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A novel splicing factor is an integral component of 200S large nuclear ribonucleoprotein (lnRNP) particles

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In previous studies we have shown that nuclear transcripts of several pre-mRNAs can be released from nuclei of mammalian cells in the form of large nuclear ribonucleoprotein (lnRNP) particles. By electron microscopy, these particles appeared as compact composite structures, 50 nm in diameter, which invariably sedimented at the 200S region in sucrose gradients. In order to identify putative protein splicing factors associated with the 200S lnRNP particles, a panel of monoclonal antibodies directed against these particles were screened for their ability to inhibit splicing of pre-mRNA *in vitro*. In this study we have focused on a nuclear protein of 88 kd in molecular weight, which is an integral component of the lnRNP complex and is recognized by monoclonal antibodies from a specific clone. This protein has been identified here as a novel splicing factor by, (i) antibody inhibition of splicing *in vitro* and (ii) depletion of splicing activity from HeLa cell nuclear extract after removing the 88 kd polypeptide by immunoadsorption, and complementation of the depleted activity with an affinity-purified 88 kd antigen. This splicing factor has further been shown to be required for the assembly of an active splicing complex.

Key words: lnRNP proteins/monoclonal antibodies/pre-mRNA splicing factor

Introduction

In the nucleus of eukaryotic cells, RNA maturation involves post-transcriptional processing events which are required to convert nuclear precursor messenger RNA (pre-mRNA) into functional mRNA by splicing, capping and polyadenylation reactions. Studies of splicing *in vitro* revealed that splicing proceeds by a two-step mechanism (reviewed in Padgett *et al.*, 1986; Green, 1986; Krainer and Maniatis, 1988). The first entails cleavage at the 5′ splice site with the concomitant formation of a 2′–5′ phosphodiester bond between the 5′ terminal guanosine residue of the intron and an adenosine residue located ~30 nucleotides upstream from the 3′ splice site. The second step involves cleavage at the 3′ splice site, ligation of the two exons, and release of the excised intron in a lariat form.

Splicing reactions *in vitro* occur only after the assembly of a multicomponent ribonucleoprotein (RNP) complex which is termed the spliceosome (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski *et al.*, 1985). The assembly of pre-mRNA into functional spliceosomes involves the direct requirement of a multitude of protein and RNA components; the most extensively characterized of these are the U1, U2, U4, U5, and U6 small nuclear RNP (snRNP) complexes (reviewed in Steitz *et al.*, 1988; Krainer and Maniatis, 1988). A useful functional assay for the identification of proteins involved in RNA splicing is the inhibition of one or more steps in the *in vitro* splicing reaction by antibodies directed against specific cognate proteins (Choi *et al.*, 1986; Sierakowska *et al.*, 1986; Fu and Maniatis, 1990).

In vivo, pre-mRNA transcripts of RNA polymerase II, often referred to as heterogeneous nuclear RNA (hnRNA), are assembled during transcription with proteins and other components to form nuclear RNP particles, as initially observed by electron microscopy (Malcolm and Sommerville, 1974). The assumption that nuclear RNP particles serve as the pre-mRNA processing machinery, motivated a large body of research to elucidate their structure. Earlier attempts to isolate nuclear RNP particles led to the isolation of complexes sedimenting at the 30–40S region in sucrose gradients. These particles are considered to be the subunits of nuclear RNP, since they are produced by the nucleolytic breakdown of nuclear RNAs (Samarina *et al.*, 1968; Pederson, 1974; LeStourgeon *et al.*, 1977; Dreyfuss, 1986). In studies of the protein component of the 30–40S particles, which constitutes more than 80% of their total mass, six proteins (A1, A2, B1, B2, C1 and C2) designated core RNP proteins were identified (Beyer *et al.*, 1977). Subsequent immunological studies of proteins in nuclear RNP particles prepared by various methods, led to the production of monoclonal antibodies (MAbs) directed against a variety of RNP proteins (Leser *et al.*, 1984; Choi and Dreyfuss, 1984a and b; Lutz *et al.*, 1988; D.Offen *et al.*, in preparation) and to the assignment of more than 20 nuclear polypeptides in the molecular weight range of 34–120 kd as nuclear RNP proteins (Piñol-Roma *et al.*, 1988).

In more recent attempts to prepare intact nuclear RNP particles, we employed mild sonication of nuclei and the presence of several potent RNase inhibitors during RNP preparation and fractionation. We have thus shown that specific nuclear transcripts of RNA polymerase II can be released from nuclei of various cells as large compact RNP particles, ~50 nm in diameter, that sediment at the 200S region in sucrose gradients (Sperling *et al.*, 1985; Spann *et al.*, 1989; Sperling and Sperling, 1990). By following the fate of several specific transcripts, it was shown that RNA transcripts that differ largely in size, such as the 7.9 kb transcript of the *CAD* gene or the 1.6 kb transcript of the β-actin gene, are packaged in particles of similar hydrodynamic properties. Furthermore, the U snRNPs U1, U2, U4, U5 and U6, which are required for splicing (reviewed in Steitz *et al.*, 1988) have been found to be

associated with the 200S large nuclear RNP (lnRNP) particles (Sperling *et al.*, 1986, and J.Sperling, unpublished results). These studies suggest that the nuclear transcripts of RNA polymerase II are packaged in the lnRNPs, and raise the possibility that the processing of nuclear RNA occurs on these particles.

To facilitate the studies on the structural and functional aspects of the lnRNP particles, we generated a panel of MAbs against a variety of RNP proteins by immunizing mice with nuclear extracts enriched by lnRNP particles. Following fusion, the antibodies produced by the hybridoma cells were screened by radioimmunoassay (RIA) against nuclear RNP preparations from several mammalian sources, and finally selected by RIA against purified 200S lnRNP particles. Immunoblotting analyses show that each of these MAbs is specifically directed against a protein from a set of polypeptides in the molecular weight range of 20−120 kd (D.Offen *et al.*, in preparation).

In this paper we describe a MAb that recognizes in spliceosomes, as well as in 200S lnRNP particles, a protein of 88 kd in molecular weight. Antibody inhibition of splicing and of spliceosome assembly, as well as immunodepletion and complementation of splicing activity by the affinity-purified antigen, identify the 88 kd protein as a putative splicing factor.

Results

Monoclonal antibodies monospecific to nuclear proteins

The MAbs described in this paper were arbitrarily selected from a panel of MAbs against nuclear RNP proteins that had been prepared in our laboratory (D.Offen *et al.*, in preparation). Briefly, SJL/j mice were immunized with HeLa cell nuclear extract enriched by large nuclear RNP (lnRNP) particles that sediment at about 200S in sucrose gradients (Sperling *et al.*, 1985; Sperling *et al.*, 1986; Spann *et al.*, 1989; Sperling and Sperling, 1990). Spleen cells were fused with NS0/1 myeloma cells, and hybrid cells producing antibodies were selected by radioimmunoassay against purified 200S lnRNP particles.

Immunoblotting experiments were performed with antibodies produced by clones designated 53/4, 85 and 36, against the total gel-fractionated proteins of HeLa cell nuclear extract (Dignam *et al.*, 1983). These experiments (Figure 1) revealed that antibodies produced by the above mentioned clones recognize nuclear proteins of molecular mass of 88, 45 and 45 kd, respectively. Identical results were obtained when the proteins of nuclear supernatants devoid of cytoplasmic contamination and enriched by 200S lnRNP particles (Sperling *et al.*, 1985) were utilized as the antigenic source. The nuclear authenticity of these antigens has been confirmed in Western blots demonstrating that neither the S100 fraction (Dignam *et al.*, 1983), nor the cytoplasmic fraction of the lnRNP particles preparation (Sperling *et al.*, 1985), contain these antigens (D.Offen *et al.*, in preparation). It should be pointed out, however, that immunoblotting with antibodies from clone 53/4 recognized, in some preparations of HeLa nuclear extracts, polypeptides of 66 and 45 kd, in addition to the 88 kd polypeptide. Whether the 45 and 66 kd species are specific products of proteolysis, or genuine nuclear RNP proteins sharing epitopes with the 88 kd protein, remains to be seen.

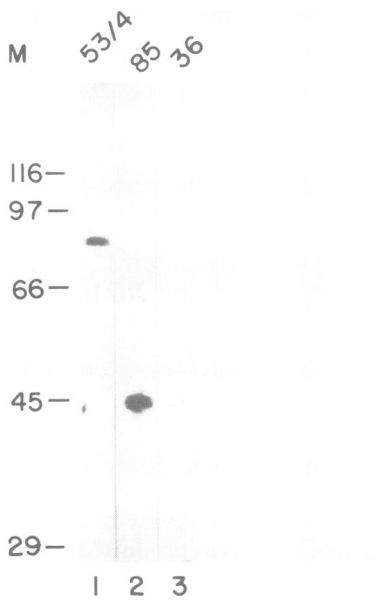

Fig. 1. Specific RNP proteins are recognized by MAbs to 200S lnRNP particles. Total HeLa cell nuclear RNP proteins (60 μg per lane) were electrophoresed on a 12% SDS−polyacrylamide gel, transferred electrophoretically to a nitrocellulose filter, probed with the indicated MAbs (purified ascites fluids diluted 1:100) and with [^{125}I]protein A, and autoradiographed (see Materials and methods). Lanes 1−3, MAbs 53/4, 85 and 36, respectively. The sizes of the molecular weight markers (lane M) are indicated in kd.

MAbs produced by clone 53/4 identify a nuclear RNP protein associated with 200S lnRNP particles

For the assignment of a MAb as directed against a nuclear RNP protein, we have utilized an RNase-sensitivity criterion previously employed by us for the analysis of specific RNA transcripts in RNP complexes (Sperling *et al.*, 1985). In this assay, we demonstrated that 200S lnRNPs containing specific nuclear RNA transcripts were converted by mild RNase treatment to particles sedimenting at 30S in a sucrose gradient, whereas free RNA or cytoplasmic RNPs (e.g. polysomes) were completely degraded under identical RNase digestion conditions.

Thus, analysis of the distribution of the 88 kd protein in sucrose gradient-fractionated RNP was performed by solid-phase RIA with MAbs produced by clone 53/4, and revealed a peak at 200S (Figure 2A, solid line). Similar results were obtained for the nuclear proteins recognized by MAbs 85 and 36 (data not shown). After mild digestion with RNase, the 88 kd protein became associated with 30S RNP particles (Figure 2A, dashed line). Furthermore, the distribution of the 88 kd protein coincided with that of β-actin pre-mRNA in the 200S and 30S regions of the respective sucrose gradients, as determined by hybridization with a synthetic oligonucleotide probe corresponding to nucleotides 2341−2359 in the fourth intron of human β-actin pre-mRNA (Figure 2B and C, see also Spann *et al.*, 1989). This observation is in accordance with experiments demonstrating that MAbs produced by clone 53/4 indirectly precipitated nuclear β-actin RNA and U snRNPs from HeLa cells nuclear extract enriched by lnRNPs (D.Offen *et al.*, in preparation). Taken together, these results identify the 88 kd polypeptide as an RNP protein, and indicate its direct association with the 200S lnRNP particles.

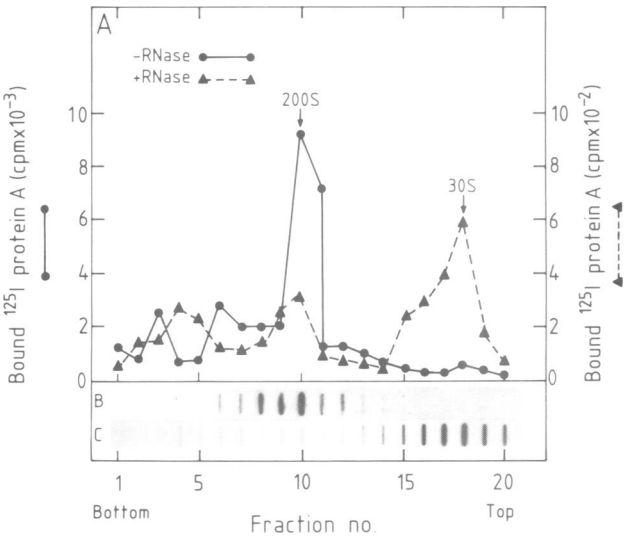

Fig. 2. Mild RNase digestion of nuclear RNP causes the appearance of a 30S RNP peak in which the 88 kd protein (**A**) and β-actin pre-mRNA (**B, C**) cosediment. HeLa nuclear RNP extract was fractionated in a 15–45% sucrose gradient before [A (●——●) and B], and after [A (▲——▲) and C] RNase digestion. The distributions of the 88 kd antigen and of β-actin pre-mRNA were analyzed by solid phase RIA and RNA blot hybridization, respectively, as described in Materials and methods. The gradients were calibrated with 200S tobacco mosaic virus (TMV) particles (fraction 10) and 30S bacterial ribosomal subunits (fraction 18) run in a parallel gradient. The apparent low recovery (~10%) of the 53/4 antigen in the 30S peak is due to the fact that these fractions are highly enriched with the bulk of nuclear proteins that interfere with the attachment of the 53/4 antigen to the solid matrix.

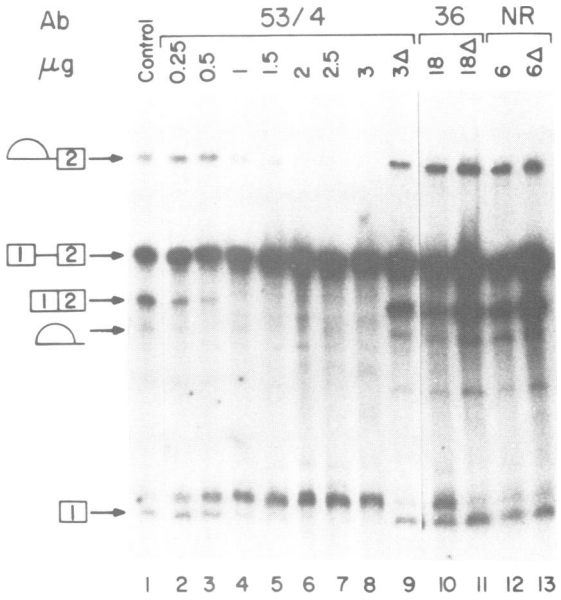

Fig. 3. The inhibitory effect of MAbs to 200S lnRNP particles on splicing *in vitro*. Splicing reaction mixtures containing HeLa nuclear extract and ^{32}P-labeled pSP64HβΔ6 β-globin pre-mRNA were incubated in the presence of the indicated amounts of the Ig fraction of the MAb. The splicing products were analyzed by electrophoresis in 10% polyacrylamide/7M urea denaturing gels, and autoradiography. Lane 1, control reaction, no antibodies added; lanes 2–8, splicing in the presence of increasing amounts of MAb 53/4; lane 9, control with heat-denatured MAb 53/4, lanes 10–13, splicing in the presence of intact (lanes 10, 12) and heat-denatured (lanes 11, 13) MAb 36 and anti-cholera toxin MAb, respectively. The pre-mRNA, splicing intermediates and products are identified by schematic drawings on the left. Top to bottom: intron–exon 2 lariat; pre-mRNA; mature mRNA; free intron lariat; free exon 1.

The protein recognized by MAb 53/4 is required for splicing in vitro

To assess the possible physiological role in mRNA processing of the 88 kd nuclear RNP protein, we examined the ability of the cognate MAb to inhibit a splicing reaction *in vitro*. Increasing amounts of purified Ig fractions of the MAb were added to a standard *in vitro* splicing reaction comprising HeLa cell nuclear extract (Dignam *et al.*, 1983) and ^{32}P-labeled human β-globin pre-mRNA transcribed *in vitro* by SP6 RNA polymerase (Krainer *et al.*, 1984). RNA was then isolated and analyzed by electrophoresis in denaturing polyacrylamide gels and autoradiography. Figure 3 shows that the addition of 1 μg (Figure 3, lane 4), or more (Figure 3, lanes 5–8), of the Ig fraction of MAb 53/4 inhibited the formation of both splicing products and intermediates. However, when heat-denatured MAb 53/4 was added (Figure 3, lane 9) no inhibition was observed, indicating that the inhibitory effect of the MAb resides in a protein and requires antigen–antibody recognition.

Interestingly, the formation of free exon 1 and intron–exon 2 lariat was not affected significantly in the presence of 0.25 and 0.5 μg of antibodies (Figure 3, lane 2 and 3), but dropped sharply at 1 μg (Figure 3, lane 4). In contrast, the formation of mature mRNA diminished gradually with increasing amounts of the MAb (compare the respective bands in Figure 3, lanes 2–4). This observation may be interpreted as resulting from the existence of at least two epitopes which have different affinities for the MAbs. One is involved in the first, and the other in the second step of the splicing reaction. The corroboration of this observation and the proposition of alternative explanations require further experiments.

MAb 36 (Figure 3, lanes 10, 11) and MAb 85 (data not shown), which recognize nuclear protein(s) of 45 kd, did not inhibit the splicing reaction. Nevertheless, the antigens recognized by the above MAbs were also demonstrated to be components of the spliceosome, as the respective MAbs immunoprecipitated ^{32}P labeled β-globin pre-mRNA from an *in vitro* splicing reaction under non-dissociating conditions (D.Offen *et al.*, in preparation). Further controls included, (i) a non-relevant anti-cholera toxin MAb TE 34 (a kind gift from Dr Jacob Anglister) which did not inhibit the splicing reaction (Figure 3, lanes 12, 13); and (ii) anti-Sm antibodies which inhibited the reaction (data not shown). We can thus conclude that the inhibitory effect on splicing *in vitro* of MAb 53/4 is specific.

In an independent experimental approach, the requirement for splicing of the 88 kd polypeptide was confirmed by immunodepletion and complementation experiments. Aliquots of HeLa cell nuclear extract were adjusted to 0.42 M NaCl (buffer C of Dignam *et al.*, 1983) and incubated with protein A–Sepharose beads coated with the MAbs. After centrifugation, the protein contents of the pellets and of the remaining solutions were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE), immunoblotting and silver-staining. Figure 4 shows the protein profile of the HeLa nuclear extract, after (Figure 4, lane 1) and before (Figure 4, lane 2) depletion with MAb 53/4. It can be seen that silver staining revealed

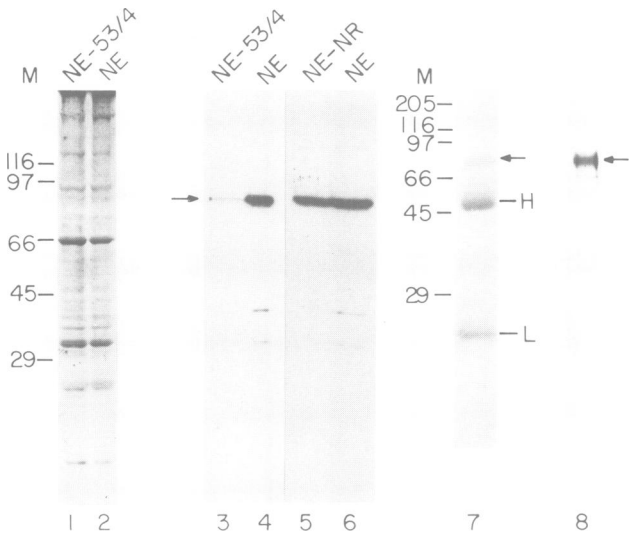

Fig. 4. Anti-200S lnRNP MAb 53/4 specifically immunoprecipitates an 88 kd nuclear protein. Aliquots of HeLa nuclear extract (NE) were incubated with immobilized MAbs under conditions that maintain the proteins fully dissociated, as described in Materials and methods. The supernatants and precipitates were then analyzed for their protein content by SDS–PAGE in 12% gels, silver staining and Western blotting. Lanes 1 and 2, silver-stained total proteins of NE after, and before immunoprecipitation with MAb 53/4 (lanes marked NE-53/4 and NE, respectively); lanes 3 and 4, Western blots with MAb 53/4 of stripes similar to 1 and 2, respectively; lanes 5 and 6, Western blots with MAb 53/4 of total proteins of NE after, and before immunoprecipitation with the control non-relevant MAb (lanes marked NE–NR and NE, respectively); lane 7, silver-stained proteins immunoprecipitated by MAb 53/4; lane 8, silver staining of the immunoaffinity-purified 88 kd protein. M, molecular weight markers (kd). H and L indicate heavy and light Ig chains, respectively.

no significant difference between these preparations. However, a comparison of similar gel-stripes by protein blot analysis with MAb 53/4 (Figure 4, lanes 3 and 4) indicates that >98% of the 88 kd protein, as measured by densitometry, was depleted from the antibody-treated extract. As a control we show that immunodepletion with the non-relevant anti-cholera toxin MAb had no effect on the content of the 88 kd protein in the nuclear extract (Figure 4, lanes 5 and 6). Furthermore, SDS–PAGE analysis and silver staining of the proteins eluted from the precipitated beads that had been coated with MAb 53/4, revealed the immunoprecipitated 88 kd protein along with the heavy and light chains of the antibody (Figure 4, lane 7). The control beads, coated with the non-relevant anti-cholera toxin MAb, precipitated neither the 88 kd polypeptide nor other polypeptides in the molecular mass ranges not covered by the heavy and light chains of the antibody (not shown). The 88 kd protein was also purified by affinity chromatography on MAb 53/4 covalently bound to Sepharose beads. The protein was recovered from the column in a virtually purified form, as determined by SDS–PAGE and silver staining (Figure 4, lane 8). It should be pointed out that the presence of high salt in the immunoprecipitation reactions was required in order to maintain the RNP proteins in a fully dissociated form, thereby avoiding the indirect co-precipitation of proteins associated with the respective target proteins (Choi *et al.*, 1986). Immunoprecipitations performed in low salt (0.1 M NaCl) resulted, as expected, in the precipitation of a multitude of proteins (data not shown).

Since immunodepletion with MAb 53/4 could cause the total removal of the cognate antigen from HeLa nuclear extracts, the concomitant loss of splicing activity of such an extract could have been expected. Indeed, this treatment rendered the nuclear extract inactive in splicing (Figure 5A, lane 3, and Figure 5B, lane 2, marked NE-53/4). The controls show that nuclear extracts that had been immunodepleted with either MAb 85 or MAb 36, which did not inhibit splicing by direct addition (see Figure 3, lanes 10 and 11 for MAb 36; data not shown for MAb 85), retained their splicing activity (Figure 5A, lanes 5 and 6, marked NE-85 and NE-36, respectively). Similarly, a nuclear extract that had been immunodepleted with the non-relevant anti-cholera toxin MAb, also retained its splicing activity following this treatment (Figure 5A, lane 2, marked NE-NR).

The immunodepleted splicing activity could be restored to defective extracts by addition of a micrococcal nuclease-treated extract [Figure 5A, lane 4, marked NE-53/4+NE(MN)], which by itself was inactive in splicing [Figure 5A, lane 7, marked NE(MN)]. Furthermore, a splicing defective extract (Figure 5B, lane 2, marked NE-53/4) could be fully complemented by addition of the immunoaffinity-purified 88 kd antigen (Figure 5B, lane 3, marked NE-53/4+88 kd) which by itself was inactive in splicing (Figure 5B, lane 4, marked 88 kd). The gradual back addition of the purified 88 kd protein to an extract depleted with MAb 53/4 (Figure 5C, lane 1) resulted in increasing the levels of splicing activity (Figure 5C, lanes 2–6). Interestingly, it can be seen that the relationship between the levels of splicing and the amounts of added protein is not linear.

The 88 kd protein is required for spliceosome assembly

The inactivation of splicing activity may have resulted from the interference of the bound 53/4 antibody with a putative splicing activity residing in the 88 kd antigen. However, this inactivation may have also been caused by the inhibition of the spliceosome assembly process that precedes the splicing reaction. To test the effect of the antibody on the assembly of the splicing complex and its intermediates, we employed a technique developed by Konarska and Sharp (1986, 1987) who analyzed the formation of RNP complexes during the splicing reaction by electrophoresis in non-denaturing polyacrylamide gels. These authors have demonstrated that a complex designated H is formed immediately after addition of pre-mRNA to the nuclear extract. The association of U2 snRNP particles with sequences upstream of the 3′ splice site results in the formation of complex A. The larger complex B is then formed by binding of U5, U4 and U6 snRNPs. This complex has been shown to contain splicing intermediates and mature mRNA and is thus designated as the functional spliceosome (Konarska and Sharp, 1987).

Figure 6A, lane 2, shows the effect of addition of MAb 53/4 to a standard splicing *in vitro* reaction mixture on the formation of the various splicing complexes. Formation of complexes H and A were not affected, whereas the formation of complex B was abolished. A control experiment using heat-inactivated MAb (Figure 6A, lane 3) revealed a pattern of RNP complexes similar to that obtained for a standard splicing *in vitro* reaction in the absence of antibodies (Figure 6A, lane 1). Further controls show that in the absence of

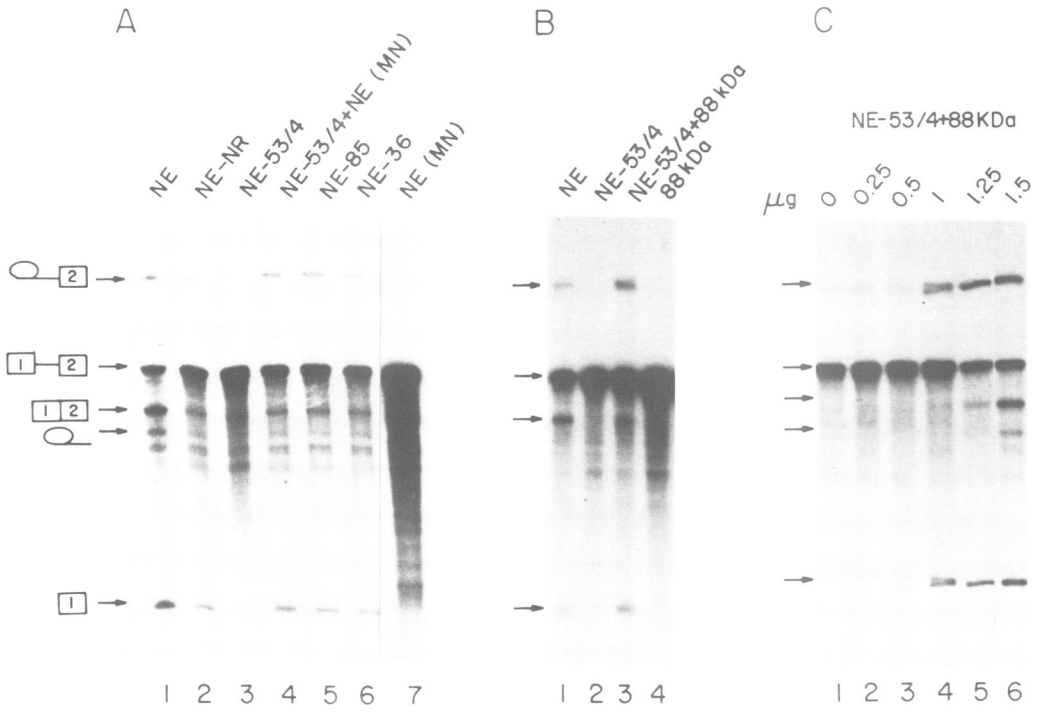

Fig. 5. The 88 kd nuclear antigen is required for splicing of pre-mRNA *in vitro*. Splicing of pSP64HβΔ6 pre-mRNA was carried out with HeLa nuclear extracts (NE) immunodepleted of specific antigens and complemented as indicated. RNA was analyzed by electrophoresis in 10% polyacrylamide/7M urea denaturing gels and autoradiography as described in Materials and methods. Panel A: lane 1, standard splicing *in vitro* reaction. lane 2, splicing with NE immunodepleted with the non-relevant (NR) anti-cholera toxin MAb; lane 3, splicing with NE immunodepleted of the 88 kd antigen with MAb 53/4 (see Figure 4, lane 3); lane 4, splicing with NE depleted with MAb 53/4 and complemented with a micrococcal nuclease-treated extract; lanes 5 and 6, splicing with NE depleted of a 45 kd antigen by either MAb 85 or MAb 36, respectively; lane 7, splicing with a micrococcal nuclease treated extract. Panel **B**: lane 1, standard splicing *in vitro* reaction. Lane 2, splicing with NE immunodepleted of the 88 kd antigen with MAb 53/4; lane 3, splicing with NE depleted with MAb 53/4 and complemented with 1 μg of the purified 88 kd protein (see Figure 4, lane 8); lane 4, splicing with the 88 kd protein alone. Panel C: lane 1, splicing with NE immunodepleted with MAb 53/4; lanes 2–6, splicing with NE depleted with MAb 53/4 and complemented with the indicated amounts of the purified 88 kd protein. The pre-mRNA, splicing intermediates and product are identified by schematic drawings as in Figure 3.

ATP, only complex H was formed—whether or not the MAb was present (Figure 6A, lanes 4 and 5).

Next, we examined the formation of splicing complexes by a nuclear extract that had been depleted of the 88 kd antigen and, hence, of its splicing activity (see Figure 5B, lane 2). Figure 6B, lane 4, shows that neither complex A nor complex B were formed by the depleted extract, while the non-specific complex H was formed. The ability to assemble both complexes A and B was restored to the defective extract by the addition of the affinity-purified 88 kd protein (Figure 6B, lane 5), concurrently with the restoration of splicing activity (see Figure 5B, lane 3). It therefore appears likely that an intact 88 kd protein is required for the formation of the active splicing complex B. However, the formation of the pre-splicing complex A, though requiring the presence of this protein, is not abolished by its binding to the antibodies. Whether or not the 88 kd protein is involved directly in the catalysis of splicing remains an open question.

Discussion

In order to identify putative protein splicing factors associated with 200S lnRNP particles, a panel of MAbs directed against these particles were screened for their ability to inhibit splicing of pre-mRNA. In this study we have focused on a MAb designated 53/4, which recognizes an 88 kd nuclear RNP protein. This protein has been identified here as a novel splicing factor by antibody inhibition-, immunodepletion- and complementation of splicing activity.

In accordance with previous studies demonstrating the conversion of the 200S particles to 30S particles upon mild RNase digestion (Sperling *et al.*, 1985), we demonstrate here that the 88 kd protein comigrates in sucrose gradients with nuclear β-actin RNA transcripts as a 200S complex and, following mild RNase digestion, appears associated with the RNA in a 30S complex. This result confirms the assignment of the MAb as directed against an RNP protein that is specifically associated with the 200S lnRNP particles.

Several lines of evidence confirm that the 88 kd protein is associated with spliceosomes, and is required for spliceosome assembly and for splicing activity *in vitro*. First, the direct inhibition of splicing *in vitro* by MAb 53/4, and the depletion of splicing activity from HeLa cell nuclear extract by removing the 88 kd polypeptide with the same antibody, identify the 88 kd antigen as a factor required for splicing. The residence of the immunodepleted splicing activity in a protein factor is confirmed by showing that splicing activity could be restored to the defective extract by the addition of a micrococcal nuclease-treated extract. Direct evidence for the involvement of the 88 kd protein in splicing is provided by restoring activity in splicing to a depleted extract by the gradual addition of the purified protein. Notably, however, the levels of splicing restored to such extracts do not appear to correspond linearly to the

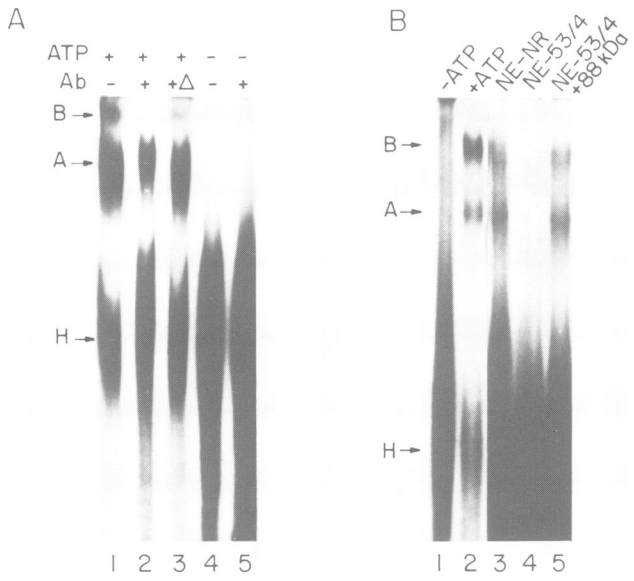

Fig. 6. The inhibitory effect of MAbs to the 88 kd protein on spliceosome assembly. (**A**) Direct inhibition. Splicing reactions of ^{32}P-labeled β-globin pre-mRNA were carried out in the presence or absence of ATP and MAbs as indicated. Splicing complexes were fractionated in a non-denaturing polyacrylamide gel and autoradiographed. Lane 1, standard splicing reaction without antibodies; lane 2, splicing in the presence of 12 μg of purified Ig of MAb 53/4; lane 3, as in lane 2 but with heat-inactivated MAb; lane 4, splicing in the absence of ATP; lane 5, splicing in the absence of ATP with 12 μg of purified Ig of MAb 53/4. (**B**) Depletion and complementation. Splicing reactions of ^{32}P-labeled adenovirus pre-mRNA were carried out as indicated and analyzed for splicing complex formation. Lanes 1 and 2, standard splicing reactions without and with ATP, respectively; lane 3, splicing reaction with an extract immunodepleted by the non-relevant anti-cholera toxin MAb; lane 4, splicing reaction with an extract immunodepleted by MAb 53/4; lane 5, splicing reaction as in lane 4, complemented by the affinity-purified 88 kd protein. The positions of complexes B, A and H are indicated to the left of each panel.

amount of protein added. Whether this observation is indicative of cooperative interactions of the 88 kd protein with other spliceosome components during splicing remains to be determined.

Second, the structural role of the 88 kd protein in spliceosome assembly is reflected by, (i) the inhibitory effect of MAb 53/4 on the formation of the active splicing complex B, (ii) the inability of a nuclear extract depleted of the 88 kd antigen to form complexes A and B and (iii) the restoration of the assembly of complexes A and B by the addition of purified 88 kd antigen to the depleted extract. Moreover, the fact that immunoblots of the gel-resolved splicing complexes show that the 88 kd protein is associated with the functional splicing complex B, as well as with complexes A and H (data not shown), indicates that the 88 kd protein is an integral component of the active splicing complex. In this context, it is interesting to note the different effect of depletion versus direct inhibition on the assembly of the splicing complexes. The depletion experiment shows that both complexes A and B were not assembled in the absence of the 88 kd protein. On the other hand, the formation of complex A was not affected in the presence of the antibody-bound 88 kd protein, while formation of complex B was abolished. The latter observation can be attributed to the masking effect of the bound antibodies on a binding site(s) for an additional factor(s) required for the conversion of the pre-splicing complex A to the active splicing complex B. Alternatively, the binding of such factor(s) may be prevented due to the conformational changes induced in the 88 kd protein upon its binding to the antibodies. In any case, it seems likely that the 88 kd protein plays an important role in the assembly of complex B—the active splicing complex.

The 88 kd protein is a component of both the 200S lnRNP particle and the spliceosome. Its association with RNA and proteins in both these complexes is salt dependent. At high salt (420 mM) the 88 kd protein alone is immunoprecipitated (Figure 4), whereas at low salt an assortment of nuclear proteins, as well as the pre-mRNA and U snRNPS, are precipitated by the 53/4 MAb (D.Offen *et al.*, in preparation). It is noteworthy that a protein of 88 kd has not been described before as belonging to the family of heterogeneous nuclear RNP proteins (Piñol-Roma *et al.*, 1988). The 88 kd protein also differs from the previously described 76–105 kd RNP proteins (Piñol-Roma *et al.*, 1988) with respect to the effect of heparin on its association with RNP complexes. Thus, the 88 kd protein was found by a Western blot to be associated with the spliceosome even in the presence of heparin (data not shown), whereas none of the hnRNP proteins in the molecular weight range of 76–105 kd is heparin resistant (Piñol-Roma *et al.*, 1988).

In this study we have identified a nuclear RNP protein, recognized by anti-200S RNP MAb 53/4, as a putative pre-mRNA splicing factor. This protein is an integral component of both the 200S lnRNP particles and the spliceosome. Additional RNP proteins, recognized by MAbs 85 and 36, are also components of the two types of the RNP complexes, but apparently are not required for splicing. The U snRNPs, which are functional components of the spliceosme (Krainer and Maniatis, 1988; Steitz *et al.*, 1988), are also integral components of the 200S lnRNP complexes (Sperling *et al.*, 1986). It is therefore interesting to compare these two kinds of RNP complexes with respect to their possible structure and function in pre-mRNA processing. Spliceosomes are assembled on short synthetic pre-mRNA-like transcripts, containing mostly two exons interrupted by an intron, upon incubation with mammalian nuclear extract or yeast cell extract highly enriched with proteins (reviewed in Padgett *et al.*, 1986; Green, 1986). The *in vivo* assembled RNP particles are in most cases more complex than spliceosomes. Their RNA transcripts contain several introns, each bearing splicing signals, as well as signals required for other RNA processing activities such as 3′-end processing. In addition, they probably contain information that controls the transport of mature nuclear RNA from the nucleus to the cytoplasm. Thus, more components are expected to be associated with the native particle *in vivo* than with the *in vitro* assembled spliceosome. It is feasible therefore that the spliceosome represents a substructure of the native processing apparatus, i.e. that part of the native particle which incorporates the structural and functional elements required for the excision of the intron and ligation of the released exons to form a mature RNA-like structure. If indeed the spliceosome is a substructure of the lnRNP particles, it can be expected that the panel of MAbs we have raised against the lnRNP particles should contain also antibodies directed against factors that are not shared with the spliceosome, but are specific to the lnRNP particle and are required for its additional functions.

Materials and methods

Cell growth

HeLa cells were grown in suspension in SMEM medium containing 5% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin sulfate and 100 u/ml sodium penicillin, at 37°C.

The preparation and selection of hybridoma cells producing MAbs to nuclear proteins associated with the lnRNP particles, is described elsewhere (D.Offen *et al.*, in preparation). For the studies reported here, we used MAbs from three hybridoma cells designated: 53/4, 85 and 36. The cells were grown in tissue culture plates as described by Eshhar (1985).

Ascites fluids

Ascites fluids were prepared by injecting $6-10 \times 10^6$ hybridoma cells in 1 ml of phosphate buffered saline (PBS, 140 mM NaCl, 10 mM sodium phosphate, pH 7.5) intraperitoneally to either BALB/c or [BALB/c × SJL]F1 mice that had been injected, at least 14 days before the hybridoma cells injection, with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane). The ascites fluids were removed by tapping a needle into the lower part of the abdomen and purified by affinity chromatography on protein A–Sepharose (Pharmacia, Upsalla). One ml of ascites fluid was mixed with 30 mg of protein A–Sepharose beads suspended in 150 μl of PBS and incubated for 2 h at ambient temperature with gentle shaking. The beads were washed six times with 15 ml of PBS and centrifugation. The bound antibodies were recovered by two successive elutions with 0.7 ml of 0.2 M glycine–HCl, pH 2.7, and neutralization to pH 8 with 1 M K_2HPO_4. The combined eluates were dialyzed against 0.02 M sodium phosphate, pH 8, and concentrated by centrifugation in a Centricon microconcentrator (10 000 mol. wt cutoff, Amicon, Danvers, MA) at 3000 r.p.m. in a Sorvall SS34 rotor. The protein concentration was determined by the method of Bradford (1976).

Immunoblotting

Total proteins of HeLa cells nucelar extract were displayed on 12% SDS–polyacrylamide gels (acrylamide: bismethyleneacrylamide weight ratio of 30:0.8) (Laemmli, 1970). The separated proteins were transferred to nitrocellulose filters by electrophoresis for 20 h at 200 mA and 4°C in a buffer containing 15 mM Tris base, 115 mM glycine and 20% methanol (Towbin *et al.*, 1979). The blots were incubated for 16 h in a blocking solution consisting of 10% low-fat milk in PBS (50 ml per 100 cm^2) at 4°C with shaking. Antibodies, diluted in blocking solution as indicated, were incubated with the blots for 16 h at 4°C with gentle shaking. The filters were washed five times for 10 min in blocking solution, and probed with [^{125}I]protein A (2×10^5 c.p.m./ml) in blocking solution for 2 h at 25°C. The filters were washed twice in 0.05% Tween 20, four times for 10 min with PBS, and autoradiographed.

Preparation and RNase digestion of 200S lnRNP particles

HeLa cell nuclear supernatant enriched for intact lnRNP particles was prepared and fractionated in a 15–45% sucrose gradient as previously described (Sperling *et al.*, 1985; Spann *et al.*, 1989). In parallel, another sample of HeLa cell nuclear supernatant was treated for 10 min at 20°C with 10^{-3} μg/ml of pancreatic RNase (Sperling *et al.*, 1985), and fractionated in a sucrose gradient as described above.

Analyses of RNP particles by solid phase radioimmunoassay (RIA) and RNA blot hybridization

Aliquots of 100 μl from each of the above fractions were attached to a 96-well microtiter plate (Falcon 3911) for 24 h at 4°C, and analyzed by RIA as described by Sperling *et al.* (1986) with minor modifications. Briefly, the plates were blocked with 2% BSA in PBS for 2 h at ambient temperature (or 16 h at 4°C), washed with 1% BSA in PBS, and incubated for 20 h at 4°C with 100 μl of ascites fluid diluted in the same solution, as indicated. The wells were washed five times with 0.5% BSA in PBS, incubated for 1 h at 37°C with [^{125}I]protein A (2×10^5 c.p.m. in 100 μl of 1% BSA in PBS per well), washed twice with 0.05% Tween 20 in PBS, five times with PBS, and counted.

RNA blot analysis was as described by Sperling *et al.* (1986). An aliquot of 50 μl from each of the above fractions was attached to a nitrocellulose sheet and probed with a ^{32}P-labeled synthetic oligonucleotide corresponding to nucleotides 2431–2359 in the fourth intron of the human β-actin gene (Gunning *et al.*, 1983).

Antibody inhibition of splicing in vitro

Standard splicing reactions were carried out as previously described by Krainer *et al.* (1984), using HeLa nuclear extracts (Dignam *et al.*, 1983), and ^{32}P-labeled β-globin pre-mRNA transcribed from the plasmid pSP64HβΔ6 cut with *Bam*HI (Krainer *et al.*, 1984; kindly provided by Dr A.R.Krainer). Splicing reactions were in a total volume of 25 μl containing 6.5 ng of ^{32}P-labeled pre-mRNA (2×10^4 c.p.m.), 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$, and 15 μl of HeLa nuclear extract.

The antibody inhibition reactions were carried out in a total volume of 31.6 μl containing 15 μl of nuclear extract. The concentrations of salts and cofactors remained the same as in the standard reaction. The splicing reaction mixture was first incubated on ice for 30 min with the indicated amount of purified MAb. The splicing reaction was then allowed to proceed for 90 min at 30°C. It was stopped by adding 174 μl of 200 mM Tris–HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% SDS and 1.2 mg/ml proteinase K, and incubation for 30 min at 30°C. After phenol extraction and ethanol precipitation, the reaction products were analyzed by electrophoresis in 10% polyacrylamide/7 M urea gels and autoradiography.

Immunoaffinity purification of the 88 kd protein

An immunoaffinity matrix was prepared by binding ammonium sulfate-precipitated ascites fluid 53/4 to cyanogen bromide-activated Sepharose 4B CL (Pharmacia, Uppsala), using a standard coupling protocol. HeLa cell nuclear extract in buffer C (Dignam *et al.*, 1983) was diluted 16-fold with 20 mM HEPES, pH 7.9, 0.5 M NaCl, and passed four times through a column containing an equal volume (packed beads) of the affinity matrix. The column was extensively washed with 20 mM HEPES, pH 7.9, 0.5 M NaCl, 1 mM DTT, 2 mM PMSF, and eluted with 0.2 M acetic acid. Each fraction was neutralized with 1 M K_2HPO_4, and the pure protein was recovered after dialysis and concentration as described under Ascites fluids.

Immunodepletion and complementation of splicing-defective extracts

150 μl of ascites fluid were mixed with 30 mg of pre-swollen protein A–Sepharose beads, the mixture was adjusted to 20 mM HEPES, pH 7.9, and 500 mM NaCl, and incubated for 3 h at ambient temperature with gentle shaking. The beads were centrifuged for 5 s at 10 000 *g*, and washed five times with 1 ml of 20 mM HEPES, pH 7.9, 500 mM NaCl. The washed beads were then incubated with 100 μl of nuclear extract in buffer C (Dignam *et al.*, 1983) for 1 h at 4°C with gentle shaking, and centrifuged as above. The supernatant was removed and dialyzed against buffer D (Dignam *et al.*, 1983). The depleted nuclear extracts were examined for their ability to splice pre-mRNA in the splicing *in vitro* system as described above.

Splicing complementation reactions (Figure 5B) were carried out in a total volume of 25 μl containing 10 μl of depleted nuclear extract and either 5 μl of a micrococcal nuclease treated nuclear extract (Krainer and Maniatis, 1985), or 1 μg of the purified 88 kd protein in 5 μl of 20 mM sodium phosphate, pH 7.5. The dose-dependent splicing complementation reactions (Figure 5C) were carried out in a total volume of 31.6 μl containing 15 μl of depleted nuclear extract and the indicated amounts of purified 88 kd protein in 6 μl of 20 mM sodium phosphate, pH 7.5. The concentrations of salts and cofactors remained the same as in the standard reaction. Reactions were incubated for 90 min at 30°C and analyzed as described above.

Analysis of immunodepleted proteins

The precipitated and washed beads from above were further washed in 20 mM HEPES, pH 7.9, 500 mM NaCl (5 × 1 ml) and treated with 0.2 M acetic acid (2 × 25 μl) to elute the bound proteins. The eluted material was fractionated in a 12% polyacrylamide gel [acrylamide:bisacryloylpiperazine (Hochstrasser *et al.*, 1988; PolyChem, Nes-Ziona, Israel) weight ratio of 27:1] in the presence of SDS, and visualized by silver staining (Wray *et al.*, 1981).

Antibody inhibition of splicing complex formation

Standard splicing reactions of ^{32}P-labeled β-globin pre-mRNA were carried out in the presence or absence of ATP and MAb 53/4 (12 μg of purified Ig) as indicated in Figure 6A. The reaction mixtures were treated with heparin and 15 μl aliquots were analyzed in a 4% polyacrylamide gel in 45 mM Tris–borate, pH 8.3, 1 mM EDTA (0.5 × TBE) as described by Konarska and Sharp (1986). Similar results were obtained when heparin was omitted and the splicing complexes analyzed in 50 mM Tris–glycine, pH 8.8 (Konarska and Sharp, 1987). The depletion and complementation experiments were carried out in a total volume of 25 μl, each containing 15 μl of either of the following, (i) untreated HeLa nuclear extract, (ii) immunodepleted extract prepared as described above and (iii) immunodepleted extract and 1 μg of the affinity purified 88 kd protein. The substrate used for these experiments was ^{32}P-labeled adenovirus pre-RNA transcribed *in vitro* from the plasmid pSP62Δil cut with *Sca*I (Frendewey and Keller, 1985; kindly provided by Dr W.Keller), and incubations were for 1 h at 30°C. Splicing complexes were analyzed in aliquots of 15 μl, loaded directly onto a 4%

polyacrylamide gel in 50 mM Tris–glycine, pH 8.8, essentially as described by Konarska and Sharp (1987). The gel was pre-electrophoresed for 20 min at 12 V/cm and electrophoresis was carried out at the same voltage gradient for 6 h at 17°C. Splicing complexes were visualized by autoradiography of the dried gels.

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