–ΔIVS which contains the Ad-2 major late transcription unit (*15–17*). The cap $m^7G(5')ppp(5')G$ dinucleotide (0.5 m*M*) was included as a primer during in vitro transcription (*17*). RNA was purified by 10 percent polyacrylamide–8*M* urea gel electrophoresis with subsequent elution of the band by diffusion in 0.5*M* amonium acetate, 10 m*M* magnesium acetate, 0.1 percent sodium dodecyl sulfate, and 0.1 m*M* EDTA (*19*). Splicing reactions were carried out in 20 m*M* Hepes (*p*H 7.6), 1.5 m*M* ATP, 5 m*M* creatine phosphate, 2 m*M* $MgCl_2$, HeLa cell nuclear extract (44 percent, by volume), and ^{32}P-labeled precursor RNA (50,000 count/min) in a 25-μl reaction (*15, 16*). HeLa nuclear splicing extract was prepared by the procedure of Dignam *et al.* (*18*). In the antibody inhibition experiments splicing extracts (11 μl each) were first incubated (on ice) with the indicated amount of monoclonal antibodies to hnRNP proteins for 30 minutes, and the splicing reactions were carried out at 30°C for 30 minutes with precursor RNA (50,000 count/min each) and necessary components in 25 μl as described (*16*). After phenol extraction and ethanol precipitation, reaction products were analyzed by denaturing gel electrophoresis on 10 percent polyacrylamide–8*M* urea gel. Monoclonal antibodies were prepared from ascites fluid by affinity chromatography on protein A–agarose (Boehringer Mannheim) and dialysis against 10 m*M* Hepes (*p*H 7.6). The protein concentration was adjusted to 5 mg/ml and the relative titers of antibodies 4F4, 2B12, and 3G6 were confirmed by immunofluorescence microscopy on HeLa cells after serial dilution.

generated, in an adenosine triphosphate (ATP)–dependent reaction, several processed products that were resolved on a 10 percent polyacrylamide–8*M* urea gel (Fig. 1). The splicing products have been characterized (*16, 19*) and are shown in Fig. 1. In addition to the spliced mRNA (E1–E2), free first exon (E1) and the lariat forms of the intron (denoted by asterisk) IVS1* and IVS1*-E2 were also detected.

The effect of several monoclonal antibodies to hnRNP proteins on pre-mRNA splicing was investigated. These antibodies include the monoclonal antibodies 4F4 and 2B12 both of which recognize the antigenically related C1 and C2 proteins. These two monoclonal antibodies recognize distinct antigenic sites on both proteins (*9, 10*). Additional monoclonal antibodies tested include: 3G6, to the 120 kD hnRNP protein (*9*); 2C5, to 68 kD hnRNP proteins (*20*); and control nonimmune antibodies from a mouse inoculated with the parent myeloma line (SP2/0) used for the preparation of the hybridomas (*9, 10*). All the antibodies were prepared from ascitic fluids and purified by affinity chromatography on protein A–agarose. Similar amounts of each antibody were added to the extracts as determined by absorbance at 280 nm and by Coomassie blue staining after SDS-PAGE. The effect of the antibodies on the splicing of the pre-mRNA in vitro was assayed by adding either 2.5 or 10 μl of the antibody to the cell-free extract 30 minutes prior to initiation of the reaction and incubating at 0°C. One of the monoclonal antibodies to the C proteins, 4F4, inhibited the splicing of the pre-mRNA (Fig. 1). The inhibition occurs at an early step of the RNA splicing reaction because neither free exon 1 (E1) nor the lariat IVS1*-E2 was detected. In contrast to 4F4, the other antibodies including another antibody to the C proteins (2B12) did not have an effect. Boiled 4F4 antibody did not inhibit the reaction, an indication that the inhibition by 4F4 is not a consequence of salt or other nonspecific effect. Furthermore, 4F4 did not cause degradation of the pre-mRNA because the amount of pre-mRNA in the gel as well as total precipitable radioactivity were not reduced. At high concentrations the antibody to the 120-kD hnRNP protein (3G6) had some, but variable, effect on the amount of spliced mRNA. Multiple bands were reproducibly detected in reactions containing 2B12. We do not know the cause for this, but it is possible that 2B12 induces aberrant cleavages of the pre-mRNA. The extent of inhibition by 4F4 was proportional to the amount of antibody added (Fig. 2). In the assay conditions used, considerable inhibition was observed with 0.5 μl of 4F4 and complete inhibition of splicing occurred with 6 μl of antibody (Fig. 2). In subsequent experiments 10 μl of 4F4 were used to effect complete inhibition of splicing.

The antibody to the C proteins does not prevent the formation of the 60*S* splicing complex. Sedimentation experiments have shown that the splicing of pre-mRNA involves the formation of a large, rapidly sedimenting multicomponent splicing complex (*15, 21, 22*). The splicing complex, which contains protein and RNA, sediments between 40*S* and 60*S*, depending on the source of the extract (yeast or HeLa, respectively) and the sedimentation conditions. Formation of the complex is probably necessary for progression of the splicing reaction (*15, 21, 22*). To examine the effect of the antibody 4F4 on the formation of the splicing complex, sedimentation experiments were carried out on splicing reactions incubated with or without 4F4 (Fig. 3). Without ATP, the pre-mRNA sediments in the 30*S* to 40*S* region (Fig. 3A). In an ATP-dependent reaction a 60*S* splicing complex forms (Fig. 3B) (*15*). In the presence of 4F4 without ATP (Fig. 3C) the pre-mRNA sediments between 40*S* and ~60*S* (peaks at fractions 12 and 9), somewhat faster than the RNA in the reaction without antibody (Fig. 3A). The nature of these peaks is not known but they result from the binding of the antibody to C proteins that are bound to the pre-mRNA. Surprisingly, 4F4 did not prevent the ATP-dependent

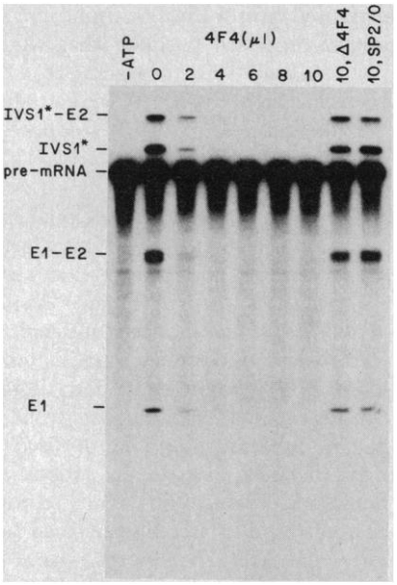

Fig. 2. Titration of inhibition of pre-mRNA splicing in vitro with monoclonal antibody 4F4. Splicing extracts (11 μl) were first incubated with various amounts (2, 4, 6, 8, and 10 μl) of monoclonal antibody 4F4 for 30 minutes on ice, and the splicing reactions were carried out as described in Fig. 1.

formation of a complex that sediments at or slightly larger than 60*S* (fraction 7) although the amount of this complex was usually reduced by up to 25 to 30 percent relative to control (Fig. 3D). Although the associated antibody caused the complexes to sediment slightly faster, no ^{32}P-labeled RNA was found in the pellet, indicating that 4F4 did not cross-link or precipitate pre-mRNA–containing structures. Therefore, in the presence of 4F4, an ATP-dependent splicing complex does form but in diminished amount; however, it is nonfunctional, and the pre-mRNA is not spliced (Figs. 1 and 2).

The hnRNP C proteins are associated with the 60*S* splicing complex. Because the antibody to the hnRNP C proteins inhibited the splicing of pre-mRNA (Figs. 1 and 2) and the C proteins interact with RNA in vivo and in vitro, it was of interest to determine whether C proteins are in the 60*S* splicing complex. Splicing reactions were carried out as described above with or without 4F4. The samples were then sedimented on sucrose gradients and gradient fractions were monitored for ^{32}P-labeled RNA. Peak fractions (Fig. 3) were collected and incubated either with protein A–agarose alone or with 4F4 and protein A–agarose, and the RNA adsorbed to the beads was extracted with phenol and analyzed by gel electrophoresis. The data in Fig. 4 show that protein A–agarose alone precipitated the pre-mRNA from the peak fractions of extracts incubated with 4F4 either without or with ATP (Fig. 4, lanes 8 and 11, respectively). In contrast, no RNA was precipitated with protein A–agarose from reactions carried out without the antibody. However, the pre-mRNA as well as the splicing products (mRNA and IVS1*) and splicing intermediates (E1 and IVS1*-E2) could be immunoprecipitated with protein A–agarose from these peak fractions after addition of 4F4 (Fig. 4, lane 6). This suggests that the hnRNP C proteins are associated with the pre-mRNA–RNP complex and with the splicing complex. Furthermore, 4F4 becomes incorporated into the pre-mRNA–RNP complex and into the splicing complex when incubated in the splicing extract and these complexes can be immunoadsorbed on protein A.

Although the above findings demonstrate that the C proteins are found in the splicing complex and suggest that they function as essential components of these RNP complexes, it should be noted that C proteins bind RNA in vitro without sequence specificity. Their association with the pre-mRNA in this complex could reflect nonspecific binding. In fact, immunoblotting experiments with 4F4 show that C proteins are found across the sucrose gradient, indicating the presence of endogenous C protein–containing RNP complexes in the extract (*20*).

Depletion of C proteins from the splicing extract results in loss of splicing activity. The role of the hnRNP C proteins in pre-mRNA splicing in vitro was further investigated by assessing the effect of depleting C proteins from the nuclear extract with monoclonal antibodies. At moderate salt concentrations (50 to 100 m*M* NaCl) the C proteins are associated with hnRNA and with other proteins in the form of hnRNP complexes (*7, 11*). This is also the case for C proteins in the splicing extract. In some depletion experiments under moderate salt concentration (50 to 100 m*M* NaCl), antibodies to the C proteins immunoprecipitated many proteins, in addition to the C proteins. To circumvent this problem, C protein depletion experiments were carried out at 420 m*M* NaCl (see legend to Fig. 5). Under these conditions, the hnRNP complex dissociates (*7*) and most of the hnRNP proteins are no longer immunoprecipitated with the C proteins–hnRNA complex (*20*). Thus only C proteins and some 120-kD protein were removed from the nuclear extract by adsorption to the complex of anti-C and

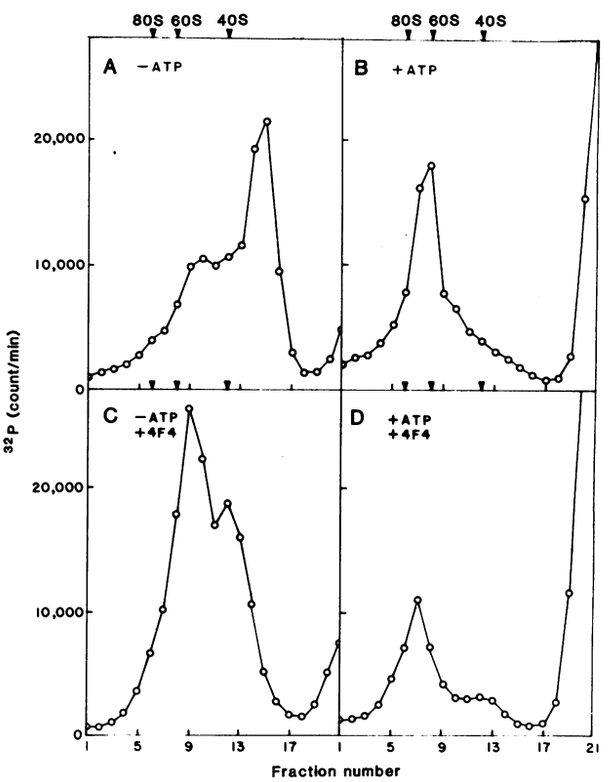

Fig. 3. Sedimentation profiles of the splicing reactions. (A) Splicing reaction without ATP. (B) Splicing reaction with ATP. (C) Same as (A) but with 4F4 monoclonal antibody in the splicing reaction. (D) Same as (B) but with 4F4 monoclonal antibody in the splicing reaction. In antibody inhibition experiments (C and D) 50 μl of monoclonal antibody 4F4 was first incubated (on ice) with 55 μl of splicing extract for 30 minutes. For each sucrose gradient, ^{32}P-labeled precursor RNA (250,000 count/min) and necessary components (*16*) were added to a final reaction volume of 125 μl, and the reaction was carried out for 30 minutes at 30°C. In reactions (A) and (C) both ATP and creatine phosphate were omitted. The reaction mixture was diluted with an equal volume of ice-cold buffer containing 20 m*M* Hepes (*p*H 7.6), 50 m*M* KCl, and 2 m*M* $MgCl_2$ and sedimented on a 10 to 30 percent sucrose gradient in the same buffer in a Beckman SW41 rotor at 38,000 rev/min for 5 hours at 4°C. Fractions (21 at 500 μl each) were collected from the bottom of the gradient, and radioactivity was measured by Cerenkov counting. Size markers (80*S*, 60*S*, and 40*S*) were determined with the cytoplasmic fraction of HeLa cells in analogous sucrose gradients sedimented in parallel.

protein A–agarose (Fig. 5, B and C). To deplete the extract of C proteins, a nuclear extract prepared according to the procedure of Dignam *et al.* (*18*), which specifies extraction with a 420 m*M* NaCl-containing buffer, was incubated with 4F4 or 2B12 monoclonal antibody and the protein A–agarose complex. After centrifugation, the antibody complex was washed at 420 m*M* NaCl and the washing was pooled with the C protein–depleted nuclear extract, dialyzed, and used in splicing reactions. The C protein–depleted nuclear extract was defective for splicing (Fig. 5A, lanes $4F4^-$ and $2B12^-$). Virtually no fully spliced product (E1-E2) was detected. Nuclear extract mock-depleted with control nonimmune mouse antibodies (SP2/0) was indistinguishable in splicing activity from untreated control nuclear extract (Fig. 5A, lanes "control" and $SP2/0^-$). This suggests that the depletion protocol followed did not nonspecifically inactivate the extract. Compared to mock-depleted extract, the first endonucleolytic cleavage reaction at 5′ splice site was reduced by about 65 to 80 percent in the C protein–depleted extract. In addition, the low ratio of free intron lariat IVS1* as compared to lariat intermediate IVS1*-E2 (1/5 by densitometric scanning), and the absence of spliced product suggest that the second endonucleolytic cleavage and ligation reaction was severely affected by removal of the C proteins from the nuclear extract.

The low level of partial splicing in the reaction may be attributed to the incomplete depletion of the C proteins from a nuclear extract (Fig. 5B, lanes $4F4^-$ and $2B12^-$). To assess the efficiency of the removal of C proteins from the extract and the level of contaminating antibody released into the depleted extract, we analyzed the C protein–depleted extract and the corresponding protein A–agarose precipitates by immunoblotting. Residual levels of C proteins were about 27 and 10 percent in 4F4- and 2B12-depleted extract, respectively, as determined by densitometric scanning of the immunoblot (Fig. 5B). Furthermore, the inhibition of splicing in the C protein–depleted extract is not a consequence of contamination of the extract with antibody leaking from the beads since no antibody was detected by immunoblotting in depleted extract (Fig. 5B). Moreover, the inhibition of splicing with 2B12-depleted extract provides evidence that the inhibition is not a consequence of contaminating 2B12 because addition of even large doses of 2B12 did not inhibit splicing (Fig. 1). Under the high salt concentration used for depletion, only a small fraction of the 120-kD protein was also removed from the extract (Fig. 5C). Therefore, it is unlikely that the loss of splicing activity is due to a reduction in the amount of this protein. As yet this has not been tested directly by removal of the 120- or 68-kD proteins by depletion experiment. Although the relative amounts of most of the major protein components in the depleted extract were the same as in control or mock-depleted extract, we cannot exclude the possibility that some minor components were depleted with C proteins by virtue of their tight association. Even if this was the case, it would still suggest that the hnRNP complex including C proteins is necessary for splicing.

A 60*S* splicing complex does not form in C protein–depleted extract. The 60*S* splicing complex contains components of the splicing machinery and C proteins. In view of the loss of splicing activity on C protein depletion, the possible role of the C proteins in the formation of the 60*S* splicing complex was examined in C protein–depleted nuclear extracts. After 30 minutes of incubation of mock-depleted nuclear extract under splicing reaction conditions, precursor RNA sedimented as a 60*S* splicing complex (Fig. 6B). However, in the nuclear extract that was treated with 4F4 and in which the C proteins were depleted, a 60*S* splicing complex did not form, and only a 30*S* complex was observed (Fig. 6A). These results suggest that the C proteins are necessary not only for the proper splicing reaction but also for 60*S* splicing complex formation.

The role of the hnRNP C proteins in RNA splicing. The hypothesis that pre-mRNA processing occurs in the nucleus in hnRNP complexes and that the hnRNP particle itself is a critical element not only in the packaging but also in processing of pre-mRNA was made with the earliest observations of hnRNP complexes. Until recently, however, it has not been possible to test the functional significance of hnRNP's because there were no definitive probes for hnRNP proteins and no adequate in vitro assay for pre-mRNA splicing. In our work, monoclonal antibodies to hnRNA-contacting proteins in vivo (hnRNP's), produced by immunizing mice with cross-linked hnRNA-protein complexes (*9, 10*), were used to study the possible roles of several hnRNP proteins in RNA splicing. The finding of specific inhibition of splicing with one of the monoclonal antibodies to the hnRNP C proteins and the loss of splicing activity upon removal of these proteins from the extract with either of two distinct monoclonal antibodies to C proteins, provides the first evidence that components of the hnRNP complex play an important role in pre-mRNA splicing. The observations that C proteins are in the splicing complex and the inability of the C protein–depleted extract to form 60*S* splicing complexes suggest that these proteins play a crucial role in both splicing complex formation and function.

The C proteins C1 and C2 are major constituents of hnRNP particles in vertebrates (*7, 9–11*). These abundant nucleoplasmic proteins are associated with the hnRNA in vivo and are efficiently cross-linked to it after ultraviolet irradiation of intact cells. The tight association of the C proteins with the hnRNA is also reflected in isolated hnRNP complexes in vitro by their relative resistance to

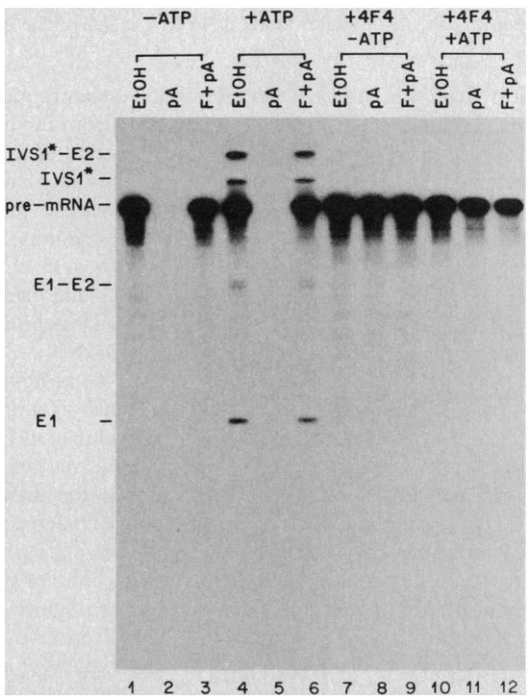

Fig. 4. Immunoadsorption of splicing complex with protein A–agarose or with 4F4 monoclonal antibody and protein A–agarose. Sample (25 μl) of peak fractions of the sucrose gradients in Fig. 3 [fraction number 15 of (A), fraction 8 of (B), fraction 9 of (C), and fraction 7 of (D), respectively] were incubated with 25 μl of protein A–agarose (lane pA) for 10 minutes in 100 μl of buffer containing 50 m*M* tris-HCl (*p*H 7.6), 150 m*M* NaCl, and 0.05 percent NP40 (*15*). After three washings with the same buffer, the adsorbed ^{32}P-labeled RNA was analyzed after phenol extraction by 10 percent polyacrylamide–8*M* urea gel electrophoresis. Another set of samples (25 μl each) were adsorbed with 2.5 μl of 4F4 antibody and 25 μl of protein A–agarose (lane F+pA) in the same condition. Ethanol-precipitated RNA (lane EtOH) from a third sample of each fraction (25 μl each) was run for comparison.

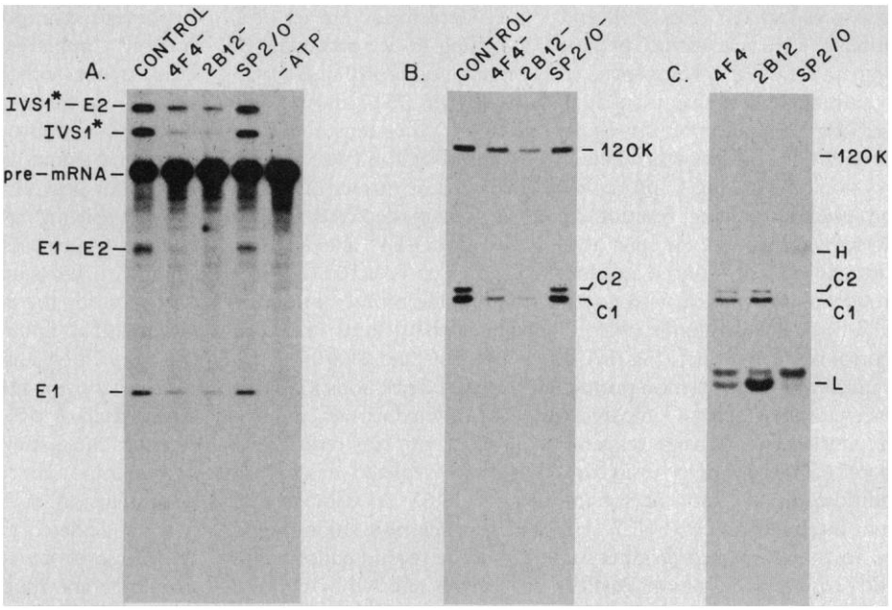

Fig. 5. Effect of depletion of C proteins from a nuclear extract on splicing in vitro. HeLa cell nuclear extract was depleted of C proteins by immunoadsorption and its splicing activity (A), and the amount of the C proteins removed (B and C) was analyzed. (A) Splicing reactions were carried out with 15 μl of depleted extract or 7.5 μl of untreated control extract in 25-μl reaction as described in Fig. 1. (Lane "Control") Nuclear extract prepared by the procedure of Dignam *et al.* (*18*). (Lanes 4F4⁻ or 2B12⁻) Nuclear extract depleted with 4F4 or 2B12 monoclonal antibody–coated protein A on agarose beads. (Lane SP2/0⁻) Nuclear extract mock-depleted with nonimmune mouse antibody-coated protein A on agarose beads. (Lane −ATP) Same as control but without ATP. (B) Immunoblot analysis of C proteins (C1 and C2) and 120-kD protein in the control and the depleted extracts used in (A). Samples of the depleted extract (5 μl) or control extract (2.5 μl) were fractionated by SDS-PAGE. After electrophoretic transfer onto nitrocellulose paper, the blots were probed with a mixture of 4F4 (1:1000) and 3G6 (1:1000) and detected with ^{125}I-labeled goat antibody to mouse F(ab')$_2$ (*9, 10*). (C) Immunoblot analysis of C proteins (C1 and C2) and 120-kD protein depleted from the extract by 4F4, 2B12, or SP2/0 antibody-coated protein A–agarose beads. The blot was probed as described in (B). Heavy and light chains of mouse antibodies are indicated as H and L on the right, respectively. The C proteins were depleted from C buffer nuclear extract (*18*) before final dialysis. Monoclonal antibody–coated protein A–agarose beads (100 μl) were prepared by incubation with 100 μl of ascites fluids of 4F4, 2B12, or SP2/0 for 1 hour in 20 m*M* Hepes (*p*H 7.6) containing 500 m*M* NaCl. After thorough washing with 20 m*M* Hepes, the beads were incubated with 100 μl of C buffer extract (*18*) for 30 minutes on ice. The supernatant was removed from the beads, and the beads were washed once with 100 μl of C buffer; both fractions were pooled and dialyzed against D buffer as described in (*18*). The beads were eluted with 200 μl of SDS-PAGE sample buffer for the analysis of the proteins depleted. Dilution of the extract was minimized at each step by brief centrifugation (13,000*g*) of the beads in punctured centrifuge tubes. The depleted extract or untreated control extract (7.5 μl) was used in each 25-μl splicing reaction for comparison. The SDS-PAGE and immunoblotting analysis were as described (*9, 10*).

dissociation from the hnRNA at high salt concentrations. For example, the A and B hnRNP proteins dissociate from the hnRNA at approximately 150 m*M* NaCl, while the C proteins dissociate at approximately 750 m*M* NaCl (*7*). Monoclonal antibodies have been used to show the presence of C proteins in widely divergent vertebrates from human to lizard (*10*). In all species examined, there are two C proteins: C1, 39 to 42 kD and C2, 40 to 45 kD. Both proteins are related immunologically and both are extensively phosphorylated (*9, 10*). They have distinct but similar peptide maps and have the same isoelectric point (*p*I = 6.0 ± 0.5) (*9*). Complementary DNA (cDNA) clones for the human C proteins have recently been isolated (*23*). Genomic blotting analysis with these cDNA's showed multiple hybridizing bands in human and mouse and homologous DNA sequences are found in various eukaryotes from human to yeast. The sequences encoding the hnRNP C proteins therefore appear to be members of a conserved gene family and the related proteins appear to be ubiquitous in eukaryotes.

The C proteins are nonsequence-specific RNA binding proteins in vitro (*20*), and therefore it is not likely that they recognize specific intron or exon sequences. How, then, might such proteins play a role in RNA splicing? Formation of a splicing complex (or spliceosome) involves recognition of sequences at both the 5′ and 3′ splice sites, probably by small nuclear RNP's (snRNP's) (*24, 25*). These sites along with the branch site must be juxtaposed for cleavage and ligation (*15, 16*). A central problem in the splicing of pre-mRNA becomes apparent in that many different pre-mRNA's undergo splicing in the nucleus at the same time. During pre-mRNA splicing, after endonucleolytic cleavage at the 5′ intron junction, the upstream exon must normally find the correct 3′ (downstream) exon within the same pre-mRNA strand to produce the correct mRNA. Thus, because the junction sequences are interchangeable and in order to prevent the random formation of alternative or chimeric mRNA's, it is necessary to organize the bipartite RNA splicing intermediates so as to form a single reaction center that will prevent diffusion of the intermediates. This will prevent chimeric mRNA formation and ensure a high reaction rate. That splicing indeed occurs efficiently and accurately both in vivo and in vitro suggests that the RNA reactants are held in a single complex for the duration

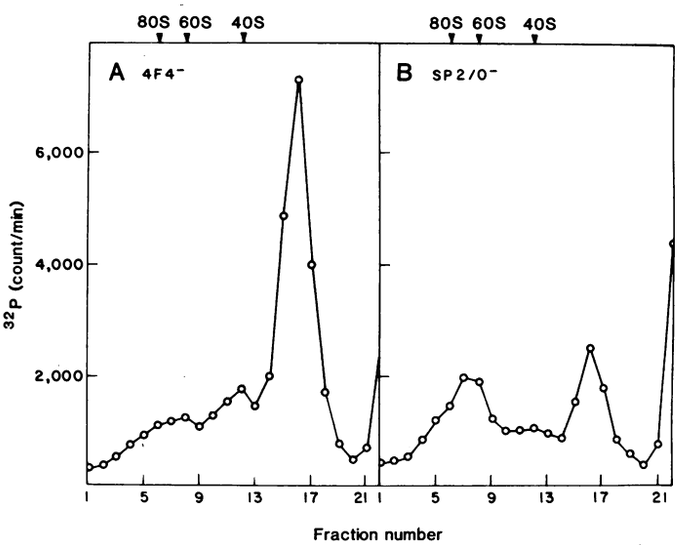

Fig. 6. Sedimentation profiles of splicing reactions in C protein–depleted extract. Nuclear extract was depleted of C proteins with (A) the monoclonal antibody 4F4 (4F4⁻) or (B) mock-depleted with nonimmune antibody (SP2/0⁻) as described in Fig. 5. Splicing reaction (50 μl) with 30 μl of each nuclear extract was sedimented on 10 to 30 percent sucrose gradients at 38,000 rev/min for 5 hours in a Beckman SW41 rotor at 4°C. Fractions (21) were collected from the bottom of the tube and the radioactivity was measured by Cerenkov counting.

of the reaction. The findings that exon 1 and the rest of the pre-mRNA chain (IVS1*-E2) are held together in one splicing complex even though there are no consensus sequences between exons indicates that the spliceosome contains such nonspecific RNA binding proteins. The nonsequence-specific RNA binding properties of the hnRNP C proteins make them excellent candidates for such a role, and they could then serve an important function in RNA splicing by holding the reactants together. The immunoprecipitation of the splicing complex with 4F4 suggests that C proteins are found in the splicing complex and that they are exposed on its surface. The C proteins are closely associated with other groups of hnRNP proteins such as the A and B proteins. Hence, these proteins may also be assembled into the spliceosome. In addition to holding the splicing intermediates together, the packaging of precursor RNA into hnRNP particles could have several important functions such as (i) suppressing the formation of intramolecular secondary structure so that specific sequences remain available in a single-stranded form; (ii) sequestering sequences within introns to reduce the likelihood of intermolecular base pairing which might result in trans-splicing of exons (*26*); and (iii) organizing and compacting long introns for efficient splicing. It is interesting that long RNA precursors are highly compacted in hnRNP particles (*27*).

The splicing complex contains, in addition to the nonspecific RNA binding proteins (hnRNP C proteins and possibly other hnRNP proteins), sequence-specific RNA binding factors such as U1 and U2 snRNP's that recognize specific sequences in the pre-mRNA (*24, 25*). It is possible that in spite of their nonspecific RNA binding capacity the C proteins are actually nonrandomly organized on the pre-mRNA chain because these snRNP's and other factors may determine the position on the RNA of the hnRNP proteins and direct the formation of a spliceosome.

It is possible that the C proteins play a fundamental role in the cleavage and ligation reactions of splicing. First, these proteins are found in the splicing complex, and second, binding of the monoclonal antibody 4F4 to the complex apparently inhibits cleavage and branch formation. Binding of the monoclonal antibody 2B12 to the C proteins does not result in a similar inhibition. The difference in effect of the two monoclonal antibodies suggests that binding to a specific site on the C proteins interferes directly with the process or disrupts the overall conformation of the complex. The results with the 2B12 antibody are a reminder that not all antibodies to a given protein necessarily interfere with its function in vitro. Therefore, the fact that the antibodies to the 120- and 68-kD hnRNP proteins do not inhibit splicing in vitro does not rule out the possibility that these proteins are also important in RNA splicing.

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