Association of the 72/74-kDa proteins, members of the heterogeneous nuclear ribonucleoprotein M group, with the pre-mRNA at early stages of spliceosome assembly

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We have investigated the role played in precursor mRNA (pre-mRNA) splicing by the protein pair of molecular size 72/74 kDa, which are integral components of a discrete subset of heterogeneous nuclear (hn) ribonucleoproteins (RNPs) named large heterogeneous nuclear RNP (LH-nRNP). This 72/74 kDa pair of proteins has been shown to belong to the hnRNP M group, and are referred to as 72/74(M). By applying specific immunoprecipitation assays in a consecutive series of splicing reactions *in vitro*, the antigenic 72/74(M) protein species were found to associate with the pre-mRNA and not the intermediate or final products of splicing. Kinetic studies, combined with isolation of pre-spliceosomal and spliceosomal complexes from the splicing reaction, indicated a loose association of 72/74(M) with both the initially formed H assembly and the first splicing-committed E

complex. Stable binding was seen at a later stage of the reaction, well in advance of the appearance of the first intermediate products of RNA splicing. Evidence is provided that supports the almost exclusive association of 72/74(M) with pre-mRNA within the pre-spliceosomal A complex. This dynamic binding appeared to involve pre-mRNA sites similar to those of spliceosomal U1 and U2 small nuclear RNP complexes. Moreover, a preferential binding to a truncated RNA containing the 5' exon–intron part, rather than the intron–3' exon part, of pre-mRNA was observed.

Key words: RNA-binding proteins, RNA processing, splicing complexes.

INTRODUCTION

The removal of intervening sequences (introns) from precursor mRNA (pre-mRNA) is a major event of mRNA maturation in higher eukaryotes. The deciphering of the mechanistic steps involved, and the complexity of the large number of associated proteins and RNA factors, followed the establishment of *in vitro* systems able to carry out precise and efficient RNA splicing. As documented by several studies (reviewed in [1,2]), splicing takes place within a large multi-factorial structure, termed the spliceosome. Assembly of active spliceosome follows the stepwise and transient sequestration of pre-mRNA into discrete splicing complexes. The first identifiable H complex forms following addition of pre-mRNA to the splicing extract, even at 0 °C and in the absence of ATP. It contains the bulk of pre-mRNA in association with the abundant group of nuclear RNA-binding proteins: the so-called heterogeneous nuclear (hn) ribonucleoprotein (RNP) species [3]. The interaction of hnRNP proteins with pre-mRNA is currently thought to be sequence- and transcript-specific, and to involve sites of low affinity, since most of them are salt-sensitive [4]. The protein composition of the *in vitro*-assembled H complex is remarkably similar to that of the immunopurified hnRNP complexes from HeLa nuclear extracts. Both have been shown to contain an almost complete set of all known hnRNP polypeptides (hnRNP A to U) [5]. Since H complex can form equally well on intronless RNA or RNA molecules lacking functional splice sites, it is considered to be an unspecific complex [6,7]. Nonetheless, sequestration of pre-mRNA into H complex precedes the association of at least part

of it with any splicing-competent complex serving as substrate for subsequent splicing.

Upon incubation of splicing extracts at 30 °C, the formation of the ATP-independent, pre-spliceosomal E complex is detected as the first splicing-committed complex. E complex requires the participation of U1-small nuclear RNP (snRNP) and U2AF splicing factor, which establish the first interaction between the 5'- and 3'-ends of the intron respectively, mediated by protein members of the arginine/serine-rich (RS domain) family of SR proteins ([8], and references therein). The important events for splicing to occur take place during the subsequent step of pre-spliceosomal A complex: the first ATP-dependent step of RNA splicing. The splicing-competent 17 S U2-snRNP [9] is the major additional participant at the stage of A complex formation. Together with U1-snRNP and a few additional protein factors, they represent the known components of A complex. The subsequent assembly of spliceosomal B and C complexes, and the appearance of the first intermediates of splicing in C complex, follow the entry of the tri-U4·U6/U5 snRNP, together with a large number of non-snRNP splicing factors, of which several have been identified to date [10–13].

Despite increasing evidence for participation of hnRNPs in many aspects of mRNA metabolism (reviewed in [14]), their specific role in splicing is as yet largely unknown. Currently, several lines of evidence support the notion that particular hnRNP proteins play a role in splicing. For instance, antibodies raised against hnRNP C and A/B-type proteins inhibit *in vitro* splicing [15,16]. In addition, specific hnRNP proteins interact with the 3' splice site within intron sequences (hnRNP A, C and

Abbreviations used: pre-mRNA, precursor mRNA; (hn)RNP, (heterogeneous nuclear) ribonucleoprotein; snRNP, small nuclear RNP; LH-nRNP, large heterogeneous nuclear RNP.

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D), bind to polypyrimidine track sequences [polypyrimidine-tract-binding protein (hnRNP I/PTB)] or function in alternative splicing (hnRNP A/B, I/PTB and F; [1,14], and references therein). The majority of hnRNP proteins participating in H complex formation is, however, found to be displaced by splicing factors within pre-spliceosomal (E and A) and spliceosomal (B and C) complexes. Nonetheless, specific interactions involving remaining hnRNP species (e.g. hnRNPs A/B, C, E, D, L, K and I) are detected in such complexes [4,10]. The observed variation in the level and isoform type of hnRNP protein species associated with specific pre-mRNA substrates within the splicing machinery strengthens the likelihood of them influencing transcript-specific binding of splicing factors, and selection of alternative splice sites [4,10,14].

We have reported previously [17] the identification of two immunologically related proteins of molecular size 72/74 kDa as integral components of a large heterogeneous nuclear RNP (LH-nRNP) entity. This protein pair has recently been shown to belong to the hnRNP M group of proteins, which we have, thereafter, referred to as 72/74(M) [18]. The set of human hnRNP M represents a group of four protein variants (M1–M4) known to date, which are abundantly present in hnRNP complexes, with an ability to bind RNA, participate in RNA splicing and cause heat-shock-induced splicing arrest [19,20].

Our previous studies [17,18] have relied heavily on the availability of antibodies directed against the mammalian 72/74(M) protein species. In the present work we have performed consecutive series of immunoprecipitation reactions aimed at defining the association of 72/74(M) with the pre-mRNA during *in vitro* RNA splicing. Their identification *in vivo* as RNA-binding protein species, and the finding that immunodepletion or antibody addition to the splicing extract could cause a strong inhibitory effect on RNA splicing [17,18], suggest such an association. Here we show the stable binding of 72/74(M) with pre-mRNA at specific stages of the *in vitro* RNA splicing reaction.

MATERIALS AND METHODS

DNA constructs

Two different ³²P-labelled pre-mRNA substrates were utilized in the present study. The first was transcribed from the pBSAL4 plasmid (provided by A. Lamond, University of Dundee, Scotland, U.K.) that corresponds to the rabbit β -globin gene, with a deleted form of intron 2 included within sequences of exons 2 and 3 [21]. The second one is an adenovirus pre-mRNA substrate, which was obtained from the pBSAd1 plasmid, as described previously [22]. Ad5' was transcribed after cleaving pBSAd1 with *Fnu*4HI, and Ad3' with *Sau*3AI. All plasmids were transcribed using T3 RNA polymerase.

In vitro splicing reaction

Splicing extracts were prepared from HeLa cells as described in [23]. Splicing reactions on β -globin pre-mRNA were in a volume of 20 μ l, of which one half was splicing extract (approx. 50 μ g of total protein), as described previously [21]. Pre-mRNA and both intermediate and final products of the reaction were analysed by electrophoresis on a denaturing 7% polyacrylamide/7 M urea RNA gel. Splicing reactions performed on adenovirus pre-mRNA substrate were as described previously [24]. The RNA products of the reaction were analysed on a 10% RNA gel, and visualized by autoradiography.

Antibodies

The antibodies with exclusive specificity for the 72/74(M) proteins were obtained by immunizing guinea pigs with the largest (74 kDa) protein, as described previously [18]. Pre-immune serum was extracted from the same animal before immunization. The anti-Sm antibody was from a standard serum obtained from the Centers for Disease Control (Atlanta, GA, U.S.A.).

Immunoprecipitation of the splicing reaction

Splicing reactions were incubated for the times indicated in the Results section, and then were diluted 25-fold in NET-2 buffer [150 mM NaCl/10 mM Tris/HCl (pH 7.5)/0.05% (v/v) Nonidet P40]. The immunoglobulins contained in 15 μ l of either anti-(72/74) or pre-immune sera, as well as in 10 μ l of the anti-Sm serum, were first bound to Protein A–Sephacryl beads, added to the splicing reaction mix, and then followed by incubation at 4 °C for 2 h. For the T1 protection experiments, 1 unit of T1 ribonuclease/ μ l of immunoprecipitation reaction was added together with the antibodies, as described in [25,26]. The immune complexes were then obtained by centrifugation (10 000 *g* for 1 min at 4 °C), and washed three times with NET-2 buffer. To extract RNA, the protein in the immune pellets was digested with proteinase K (0.5 mg/ml) before phenol extraction. The RNA from the supernatant of the immunoprecipitation reaction was recovered concomitantly; this was then ethanol-precipitated, resolved on a denaturing RNA gel and visualized by autoradiography.

Isolation of splicing complexes

Purification of splicing complexes was accomplished essentially as described previously [10]. A 300 μ l splicing reaction was incubated with the adenovirus pre-mRNA substrate at 30 °C for 5 or 30 min for the assembly of A and B complexes respectively, or for 15 min in the absence of ATP for E complex assembly. Formation of H complex was achieved by mixing the reaction components on ice. Complexes were resolved by gel filtration on a Sephacryl S-500 high-resolution column (Pharmacia, Piscataway, NJ, U.S.A.). Fractions of 0.5 ml were collected, and one-fifth of each (0.1 ml) was counted for radioactivity by liquid scintillation. Peak fractions were pooled before use in immunoprecipitation reactions.

UV cross-linking

In vitro splicing reactions used in UV cross-linking experiments were assembled with the adenovirus pre-mRNA, as described above. The only modification was that a 4-thiouracil-substituted pre-mRNA was instead applied {synthesized at a ratio of 1:5 thio-UTP (from USB; Cleveland, OH, U.S.A.) to UTP, as described in [10]}. Cross-linking was performed on ice by irradiation of the splicing reaction for 40 min with a Black Ray lamp (Model B100 AP, long-wave UV; UVP San Gabriel, CA, U.S.A.). The cross-linked mixtures were used in immunoprecipitation reactions, as outlined above. The identification of cross-linked proteins in the immune pellets followed treatment with RNase A at a concentration of 0.3 mg/ml, as described by Staknis and Reed [27], and protein resolution by SDS/PAGE and autoradiography.

Figure 1 Ability of the anti-(72/74) serum to precipitate the pre-mRNA in the process of *in vitro* RNA splicing

A series of *in vitro* splicing assays were set up on a radiolabelled β -globin pre-mRNA and allowed to proceed from 0 to 60 min. (A) The kinetics of the splicing reaction are shown by the RNA species recovered from each reaction point applied, following resolution on a denaturing RNA gel. The lanes shown correspond to an assay incubated at 30 °C in the absence of ATP (lane 1; ATP 60') and assays in the presence of ATP, either kept on ice (lane 2; 0') or incubated for 15 min (lane 3; 15'), 30 min (lane 4; 30') and 60 min (lane 5; 60'). Lane M is a 32 P-labelled pBR322/HpaII DNA marker. (B) The RNA species recovered from a duplicate set of the splicing reaction shown in (A) that had been subjected to immunoprecipitation with either the anti-(72/74) (α -72/74; lanes 1–5) or pre-immune (PI; lanes 1'–5') guinea pig sera are presented. The symbols, from top to bottom, refer to the intron lariat–3'exon and intron–lariat intermediates, the pre-mRNA and the ligated 5'exon–3'exon product of the splicing reaction.

RESULTS

Binding of 72/74(M) proteins to pre-mRNA during *in vitro* RNA splicing

To investigate the role of 72/74(M) in pre-mRNA splicing, we considered whether antibodies against these protein components could precipitate pre-mRNA or products of the splicing reaction. A radiolabelled β -globin pre-mRNA was incubated in HeLa splicing extracts for increasing intervals of time [from zero time (reaction kept on ice) up to 60 min], as well as for 60 min in the absence of ATP. The process of splicing was monitored following analysis of the RNA on a denaturing gel. Figure 1(A) shows the kinetics of the RNA splicing reaction applied here. In agreement with published data [21], only pre-mRNA is observed upon incubation of the reaction in the absence of ATP (Figure 1A, lane 1) or when the reaction is kept on ice (lane 2). The easily identifiable intermediate products of RNA splicing (intron lariat–3'exon and intron lariat alone) did not appear during the reaction until after 30 min (lane 4). At $t = 60$ min, the final product of splicing (ligated 5'exon–3'exon) was first detected clearly (lane 5). As judged from the sustained presence of pre-mRNA and intermediate products, splicing had not reached completion after 60 min of incubation. As shown previously [17,28], an almost complete disappearance of intermediate products is not seen until 120 min after the start of the splicing reaction.

In Figure 1(B), the RNA species recovered from the splicing reaction shown in Figure 1(A) are presented following their immunoprecipitation with guinea anti-(72/74) (lanes 1–5) and

Figure 2 Salt-resistant binding of 72/74(M) proteins to pre-mRNA

RNA splicing assays that had been allowed to proceed for 30 min were subjected to immunoprecipitation with α -(72/74) (lanes 1 and 2) or pre-immune (PI; lanes 3 and 4) sera in increased salt conditions (300 and 500 mM NaCl, as indicated). Analysis of the RNA of the immune pellets, as well as of two of their corresponding supernatants (Supern.; lanes 5 and 6), is presented. Symbols are as described in the Figure 1 legend.

control pre-immune (lanes 1'–5') sera. As shown, pre-mRNA alone, but not intermediates or products of splicing, was specifically precipitated by the anti-(72/74) serum. By densitometric estimation of the amount of RNA recovered on the gel, the immunoselected pre-mRNA for each reaction time point relative to that in the corresponding pre-immune pellet was found to increase from an undetectable level at zero time (lane 2) to a maximum observed at 30 min (lane 4). As a rough approximation, this maximum was estimated to be approx. 6% of the total pre-mRNA input. A level that was close to the maximum had been already reached after 15 min of incubation, well before the appearance of the first intermediate products (Figure 1A, lane 3).

To determine the stability of the association of 72/74(M) with pre-mRNA, we then examined whether this interaction was salt-resistant. The immunoprecipitation assay of the 30 min RNA-splicing-reaction time point was repeated in NET-2 buffer with 300 or 500 mM NaCl, instead of the standard concentration of 150 mM. As shown in Figure 2 (lanes 1 and 2), the ability of the anti-(72/74) serum to selectively precipitate a fraction of the pre-mRNA, again in the obvious absence of the intermediate products of the reaction seen in lanes 5 and 6, remained unaffected up to a concentration of NaCl of 500 mM. This high stability of binding of 72/74(M) to pre-mRNA agrees well with the previously established salt-resistance of the LH-nRNP complex itself, of which the antigenic 72/74(M) protein species are major constituents [17].

Figure 3 Identification of binding of 72/74(M) to pre-mRNA during early stages of *in vitro* spliceosome assembly

Large-scale RNA splicing assays were set up on a radiolabelled adenovirus pre-mRNA substrate and allowed to proceed under conditions required for pre- and spliceosomal complex formation, as outlined in the Materials and methods section. The assembled H, E, A complexes, and a mixture of the A/B complexes, were then isolated from the splicing reactions by gel filtration. (A) RNA recovered from each isolated complex (as shown) following immunoprecipitation with either α -72/74 (lanes 2–5) or pre-immune (PI; lanes 6–9) sera is shown. Lane 1 in the left part of the panel refers to the RNA species present in a 30 min splicing assay, from which the mixture of A/B was obtained. The double-box symbol marks the position of pre-mRNA. (B) Protein species recovered in the α -72/74 (lanes 1 and 2) and PI (lanes 3 and 4) precipitates of A and A/B complexes were identified by their UV cross-linking to pre-mRNA, following resolution by SDS/PAGE and autoradiography. The arrow points to protein species corresponding by size to the 72/74(M), as referenced by the molecular-mass standards shown in the right.

Direct association of 72/74(M) proteins with pre-mRNA during pre-spliceosome assembly

In order for the observed differential association of 72/74(M) with pre-mRNA during *in vitro* RNA splicing to be firmly established, the ability of the anti-(72/74) serum to precipitate the pre-mRNA from chromatographically purified pre-spliceosomal and spliceosomal complexes was investigated. Large-scale RNA splicing reactions were set up, as described in the Materials and methods section. A radiolabelled adenovirus pre-mRNA was used in these experiments as the splicing substrate, in place of the β -globin pre-mRNA used in the experiments shown in Figure 1. Both denaturing RNA gel analysis and native gel electrophoresis, performed on aliquots taken from each reaction point, were used to monitor the progress of splicing (results not shown). The splicing reactions were then applied on to a Sephacryl S-500 column to resolve specific fractions corresponding to single H, E or A complexes, or a mixture of A/B complexes (as verified by native gel electrophoresis), as described by Bennett et al. [10]. Purified complexes were then subjected to immunoprecipitation by anti-(72/74) or pre-immune antisera, and the RNA recovered in the immune pellets was visualized by gel analysis.

As shown in Figure 3(A), an appreciable amount of pre-mRNA was recovered in the immune pellets corresponding to the purified A/mix of A and B complex(es). This also shows that, as in the case of β -globin, the adenovirus pre-mRNA was efficiently precipitated by the anti-(72/74) serum. Relatively higher amounts of pre-mRNA were recovered in the fraction of pre-spliceosomal A complex alone than in the mixture of A and B complexes, whereby the same number of input counts was applied (compare lanes 4 and 5). This pattern of 72/74(M) binding to pre-mRNA in isolated complexes agrees quite well with the findings presented in Figure 1(B). As for the $t = 0$ incubation time point (lane 2), there was no evidence to support binding to pre-mRNA within the H complex, whereas the weak association observed when incubating the splicing reaction in the absence of ATP (Figure 1B, lane 1) was not reproduced with the purified E complex. Taken together, our results confirm the

preferential and stable association of 72/74(M) proteins with pre-mRNA during pre-spliceosomal A complex formation.

As demonstrated previously [18], the 72/74(M) proteins can be efficiently UV cross-linked to the RNA *in vivo*. We then sought to investigate whether the proteins were, indeed, in close proximity to the pre-mRNA within the chromatographically purified A and A/B complexes. To meet this aim, 4-thiouracil incorporated into pre-mRNA was applied as a general labelling probe to enhance subsequent UV cross-linking of RNA-binding proteins within the assembled splicing complexes. Immunoprecipitation was then performed, using the anti-(72/74) and pre-immune sera, concomitantly with RNase digestion to remove the bulk of the RNA. The presence of RNA-binding proteins in the immune pellets was revealed on an SDS/polyacrylamide gel by their indirect labelling due to the remaining cross-linked nucleotides of pre-mRNA ([27], and references therein). As shown in Figure 3(B), labelled protein components in the range of the expected 72/74(M) proteins, clearly absent from the pre-immune pellet, were resolved in the immunoselected A complex. The lack of a complete resolution of the 72/74(M) protein pair was anticipated, as a consequence of the UV cross-linking. In agreement with the RNA analysis shown in Figure 3(A), a similar band of 72/74 kDa was observed in the A/B mixture with a lower intensity than that of A complex alone. Additional co-precipitated faint bands with molecular masses of approx. 50 and 90–200 kDa were also present within the A complex. This is in accordance with previously published data showing that a number of protein species are associated with affinity-purified A complex [10]. We thus conclude that the 72/74(M) proteins associate predominantly with the pre-mRNA in the isolated pre-spliceosomal A complex.

Specificity of binding of the 72/74(M) proteins to pre-mRNA

To investigate further the nature of the association of 72/74(M) with pre-mRNA, we combined immunoprecipitation and RNase T1 protection assays to examine the pattern of the immunoselected RNA fragments. This type of experiment has been

Figure 4 RNase T1-protection analysis of the binding of 72/74(M) to pre-mRNA

Early (15 min) RNA splicing reactions on β -globin pre-mRNA were reproduced as in Figure 1 and, subsequently, immunoprecipitated with α -72/74 (lanes 1 and 2) or α -Sm (lanes 3 and 4) sera in the presence (+) or absence (–) of T1 enzyme. The RNA species recovered in the immune pellets are presented here, following their analysis on an RNA gel. The double-box symbol marks the position of pre-mRNA, and the arrows point to the presence of T1-protected RNA fragments.

successfully applied in the past on β -globin pre-mRNA, using the well-known anti-Sm and anti-U1RNP antibodies, and has provided important information on the sites of binding of the spliceosomal snRNP complexes to pre-mRNA during *in vitro* RNA splicing [25]. In our studies, we performed a similar type of analysis using the anti-(72/74) serum in parallel with a standard anti-Sm antibody [28]. The $t = 15$ min splicing reaction was selected as the time point for analysis, since this represents an early reaction time that combines the absence of intermediate products of splicing (Figure 1A, lane 3) with the relatively high level of immunoselected β -globin pre-mRNA (Figure 1B, lane 3). In Figure 4, the results of such an analysis are shown following immunoprecipitation in the absence or presence of T1 enzyme, with either anti-(72/74) (lanes 1 and 2) or anti-Sm (lanes 3 and 4) sera. To improve the resolution of the image of the RNA gel, longer-than-normal exposure times of the X-ray film were applied, which resulted in the increased background seen in the immunoprecipitated pre-mRNA in the absence of T1 digestion (lanes 1 and 3). From a direct comparison of the RNA pattern in the anti-(72/74) and anti-Sm immune pellets, it was clear that strikingly similar T1-protected RNA fragments were recovered (compare lanes 2 and 4). In agreement with the anti-Sm study published previously [25], no RNA fragments were observed in a control lane into which an amount of T1-digested pre-mRNA equivalent to that loaded in lanes 2 and 4 had been applied prior to the immunoprecipitation (results not shown). The three major immunoselected T1-protected RNA fragments seen in Figure 4 (lanes 2 and 4) corresponded very closely in terms of their size to those reported in the literature as being Sm-associated pre-

Figure 5 Differential binding affinity of the 72/74(M) proteins to an RNA substrate containing the 5' or 3' splice site part of pre-mRNA

An adenovirus pre-mRNA corresponding to full size (Ad) or to truncated molecules with either the 5' or the 3' splice site half (Ad5' and Ad3' respectively) were applied in parallel 15 min splicing assays. Following immunoprecipitation with either α -72/74 or pre-immune (PI) sera, the RNAs recovered in the immune pellets (lanes 1–3 or 4–6), as well as in an aliquot (one-tenth) of their corresponding supernatants (lanes 7–9 or 10–12) were analysed on an RNA gel. The symbols, from top to bottom, designate the position of the full size and truncated 5' and 3' halves of the pre-mRNA.

mRNA regions [25]. These results therefore suggested that 72/74(M) had associated with the pre-mRNA at sites similar to the Sm antigen. An interesting observation was made here relating to the amount of pre-mRNA, as well as T1-protected fragments, recovered by anti-(72/74) and anti-Sm antibodies. An appreciable degree of association of the 72/74(M) polypeptides can be envisaged when compared (within the same experimental set-up) with the well-established high affinity of Sm antigens for pre-mRNA.

To determine whether the 72/74(M) proteins exhibit any preference in their binding to discrete parts of the adenovirus pre-mRNA substrate (used in the experiment shown in Figure 3), the following protocol was followed. Full-length pre-mRNA, as well as truncated fragments of it containing either the 5'- or 3'-splice-site-containing half of pre-mRNA (Ad5' and Ad3' respectively), were used as splicing substrates in the reaction leading to pre-spliceosomal A complex formation (as in Figure 3A, lane 4), followed by subsequent immunoprecipitation assays. In Figure 5, the RNAs resolved on a gel from either the immune pellets or the supernatants of the anti-(72/74) and pre-immune reactions are shown. The efficiency of immunoprecipitation of full-size and truncated RNA species (lanes 1–3) was compared by normalizing the values with the input of the RNA in each splicing reaction, and taking into account the amount of radioactivity present in the pre-immune pellets (lanes 4–6). Surprisingly, the Ad5' substrate was precipitated very efficiently compared with Ad3'. The preferential precipitation of Ad5' compared with Ad3' is also evident from the corresponding decrease in the radioactivity remaining in the supernatant of the anti-(72/74) immune reaction compared with that of pre-immune serum (lanes 9, 12 versus 8, 11). This indicates a strong binding preference of 72/74(M) proteins for sequences within the 5'-splice-site-containing half of the pre-mRNA.

DISCUSSION

We have shown in the present work a series of immunochemical studies that support the direct and dynamic association of the 72/74 kDa pair of proteins with the pre-mRNA within discrete splicing complexes. They have been referred to before as 72/74 (M) [18], since they are found to be discrete species belonging to the abundant constituents of hnRNP complexes, the hnRNP M group of proteins [19,20]. In addition, 72/74(M) are major components of the LH-hnRNP complex, which has an overall distinct protein and RNA composition compared with the abundant hnRNP and snRNP complexes [17]. The general features of LH-hnRNP, mainly concerning its relative stability to salt and RNase digestion, have related it to the salt-resistant RNP fibrils that are considered to be in association with the nuclear matrix. In this respect, the 72/74(M) proteins have also been considered before to have a tight, RNA-dependent association with the nuclear matrix ([18], and references therein). The present findings on their active participation in RNA splicing are therefore in agreement with a number of reports that suggest a role for nuclear matrix proteins in RNA splicing [29,30].

In the *in vitro* splicing reaction, the earliest identifiable association of 72/74(M) with pre-mRNA occurred at a time corresponding to formation of the first splicing-committed E complex. This finding was not, however, reproduced with the isolated E complex, suggestive of a rather unstable binding. It is striking that no association with the pre-mRNA within the highly abundant H complex could be identified, a finding also seen with purified H complexes. Since H complex is considered to be an assembly of the bulk of the pre-mRNA with hnRNP proteins that is based on a network of salt-sensitive interactions [4], a loose association of 72/74(M) with the pre-mRNA would have been anticipated to take place. However, such interactions should not sustain immunoprecipitation with the anti-(72/74) antibody, unless they are stabilized by other means. In line with other studies showing efficient UV cross-linking of hnRNP proteins within H complex [27] is our finding that binding of 72/74(M) to pre-mRNA can be seen in chromatographically purified H complex that had been UV-irradiated prior to immunoprecipitation (P. Kafasla and J. D. Lewis, unpublished work).

As shown in the Results section, the initial weak interaction of 72/74(M) with pre-mRNA in early splicing E complex formation was stabilized during the subsequent stage of pre-splicing A complex assembly. Moreover, their presence was directly demonstrated within the isolated A complex by UV cross-linking studies. The most straightforward interpretation of our findings is to assume that the association of 72/74(M) with pre-mRNA is restricted to the A complex. This argument is mainly supported by our studies on purified complexes, whereby the bulk of the immunoselected pre-mRNA, as well as the 72/74(M) antigenic species themselves, were seen in isolated A complex. As indicated by native gel analysis of the chromatographically obtained mixed A/B fraction, the pre-spliceosomal A complex was a relatively minor fraction compared with B (results not shown). Thus the lower level of 72/74(M) detected in the A/B fraction, compared with the A fraction alone, does not provide evidence to support the stabilization of the proteins within the B complex. This conclusion is also supported by the observed decline in the immunoselected pre-mRNA at late (60 min) compared with early (up to 30 min) reaction times shown in Figure 1(B). This pre-mRNA fraction sustained at late stages of RNA splicing is most probably attributable to remaining pre-spliceosomal A complex unable to advance to the final B and C splicing complexes, owing to some limiting factor(s) exhausted from the

splicing reaction. The possibility that the observed decline could be due to masking of the antigenic 72/74(M) proteins by the entry of additional splicing factors and/or conformational changes taking place during spliceosomal B and C complex formation cannot be excluded. Nevertheless, it remains that the detectable stable association of 72/74(M) with pre-mRNA is mainly confined to the pre-spliceosomal A complex.

The findings outlined above support the following model for a dynamic participation of 72/74(M) in RNA splicing. It is assumed that their first specific contact with pre-mRNA is made at the stage of pre-splicing E complex assembly. This association is subsequently stabilized within the pre-spliceosomal A complex, followed by destabilization at the time of assembly of B and C splicing complex formation. According to this model, the functioning of 72/74(M) in RNA splicing is envisaged to take place within the A complex concomitantly with the binding of U2-snRNP at the branch site region of the pre-mRNA and the pre-existing association of U1-snRNP with the 5' splice site area [10–13]. A noteworthy feature, at this point in the Discussion, is our work published previously on U2-snRNA, being the major spliceosomal snRNA co-precipitated from HeLa nuclear extracts by the anti-(72/74) serum. U1-snRNA is also seen in the immune precipitate, but at lower amounts relative to U2-snRNA [17].

In the experiment in which immunoprecipitation analysis was combined with RNase T1 protection assays, the application in parallel of anti-(72/74) and anti-Sm sera showed strikingly similar T1-protected RNA fragments. The Sm antigen is known to represent common core protein species of U1, U2, U4 and U5 spliceosomal snRNPs. Also known is the sequence of the T1-protected fragments by Sm binding to pre-mRNA during the entire course of the splicing reaction [25]. At the early splicing times investigated in the present analysis (15-min reaction time point), the major protected fragments refer to a band containing equimolar amounts of the regions of 5' splice and branch-point sites, as well as two smaller fragments of the 5' splice area alone [25]. Although not proven directly, we believe that the three bands clearly seen in our gel most probably correspond to the expected protected fragments. Our findings therefore strongly suggest the sharing or close proximity of such sites between 72/74(M) proteins and the Sm antigen, during the assembly of the pre-spliceosomal A complex. Since U1- and U2-snRNPs are the only spliceosomal complexes participating in A complex formation, establishing interactions with the 5' splice site and the branch point of intron sequences respectively ([1,2], and references therein), these features should also be common to the 72/74(M) protein species. It is also worth taking into consideration the findings obtained with truncated pre-mRNA substrates in the *in vitro* RNA splicing (Figure 5). At incubation times corresponding to A complex formation, the preferential binding of 72/74(M) to the RNA containing the 5' exon-intron portion, relative to the 3' counterpart, was clearly seen. To what extent this interaction would involve the 5' splice site itself is not currently known, as our experimental approach did not address this question. Our current findings suggest, nonetheless, the initial participation of 72/74(M) in splicing during E complex assembly, concomitantly with U1-snRNP interaction at the 5' splice site region of pre-mRNA. The subsequent stabilization of their binding at the stage of A complex formation should somehow relate to U2-snRNP binding to the branch point. It is proposed that the 72/74(M) proteins assist in establishing an array of important RNA-protein and protein-protein interactions at this stage of splicing. In this respect, their role may be analogous to that proposed for the binding of p80 protein in the vicinity of the branch point region during E complex formation, which is thought to assist in the communication of U1-snRNP at

the 5' splice site with the branch point [12]. The binding of p80 is relaxed at the stage of A complex formation, which parallels the destabilization of the 72/74(M) proteins during progression from A to B and C splicing complexes.

The findings on the 72/74(M) proteins presented here have strengthened the view on the anticipated role of hnRNP M in RNA splicing. As pointed out above, opposite to H complex formation, few hnRNP proteins (mainly, hnRNP A/B, C and I) have been found associated with the splicing machinery, and their exact kinetics of binding have not been yet determined [4,10,14]. In this context, it is worth referring to an earlier study in which a large number of spliceosome-associated proteins were identified [31]. Interestingly, the only relevant hnRNP protein species identified were hnRNP M, A1, A2/B1 and C. Since the spliceosomal complex analysed in that study was a mixture of A, B and C splicing complexes, the preferential association of the hnRNP M protein species with the pre-spliceosome complex A, shown in our study, could not be verified.

We believe that future analysis on this specific group of hnRNP M protein species will provide insights into their envisaged important functioning in RNA splicing and, most likely, other stages of RNA maturation.

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