

Association of Nuclear Matrix Antigens with Exon-containing Splicing Complexes

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Abstract. mAbs raised against the human nuclear matrix (anti-NM)¹ mAbs have been used to investigate the role of nuclear matrix antigens in pre-mRNA processing. The three anti-NM mAbs used in this study recognize antigens that are highly localized to nuclear matrix speckles. Surprisingly, all three of these mAbs preferentially immunoprecipitate splicing complexes containing exon sequences. The anti-NM mAbs efficiently immunoprecipitate the exon product complex but not complexes containing the lariat product after the second step of splicing. Two of the anti-NM mAbs completely inhibit pre-mRNA splicing in vitro.

However, none of the anti-NM mAbs appear to recognize factors stably associated with splicing snRNPs. The three anti-NM mAbs predominantly react with distinct high molecular weight antigens, which belong to a class of nuclear proteins that selectively precipitate with Ser-Arg protein-splicing factors in the presence of high Mg²⁺ concentrations. Immunological, biochemical, and cell biological data indicate that two of the NM antigens are related to the defined set of Ser-Arg proteins. The results suggest the existence of an extended Ser-Arg family as a component of the nuclear matrix.

SINCE the discovery of pre-mRNA splicing in 1977, considerable progress has been made towards elucidating the mechanism underlying this process. A spliceosome containing multiple small nuclear ribonucleoprotein particles (snRNPs)¹ and non-snRNP protein factors executes the precise cleavage and ligation steps generating mature mRNA (reviewed in Guthrie, 1991; Moore et al., 1993). However, much less is known about the factors which specify splice site selection in pre-mRNA and the nature of the changes in this process involved in the recognition and regulation of alternative spliced pre-mRNAs. Furthermore, it is not known whether the pre-mRNA splicing process is co-ordinated with other processes of gene expression, in particular, transcription and RNA transport. Insight into these questions may emerge from the determination of precise relative locations in the nucleus of structures involved in RNA processing.

Factors involved in the transcription and processing of precursor RNA are highly localized to distinct subnuclear structures. The largest and best studied of these are the nucleoli in which ribosomal precursor RNA is transcribed, processed and packaged into ribosome subunits. Recent studies using fluorescent-tagged antibody and nucleic acid

probes show that pre-mRNA splicing factors are enriched in at least two distinct types of nuclear structure. The most prevalent of these correspond to "speckles" of which there are typically 20–50 present in a mammalian cell nucleus (reviewed in Spector, 1993). There are also one to five "coiled bodies" detected in the majority of mammalian nuclei (reviewed in Lamond and Fonseca, 1993). Both structures are sites of enrichment of U2, U4/6, and U5 snRNPs and also non-snRNP splicing factors. U1 snRNP is also present in these structures although it is also widely distributed throughout the nucleus. Also, speckles, but not coiled bodies, are sites of polyA⁺RNA accumulation, as detected by hybridization in situ with oligo-dT (Carter et al., 1991, 1993).

The immunofluorescent speckled staining pattern corresponds to the interchromatin granule clusters and perichromatin fibril network seen in conventional embedded EM sections (Spector et al., 1991). Resinless EM sections have shown that the speckled immunofluorescence pattern corresponds to dense assemblies located within the matrix filament network (Nickerson et al., 1992; Wan et al., 1994). While the precise relationship of these nuclear structures to RNA processing is not clear, newly synthesized RNA, detected by labeling with a short pulse of [³H]uridine, primarily localizes to the perichromatin fibril region and not the interchromatin granules (Fakan et al., 1976). Most introns are spliced while the nascent RNA is still in the process of elongation and therefore proximal to the gene (Beyer and Osheim, 1988). Based on these observations, it has been pro-

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1. *Abbreviations used in this paper:* AEBSF, 4-(2-aminoethyl) benzenesulfonfyl fluoride; NGS, normal goat serum; NM, nuclear matrix; RRM, RNA recognition motif; snRNP, small nuclear RNP.

posed that splicing of nascent transcripts primarily occurs in the perichromatin fibril region whereas posttranscriptional splicing, as well as splicing factor storage, assembly, and/or recycling may occur in interchromatin granules.

Recent studies, using high-resolution immunofluorescent microscopy of *in situ* hybridization probes, have revealed that certain viral and cellular pre-mRNA transcripts are localized within the confines of curvilinear "tracks" (Lawrence et al., 1989; Huang and Spector, 1991; Xing et al., 1993). These tracks emerge from the corresponding gene and generally extend out towards the nuclear periphery. In instances where tracks are observed, the corresponding gene is co-incident with or located near to one of the nuclear speckles (Huang and Spector, 1991; Xing et al., 1993). The RNA track emerging from this focus usually contains intron sequences only in the gene-proximal part of the track, whereas exon sequences are detected throughout the track, thus it is possible that certain pre-mRNA transcripts are processed during nuclear export along a vectorial pathway.

The nucleus is highly organized, with factors sorted to discrete domains, usually according to their particular functional association. In addition to speckles and coiled bodies, other localized structures include those implicated in transcription (Jackson et al., 1993; Wansink et al., 1993; Xie et al., 1993) DNA replication (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Hozák et al., 1993), as well as domains of unknown function (Ascoli and Maul, 1991; Saunders et al., 1991; Stuurman et al., 1992). The organizational principles of subdomains in the nucleus are not well understood. Most of these domains remain associated with a subnuclear structure after the majority of chromatin and soluble nuclear material has been removed. This "nuclear matrix" has been generated under a variety of different experimental conditions (reviewed in Nickerson and Penman, 1992). The nuclear matrix retains over 70% of nuclear RNA, including the majority of hnRNA (Herman et al., 1978; Miller et al., 1978; van Eekelen et al., 1981; He et al., 1990). The earliest pulse-labeled RNA transcripts that can be detected are associated with the matrix (Jackson and Cook, 1985). Unspliced and spliced mRNAs of specific genes have also been detected in the matrix suggesting that pre-mRNA processing occurs on this structure (Mariman et al., 1982; Schröder et al., 1987a,b). Direct support for this was provided by Zeitlin et al. (1987, 1989) who showed that a β -globin pre-mRNA associated with isolated matrix is rapidly processed following the addition of a soluble nuclear fraction. Furthermore, it has been shown that components in speckles, and also RNA tracks, remain quantitatively associated with the nuclear matrix (NM) following nuclear extraction (Vogelstein and Hunt, 1982; Spector et al., 1983; Xing and Lawrence, 1991).

The identification of specific roles for matrix factors will be important for understanding the role of nuclear substructure in the organization, and potentially the regulation, of pre-mRNA processing. Several mAbs that were raised against nuclear matrix preparations identify a class of integral nuclear matrix proteins which preferentially associate with a specific subset of pre-mRNA splicing complexes. These antigens are non-snRNP splicing factors, two of which may belong to the Ser-Arg family of proteins. This study suggests a fundamental role for specific nuclear matrix proteins in the nuclear organization of pre-mRNA processing.

Materials and Methods

Microscopy

CaSki cells (a human cervical carcinoma cell line: CRL 1550; American Type Culture Collection, Rockville, MD) were grown on glass coverslips. Cells were washed with PBS at 4°C and then extracted at 4°C in CSK Buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA) containing 0.5% (wt/vol) Triton X-100, 20 mM vanadyl riboside complex, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF). For immunofluorescent staining of cells with the HIB2 mAb, chromatin was first removed by digestion for 50 min at 32°C with 400 U/ml DNaseI in digestion buffer (10 mM Pipes, pH 6.8, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl₂, 1 mM EGTA) containing 0.5% (wt/vol) Triton X-100, 20 mM vanadyl riboside complex, and 1 mM AEBSF, followed by extraction in 0.25 M ammonium sulfate as described previously (Nickerson et al., 1992). This step was included to expose the HIB2 antigen which is partially masked by chromatin. Cells were fixed with 4% paraformaldehyde in CSK buffer for 20–40 min at 4°C. After fixation, cells were washed with PBS and blocked in 10% normal goat serum (NGS) in PBS for 30 min at 32°C. Antibody incubations were performed in PBS containing 2% NGS or in tissue culture supernatant (for anti-SC35 mAb) for 1 h at 32°C. After three to four washes in PBS, samples were incubated with fluorochrome-coupled second antibodies in PBS containing 2% NGS. For double-label experiments using two mAbs, the second Abs were Ig class-specific goat anti-mouse IgG coupled to fluorescein, goat anti-mouse IgM coupled to rhodamine, or anti-mouse IgM coupled to Texas Red. Following extensive washing in PBS, coverslips were mounted using VectaShield mounting medium (Vector Laboratories, Burlingame, CA).

In the immunostaining-competition experiment (see Fig. 10), each anti-NM mAb was titrated to determine the minimum concentration required for clear immunofluorescent detection. mAbs at these dilutions were pre-incubated with or (for control samples) without 40 μ g/ml of purified SR proteins in PBS containing 2% NGS. The pre-incubated mAbs were then used for immunofluorescent staining of cells as described above.

For resinless section electron microscopy and immunogold labeling, CaSki cells were extracted to uncover core filaments of the nuclear matrix as previously described (He et al., 1990). Immunogold labeling of the matrix preparation using the B4A11 mAb and a colloidal gold-conjugated second antibody was performed as described by Nickerson et al. (1992).

HeLa Cell Nuclear Splicing Extracts and SR Protein Purification

HeLa nuclear extracts were prepared essentially as described by Dignam et al. (1983). Depletion of SR proteins from HeLa nuclear extracts was performed during nuclear extract preparation, before the final dialysis step against buffer D. Nuclear extract in buffer C was aliquoted into 0.5-ml amounts in microdialysis vessels (Sartorius Balances, Westbury, NY) and dialyzed against 3 \times 1 liter changes of dialysis buffer (DB buffer: 65 mM KCl, 15 mM NaCl, 10 mM Hepes, pH 7.6, 1 mM Na₂EDTA, 2 mM DTT, 5 mM KF, 5 mM β -glycerophosphate, 0.2 mM PMSF; Zahler et al., 1992) in a beaker surrounded by a jacket of ice. Dialyzed extracts were microfuged for 5 min at 4°C to pellet non-specific precipitate and the cleared supernatants transferred to new tubes. The dialyzed extract was incubated on ice for 1 h with MgCl₂, added from a 1 M stock solution, to a final concentration of 20 mM. One half of the nuclear extract was microfuged twice at 4°C (1 \times 30 min, then in a new tube, 1 \times 15 min) to pellet SR proteins (=Spin + NE). The other half was left on ice (=Spin - NE). The Spin + and Spin - extracts were dialyzed against 3 \times 1 liter changes of buffer D (Dignam et al., 1983), aliquoted into 0.1 ml amounts, and then snap frozen in liquid nitrogen. The Spin + NE pellet fraction was washed twice in DB buffer containing 20 mM MgCl₂, solubilized in buffer D (Dignam et al., 1983) containing 5% glycerol, and then snap frozen in liquid nitrogen.

SR proteins used in splicing assays were purified from HeLa cells essentially as described by Zahler et al. (1992).

In Vitro Splicing Assays

Splicing assays containing regular HeLa cell nuclear extracts were performed using *in vitro* transcribed, capped and uniformly ³²P-labeled pre-mRNA substrates, essentially as previously described (Grabowski et al., 1984). Splicing assays using Spin + NE and Spin - NE were performed without additional MgCl₂ added, but otherwise under identical conditions as for assays using regular HeLa nuclear extract. RNA recovered from

splicing/immunoprecipitation assays carried out with the PIP85A pre-mRNA (Moore and Sharp, 1992) was analyzed on 15% polyacrylamide (1:30, bisacrylamide/acrylamide)/8 M urea denaturing gels run in 1 × TBE buffer. Splicing/immunoprecipitation assays with the β -globin pre-mRNA (AL4; Lamond et al., 1987) were analyzed on 8% polyacrylamide (1:30, bisacrylamide/acrylamide)/8 M urea denaturing gels.

Immunoprecipitations

Immunoprecipitation of splicing complexes was carried out in "IP100" buffer (100 mM NaCl, 2 mM MgCl₂, 0.5 mM DTT, 0.05% NP-40, 50 mM Tris/HCl, pH 7.6). Protein A beads (Pharmacia Fine Chemicals, Piscataway, NJ) (pre-swollen overnight in IP100 containing 0.05% azide) were washed twice in fresh IP100 before use. Beads were pre-coated with 10 μ g rabbit anti-mouse Ig's (Pierce Chemical Co., Rockford, IL) for 1 h in 150 μ l IP100, then washed three times in IP100 before coupling monoclonals. Beads were incubated with the monoclonals for 2–3 h, washed three times, and then resuspended in 300 μ l IP100. Splicing reactions (typically 60 μ l containing 30% nuclear extract, vol/vol) were incubated with the Ab beads for 1.5 h. Beads were washed three times (quick washes) in IP100 and immunoprecipitated RNA eluted by incubation for 40 min at 65°C in 300 μ l proteinase K buffer (0.5% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 7.6) containing 15 μ l proteinase K (20 mg/ml) and 1.5 μ l glycogen (20 mg/ml). 30 μ l of protein A beads (packed bead volume) was used per immunoprecipitation. Protein A beads were centrifuged at all times at 3–4K in a bench-top microfuge, and all incubation steps were carried out at 4°C, unless stated otherwise.

Immunoprecipitation of snRNPs from HeLa nuclear extracts was carried out exactly as described previously (Blencowe et al., 1993).

Northern Analysis

Immunoprecipitated RNA was separated on 10% polyacrylamide (1:30 bis acrylamide/acrylamide)/8 M urea denaturing gels run in 1 × TBE buffer. Electrophoresed RNA was electroblotted and then UV cross-linked onto Hybond "N" nylon membrane (Amersham Corp., Arlington Heights, IL). Detection of snRNAs was carried out using snRNA-specific riboprobes as described by Blencowe et al. (1989).

SDS-PAGE and Western Blot Analysis

For Western blots probed with the BIC8 and B4A11 mAbs, proteins separated in SDS-polyacrylamide gels were transferred to nitrocellulose membrane using a semi-dry electrotransfer apparatus (Bio-Rad Laboratories, Cambridge, MA). For blots probed with the HIB2 mAb, proteins were transferred onto nitrocellulose by wet transfer for 6 h, and then incubated under conditions to denature then renature proteins, as described previously (Vinson et al., 1988). All blots were pre-blocked in 3% nonfat milk powder in PBS and then incubated with primary mAbs overnight at 4°C. Detection using a 2nd antibody conjugated to alkaline phosphatase was carried out as previously described (Harlow and Lane, 1988).

Results

Nuclear Matrix Antigens BIC8, HIB2, and B4A11 Co-localize with the Non-snRNP Splicing Factor SC35

Murine mAbs raised against isolated NM preparations (anti-NM) from human cells were used to visualize intranuclear staining patterns in CaSki cells (a human cervical carcinoma cell line). Three anti-NM mAbs were selected which gave prominent punctate immunofluorescent staining patterns in interphase nuclei. Two of the anti-NM mAbs used in the present study, HIB2 and BIC8, have previously been shown to recognize antigens concentrated in nuclear matrix-associated structures which appear as speckles in immunofluorescence (Nickerson et al., 1992; Wan et al., 1994). The B4A11 mAb has also been shown to recognize an antigen retained in nuclear matrix preparations in a distinct speckled staining pattern (Fig. 1, see below).

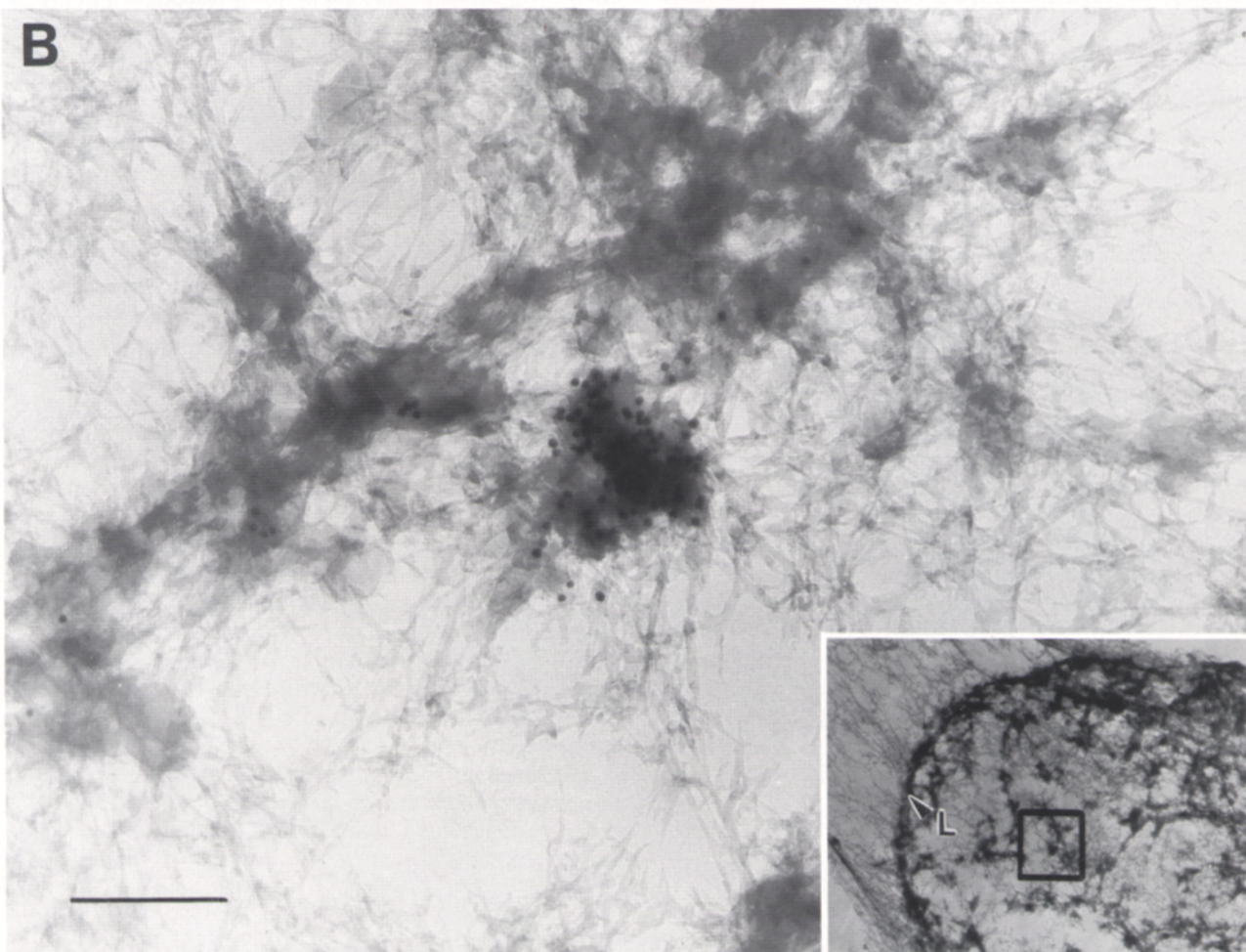
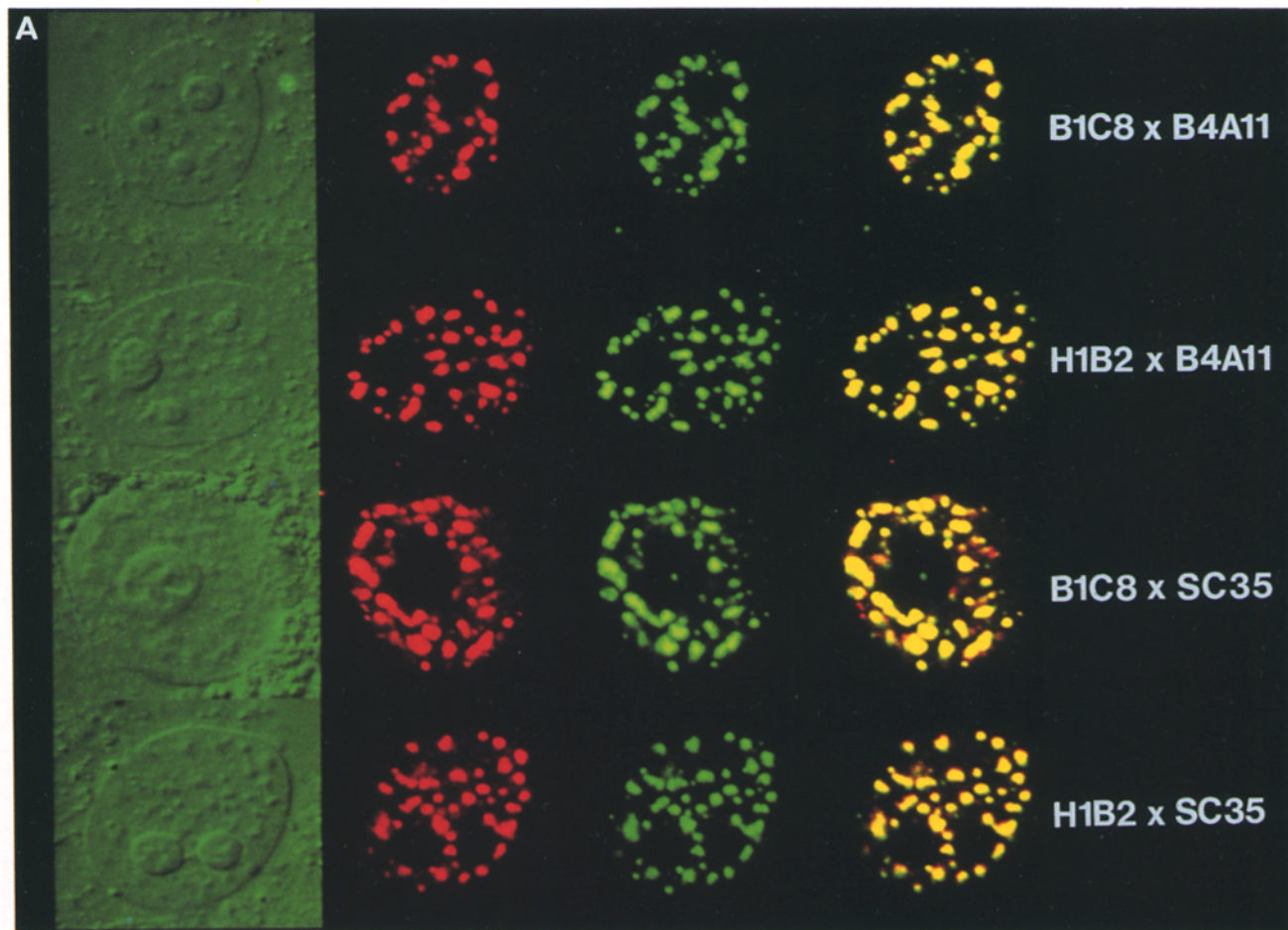
In the previous studies, the BIC8, HIB2, and B4A11 anti-

gens were also shown to have strikingly different localization patterns during mitosis (Nickerson et al., 1992; Wan et al., 1994; J. A. Nickerson and S. Penman, unpublished data). Both the HIB2 and BIC8 mAbs stain distinct regions of the mitotic spindle apparatus whereas the B4A11 antigen apparently becomes soluble during mitosis and cannot be detected in detergent-extracted cells. The very different behavior of these antigens during mitosis indicates that these three anti-NM mAbs recognize different proteins, even though they have similar nuclear distributions during interphase. It is interesting to note that the dynamic redistribution of the B4A11 matrix antigen is similar to that observed for antigens recognized by the mAb SC35, which also are not detected from prophase to late telophase (Spector et al., 1991). In total nuclear extracts this mAb reacts predominantly with SC35 as well as with other members of the Ser-Arg (SR) family of protein splicing factors (Fu and Maniatis, 1990; Fu et al., 1992). As will be expanded upon below, the SR family of proteins contain an NH₂-terminal RNA recognition motif (RRM) and a COOH-terminal domain that is rich in Serine-Arginine diamino acid repeats (Zahler et al., 1992). It has been previously shown that the SC35 mAb stains interphase nuclei in a speckled pattern (Fu and Maniatis, 1990).

In the present study, all three of the anti-NM mAbs were tested for recognition of sites of antigen concentration that are co-incident with nuclear speckles enriched in pre-mRNA splicing factors. Cells were stained with each of the anti-NM mAbs and then co-stained with a patient serum specific for Sm antigens of snRNPs. The anti-Sm serum gave a typical widespread intranuclear staining with sites of highest concentration in speckled structures, whereas the anti-NM mAbs displayed much more restricted punctate staining patterns, co-localizing with Sm antigens predominantly in the speckled structures (data not shown). This suggests that all three anti-NM mAbs recognize antigens that are located in the subnuclear structure in which RNA splicing occurs.

A direct comparison of the relative subnuclear locations of the anti-NM antigens and splicing factors was made using the SC35 mAb. Pairwise comparisons of the staining patterns obtained with the anti-NM mAbs, and with the SC35 mAb, are shown in Fig. 1. This comparison used fluorochrome conjugated 2nd antibodies that are specific for different mAb isotypes (BIC8 and HIB2 are IgM's whereas B4A11 and SC35 mAbs are IgG's). Confocal images of cells co-stained with the different anti-NM mAbs are shown in red and green in the second and third columns. Combinations of mAbs used are indicated to the right of the figure. Superimposition of the red and green images is shown in the fourth column. Precise colocalization of the anti-NM and SC35 mAb staining, represented by areas of yellow coloration, was detected in the speckled structures. This comparison revealed a similar but nonidentical staining pattern for the different anti-NM mAbs. For example, every speckled domain stained by the HIB2 mAb was also stained by B4A11, although B4A11 also stained several additional sites that were not stained by HIB2 (note the additional sites labeled by B4A11 in green in the overlay with HIB2). By contrast the BIC8 and SC35 mAbs, while displaying extensive colocalization with each other, both stained additional, nonoverlapping, speckled domains detected as the separate red and green speckles in the overlay.

The structures underlying speckles are integral to the nu-



clear matrix and remain in the nucleus after the complete removal of chromatin. Resinless section electron microscopy of chromatin depleted nuclei shows the nuclear matrix to be comprised of a dense, highly interconnected network of filaments (termed core filaments) bounded by the nuclear lamina. The filament network, seen in Fig. 1 *B*, enmeshes numerous dense structures. These structures have been decorated by immunogold labeling with the B4A11 mAb and therefore must correspond to the speckles observed in immunofluorescence. Previous studies have shown that the H1B2 and B1C8 mAbs give similar results (Nickerson et al., 1992; Wan et al., 1994).

The data in Fig. 1 show that antigens recognized by the anti-NM mAbs B1C8, H1B2, and B4A11 are highly localized to nuclear speckles in which the pre-mRNA splicing factor SC35 is also concentrated. While the antigens recognized by these antibodies extensively co-localize with each other, the overlap does not extend to every nuclear speckle. This indicates that individual nuclear speckled domains may differ from each other in their specific protein composition. The different staining patterns observed further supports the notion that the anti-NM mAbs recognize distinct nuclear matrix antigens. To address whether the anti-NM mAbs recognize antigens that are involved in RNA processing, each was assayed for interactions with pre-mRNA splicing complexes assembled *in vitro*.

Association of Antigens of the Nuclear Matrix with Pre-mRNA Splicing Complexes

Fig. 2 shows the results of using each anti-NM mAb to immunoprecipitate splicing complexes assembled *in vitro* on different radiolabeled pre-mRNA substrates. An efficiently spliced pre-mRNA substrate, PIP85A, was first incubated in splicing reactions to generate splicing complexes. Immunoprecipitations were then carried out from these reactions using beads pre-coupled to each of the different anti-NM mAbs (Fig. 2 *A*, lanes 7–9). A mAb (Y12; Lerner et al., 1981) specific for the common (Sm) snRNP-associated antigen was assayed in parallel as a positive control for splicing complex immunoprecipitation (Fig. 2 *A*, lane 6). RNA recovered following immunoprecipitation was separated alongside a sample of RNA extracted directly from a parallel splicing reaction (Fig. 2 *A*, lane 3). In the absence of mAb, or in the presence of a nonspecific control mAb, little or no immunoprecipitation of RNA was observed (Fig. 2 *A*, lanes 4 and 5). By contrast, the anti-Sm mAb resulted in efficient immunoprecipitation of unspliced pre-mRNA and both the intermediates and products of the splicing reaction (Fig. 2 *A*, lane 6). Strikingly, all three of the anti-NM mAbs also

efficiently immunoprecipitated different RNA species from the splicing reaction. However, there was a significant difference in the ratios of splicing intermediates to products immunoprecipitated by anti-NM mAbs compared to anti-Sm mAb. The anti-NM mAbs consistently immunoprecipitated more of the splicing intermediates relative to lariat product than did the anti-Sm mAb. This was particularly striking in the case of the B1C8 and B4A11 mAbs. A quantitative comparison of immunoprecipitation levels in Fig. 2 *A* showed the ratio of 3' exon-lariat intermediate to lariat product to be 2.9-fold higher for B1C8 mAb than for anti-Sm mAb and, in the case of B4A11, this ratio was 2.5-fold higher (Fig. 2 *D*). H1B2 also preferentially immunoprecipitated the lariat intermediate versus lariat product compared to Sm, although the difference in the ratios of these reaction species is less pronounced (1.6-fold higher; Fig. 2 *D*) than for the B1C8 and B4A11 mAbs, (Fig. 2 *A*, compare lane 9 with lane 6).

The data in Fig. 2 *A* show that the different anti-NM mAbs recognize antigens that are preferentially associated with unspliced pre-mRNA and the RNA intermediates of the *in vitro* splicing reaction. To determine the specificity of these antigen-RNA interactions in more detail, the ability of each anti-NM mAb to immunoprecipitate two RNAs of completely different sequence was tested (Fig. 2 *B*). Immunoprecipitations were carried out using the same mAbs as in Fig. 2 *A*, but from splicing reactions incubated with either a β -globin pre-mRNA substrate (T3 RNA; Lamond et al., 1987) or a control RNA complementary to the β -globin pre-mRNA (T7 RNA). Splicing reactions were incubated with equivalent amounts of the two different RNAs before immunoprecipitation. In reactions incubated with the β -globin pre-mRNA, the three anti-NM mAbs all show a pattern of immunoprecipitation similar to that of the PIP85A substrate. As noted previously for all three mAbs, a higher ratio of splicing intermediates to lariat product was immunoprecipitated compared to the ratio obtained with the anti-Sm Y12 mAb (Fig. 2 *B*, compare lanes 8–10 with lane 5). However, the ratios of lariat 3' exon intermediate/lariat product immunoprecipitated by the anti-NM mAbs were typically higher (approximately threefold) for the β -globin pre-mRNA substrate than for the PIP85A pre-mRNA (compare Fig. 2 *B* with *A*; see Fig. 2 *D*). The three anti-NM mAbs also efficiently immunoprecipitated the unspliced β -globin pre-mRNA. In marked contrast to results obtained with the PIP85A and β -globin pre-mRNA substrates, none of the anti-NM mAbs significantly immunoprecipitated the β -globin-complementary RNA above background levels (compare Fig. 2 *B*, lanes 12–14 with lane 11). In separate experiments, a similar pattern of immunoprecipitation was also observed for a third pre-mRNA substrate derived from

Figure 1. Immunolocalization of nuclear matrix antigens B1C8, H1B2, and B4A11. (*A*) B1C8, H1B2, and B4A11 colocalize with each other and with the non-snRNP splicing factor SC35. CaSki interphase nuclei are shown double labeled with combinations of the different anti-NM mAbs and with anti-SC35 mAb, as indicated on the right of the figure. The red and green panels show the localization pattern of each individual mAb. Colocalization, which appears as a yellow coloration in the superimposed images, is shown in the fourth column. A phase contrast image of each cell nucleus is shown in the first column. (*B*) High-resolution immunolocalization of the B4A11 antigen in the nuclear matrix. CaSki cells were extracted to expose core filaments of the nuclear matrix which consist of a network of highly branched ~ 10 -nm filaments connected to the nuclear lamina (*L*) (see low-magnification insert). The core filaments of the matrix suspend numerous dense structures which correspond to speckles detected by immunofluorescent staining. A subset of these structures are specifically decorated by the B4A11 mAb (see high-magnification micrograph, orientated sideways with respect to the insert). The sites of B4A11 antigen localization were detected in the micrograph using a colloidal gold-conjugated second antibody. Bar, 0.2 μ m.

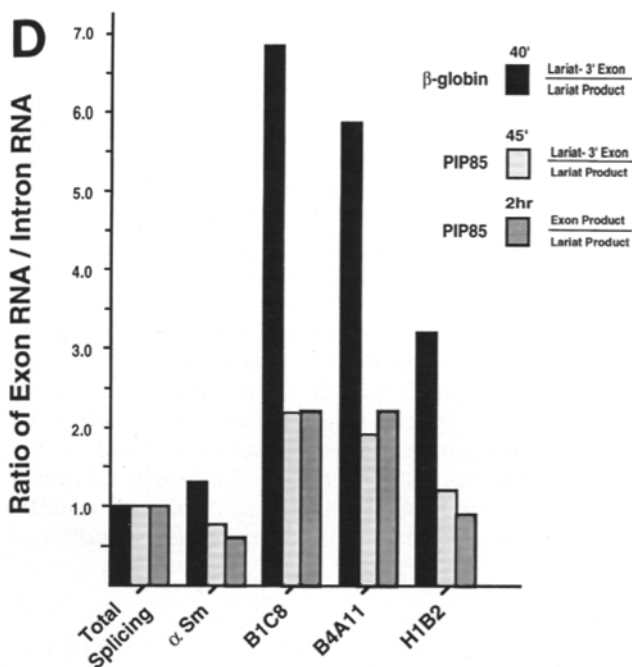
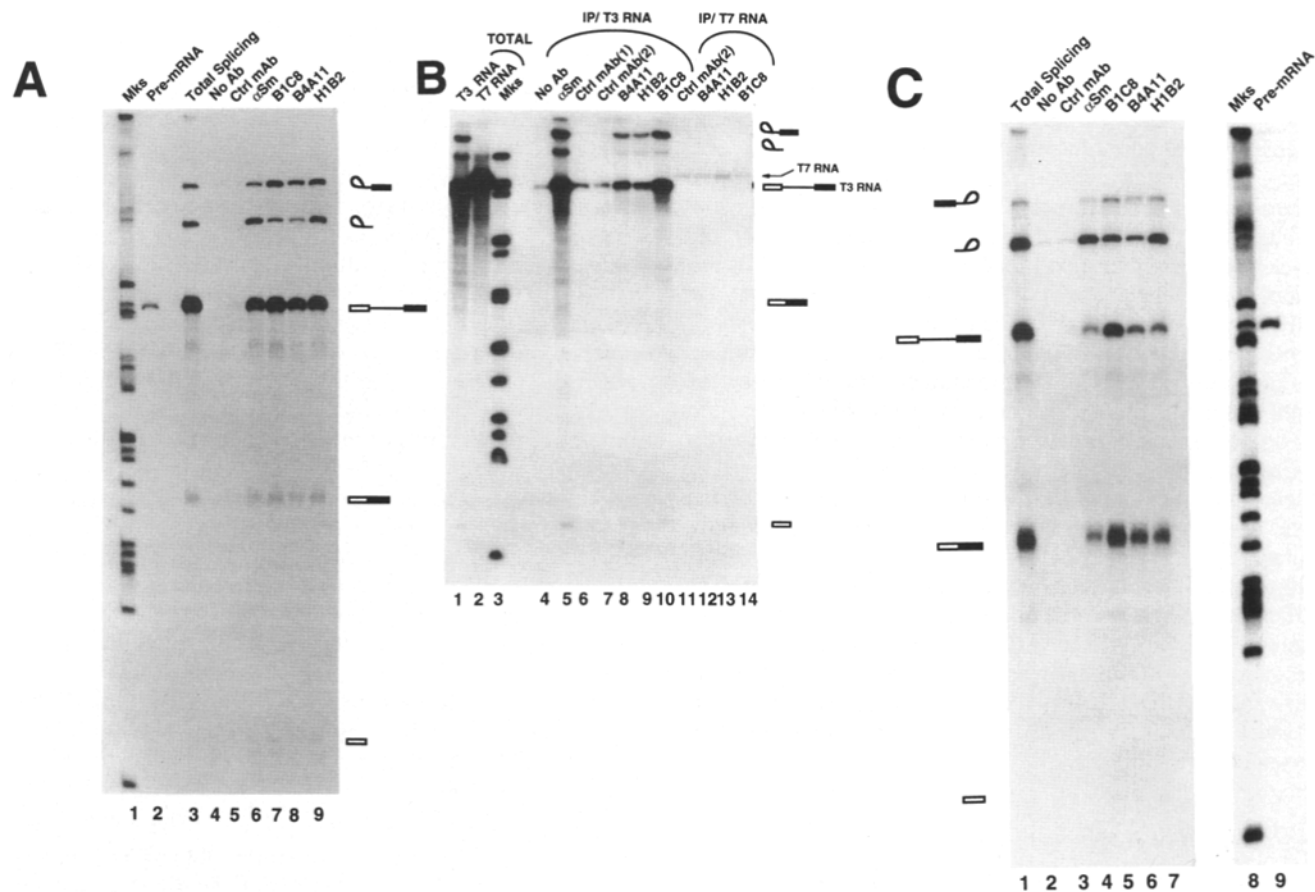


Figure 2. The B1C8, B4A11, and H1B2 mAbs preferentially immunoprecipitate exon-containing complexes from in vitro splicing reactions. (A) Pre-mRNA splicing reactions were incubated with a 32 P-labeled PIP85A RNA substrate for 45 min before immunoprecipitation. RNA recovered following immunoprecipitation is shown in lanes 4–9. RNA recovered directly from a parallel splicing reaction is shown in lane 3 (represents 50% of the total RNA recovered; RNA from each immunoprecipitation represents all of the RNA recovered). Antibodies used are monoclonal anti-Sm (Y12) (lane 6), B1C8 (lane 7), B4A11 (lane 8), and H1B2 (lane 9). Non-specific control immunoprecipitations are with beads alone (lane 4) and with a monoclonal specific for the nuclear mitotic apparatus protein (*NuMA*) (lane 5). Markers are unlabeled PIP85A RNA (lane 2) and end-labeled phiX174-HaeIII fragments (lane 1). (B) Immunoprecipitation of β -globin pre-mRNA splicing complexes. Globin sense (T3) and antisense (T7) RNAs were incubated in splicing reactions for 40 min before immunoprecipitation. Antibodies used for immunoprecipitation are as in A. Two different nonspecific control mAbs (to NuMA protein) are shown in lanes 7 and 11. RNA recovered directly from the T3 and T7 RNA splicing reactions is shown in lanes 1 and 2, respectively; the amounts shown correspond to 50% of the total sample whereas each immunoprecipitation lane represents the total RNA recovered. Markers are end-labeled pBR322-MspI fragments (lane 3). (C) Anti-NM mAbs (B1C8, H1B2, and B4A11) preferentially immunoprecipitate the ligated exon product after the second step of pre-mRNA splicing.

Immunoprecipitations were carried out as in A, but from splicing reactions that had been incubated for 2 h. Lanes 1–7 correspond directly to lanes 3–9 in Fig. 3 A. Markers (lanes 8 and 9) are as in Fig. 3 A. (D) Quantitation of RNA immunoprecipitation levels. Shaded bars represent (in relative units) the ratio of exon-containing RNA to intron-lariat RNA immunoprecipitated by anti-Sm and anti-NM mAbs. All values have been normalized to the corresponding exon RNA/intron RNA ratio in the total splicing reaction (=1). Relative ratios of β -globin lariat 3' exon/lariat product immunoprecipitated from a 40' splicing reaction are shown in black. Ratios are also shown for the PIP85A lariat 3' exon/lariat product immunoprecipitated from a 45' reaction (light grey bars) and PIP85A exon product/lariat product immunoprecipitated from a 2-h reaction (dark grey bars). The data were collected from the immunoprecipitation experiments in A–C, which are representative of levels observed in several repeat experiments. We note however that in many experiments, the ratios observed for the PIP85A pre-mRNA were higher than those shown here. The amount of each splicing reaction RNA species was determined using a Molecular Dynamics PhosphorImager.

adenovirus (Ad1; Konarska and Sharp, 1987) (data not shown). These data show that the anti-NM mAbs specifically and stably bind to antigens that are associated with pre-mRNA splicing substrates of different sequence. Furthermore, an RNA molecule that does not contain functional splice sites, or assemble into pre-mRNA splicing complexes, is not immunoprecipitated by the anti-NM mAbs.

The above data address interactions between antigens recognized by anti-NM mAbs and splicing complexes that are assembled during the first 40–45 min of a splicing reaction *in vitro*. Previous analyses of the time course of splicing *in vitro* have shown that formation of spliceosomes is maximum at about 40 min. With further incubation, spliceosomes are largely resolved into complexes containing the reaction products. After the second step of splicing, the lariat product remains in a stable complex containing splicing snRNPs (Konarska and Sharp, 1987). By contrast, specific factors that remain associated with the exon product RNA of the splicing reaction have not been identified.

The association of the NM antigens with the splicing reaction products was tested by immunoprecipitation after a 2-h incubation. Fig. 2 C shows immunoprecipitations from 2-h splicing reactions containing the PIP85A pre-mRNA, using the same mAbs as in Fig. 2 A. In the total reaction ~50% of the unspliced pre-mRNA has been converted to reaction products (Fig. 2 C, lane 1). The Y12 mAb preferentially immunoprecipitated the lariat product relative to exon-product RNA (Fig. 2 C, lane 4). This is consistent with the known association of snRNPs with a lariat-product complex. Interestingly, the anti-NM mAbs show the opposite relative affinity for the products of the splicing reaction. All three mAbs preferentially immunoprecipitated the exon-product RNA compared to lariat product species (Fig. 2 C, compare lanes 5–7 with lane 4; see Fig. 2 D). As expected, a higher level of the splicing intermediates relative to lariat product was also immunoprecipitated by the anti-NM mAbs as compared to the Y12 mAb. These data suggest that the B1C8, H1B2 and B4A11 antigens are preferentially associated *in vitro* with splicing complexes that contain exon sequences. Furthermore, the ability of these mAbs to efficiently immunoprecipitate the exon-product RNA shows that these antigens remain assembled on exons following the second step of the splicing reaction.

Inhibition of Splicing by Anti-NM mAbs

The NM antigens recognized by the three mAbs could be essential pre-mRNA splicing factors. The potential role of these proteins in splicing was tested by incubating each anti-NM mAb in splicing reactions containing the PIP85A pre-mRNA (Fig. 3). Interestingly, both the B1C8 (Fig. 3, lanes 7–9) and B4A11 (lanes 13–15) mAbs completely inhibited pre-mRNA splicing, whereas the H1B2 mAb (lanes 10–12) gave little or no inhibition, even when present at higher concentrations than the other two mAbs. Two different control mAbs, 3C1 (lanes 16–18) and anti-actin (lanes 19–21), both of which are IgM's, gave little or no inhibition of pre-mRNA splicing. 3C1 is a mAb that labels a nuclear matrix antigen with a diffuse distribution (J. A. Nickerson and S. Penman, unpublished observations). In parallel experiments, addition of inhibitory concentrations of B4A11 and B1C8 mAbs at the beginning of a splicing reaction, prevented subsequent immunoprecipitation of the unspliced pre-mRNA by these

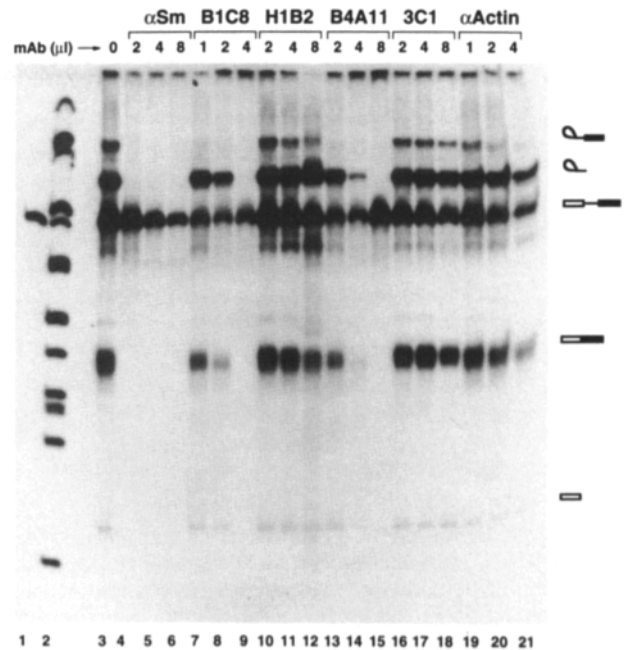


Figure 3. Effect of anti-nuclear matrix antibodies (B1C8, H1B2, and B4A11) on pre-mRNA splicing. Splicing reactions containing PIP85A RNA were incubated for 1 h in the presence of purified mAbs (lanes 4–21). Three different amounts of each mAb were used, as indicated. Negative controls for splicing inhibition include two IgM mAbs: 3C1 (lanes 16–18) and anti-actin (19–21). Anti-Sm (Y12) mAb was included as a positive control for splicing inhibition (lanes 4–6). Lane 3 shows splicing in the absence of mAb. Concentrations of mAbs are: Y12, H1B2, 3C1, and anti-actin ~1 mg/ml; B1C8 = 0.85 mg/ml, B4A11 = 0.71 mg/ml. Markers are as in Fig. 2.

mAbs (data not shown). By contrast, the H1B2 mAb, when incubated in splicing reactions at similar concentrations, still allowed efficient immunoprecipitation of splicing complexes. This suggests that the H1B2 mAb, but not the B1C8 and B4A11 mAbs, binds stably to an antigen without preventing its assembly into splicing complexes, or interfering with the splicing reaction.

Immunoprecipitation experiments were performed to determine if the NM-antigens are stable components of snRNPs involved in splicing. Fig. 4 shows a Northern analysis of spliceosomal snRNAs immunoprecipitated from a HeLa nuclear extract by the different mAbs. While mAbs against shared snRNP-associated structures (m3G cap and Sm antigens), efficiently immunoprecipitate all four spliceosomal snRNPs (U1, U2, U4/6, and U5), the anti-NM mAbs did not significantly immunoprecipitate any spliceosomal snRNP species (compare lanes 3 and 4 with lanes 5–7). In related experiments, total immunoprecipitates prepared from HeLa cell nuclear extracts using anti-m3G and anti-Sm Abs did not contain proteins that could be detected on western blots probed with the anti-NM mAbs (data not shown). We conclude that antigens recognized by the B1C8, B4A11 and H1B2 mAbs are not stable components of spliceosomal snRNPs.

Immunoreactivity of Anti-NM mAbs with SR Protein Splicing Factors

The B1C8, B4A11, and H1B2 mAbs recognize antigens that

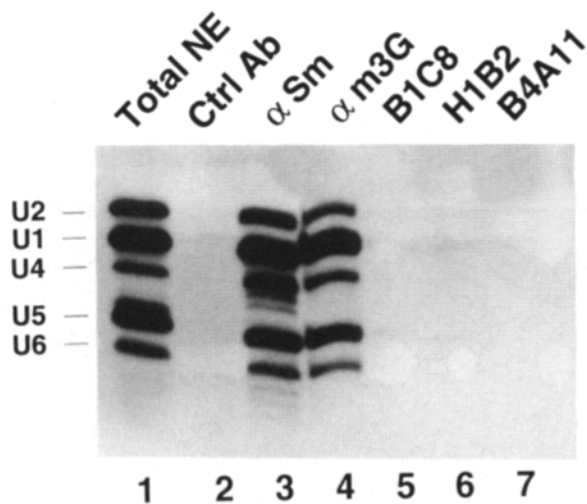


Figure 4. B1C8, H1B2, and B4A11 antigens are not associated with splicing snRNPs. RNA immunoprecipitated from HeLa cell nuclear extract with the anti-NM mAbs was probed on northern blots using ^{32}P -labeled riboprobes specific for each of the spliceosomal snRNAs. RNA immunoprecipitated by the B1C8, H1B2, and B4A11 mAbs is shown in lanes 5, 6 and 7, respectively. Anti-Sm (Y12) (lane 3) and anti-trimethylguanosine (α m3G) cap (lane 4) mAbs were used as positive controls for snRNP immunoprecipitation. Level of nonspecific immunoprecipitation by a control mAb (anti-NuMA) is shown in lane 2. A sample of total HeLa RNA is shown in lane 1. Approximately 0.25 mg nuclear extract was used in each immunoprecipitation.

could correspond to splicing factors that assemble separately of snRNPs into spliceosomes. In this regard, it is interesting to note that the pattern of subnuclear localization of these antigens on the nuclear matrix resembles closely that observed for antigens recognized by the SC35 mAb (see Fig. 1). In addition to having a similar nuclear distribution, it was found that the SC35 mAb resulted in a near identical pattern of splicing complex immunoprecipitation compared to the anti-NM mAbs (see Fig. 7 below). The SC35 mAb preferentially immunoprecipitated exon-containing RNA intermediates and products from a splicing reaction. It is therefore possible that the anti-NM mAbs also recognize members of the SR protein family.

Immunoblot analysis has shown that B1C8 mAb reacts principally with a 180-kD nuclear matrix protein and H1B2 with a nuclear matrix protein of at least 240 kD (Nickerson et al., 1992; Wan et al., 1994). More recently, the B4A11 mAb was also found to react with a high molecular weight (~300 kD) nuclear matrix antigen (J. A. Nickerson and S. Penman, unpublished observations). Consistent with these observations, proteins of the same high molecular weights are also detected by the anti-NM mAbs in HeLa cell nuclear extracts (Fig. 5). In addition to these antigens however, B1C8 shows strong reactivity with another protein, which typically appears as a diffuse band of 160 kD, and B4A11 shows minor reactivity with two proteins of around 80 kD (Fig. 5). Upon longer development of the immunoblots, it was found that the B1C8 and H1B2 mAbs also detect additional proteins in HeLa nuclear extract, including those with molecular weights typical of the abundant SR proteins.

Many SR proteins share a common phosphoepitope recognized by mAb 104. Six of the mAb 104 SR proteins (SRp's:

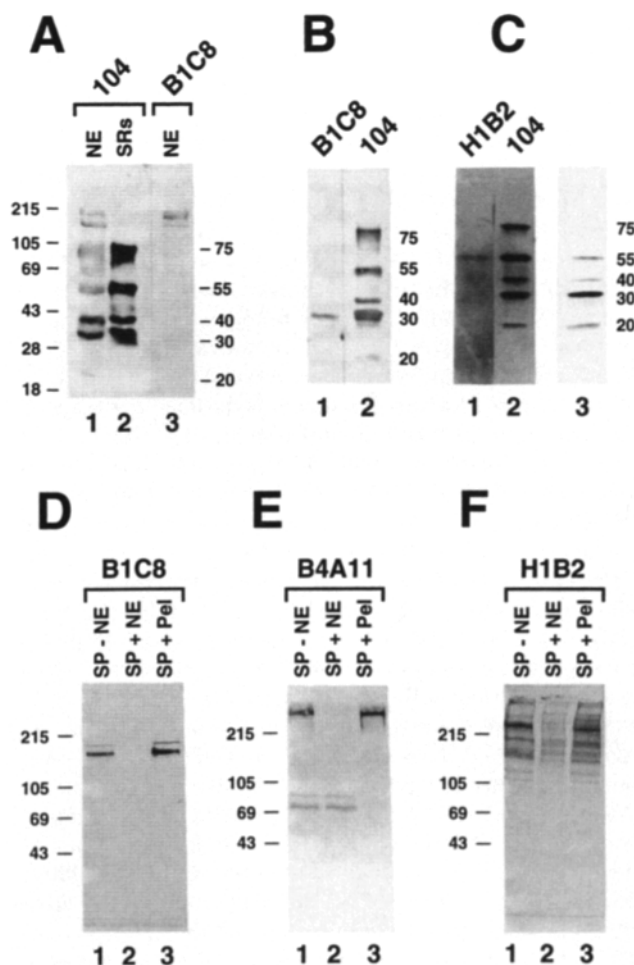


Figure 5. Detection of SR proteins by anti-NM mAbs. (A) HeLa nuclear extract proteins were separated in a 12.5% SDS gel, transferred to nitrocellulose, and probed with mAb 104 (lane 1) and B1C8 (lane 3). A sample of purified SR proteins probed with mAb 104 is shown as a marker in lane 2. (B and C) Purified SR proteins transferred to nitrocellulose were probed separately with B1C8 (B, lane 1), H1B2 (C, lane 1), and mAb 104 (B and C, lanes 2). A sample of purified SR proteins stained with coomassie is shown in C, lane 3. (D-F) Proteins from Mg^{2+} -treated HeLa nuclear extracts were separated in 10% SDS gels, transferred to nitrocellulose, and probed with the anti-NM mAbs. A nuclear extract treated with high Mg^{2+} but not centrifuged is shown in lanes 1. A centrifuged Mg^{2+} nuclear extract is shown in lanes 2. The pellet fraction of the centrifuged Mg^{2+} nuclear extract is shown in lanes 3. D was probed with B1C8, E with B4A11, and F with H1B2.

75, 55, 40, 30a, 30b, and 20 kD) can be rapidly purified to homogeneity by two precipitation steps, one of which uses a high concentration of Mg^{2+} (Zahler et al., 1992). Besides these six SR proteins, mAb 104 also reacts with several additional proteins in nuclear extract (see Fig. 8; Zahler et al., 1993). These other mAb 104-reactive proteins are separated from the six SR proteins at an early step in the purification procedure. Significantly however, they are also efficiently precipitated by high Mg^{2+} concentrations and may therefore be related to SR proteins (Fig. 8; see below). It was of interest to note that one of the additional mAb 104 antigens is a 180-kD protein which comigrates with the B1C8 antigen (Fig. 5 A, compare lanes 1 and 3). To address whether the

B1C8 and other NM antigens are related to the mAb 104 SR proteins, all three anti-NM mAbs were used to separately probe preparations of purified SR proteins and also precipitates of nuclear extracts prepared with high Mg^{2+} .

Both the B1C8 and H1B2 mAbs reacted with individual members of the SR family. The B1C8 mAb selectively recognized the 30-kD SR protein species (Fig. 5 B, lane 1) and the H1B2 mAb detected the 55-kD SR protein (Fig. 5 C, lane 1). The 30-kD SR species consists of at least two different proteins of similar size, SC35 and the related splicing factor SF2/ASF (Zahler et al., 1992). The B4A11 mAb showed no immunoreactivity with any of the purified SR proteins (data not shown). In contrast to the reactivities with the large proteins in nuclear extracts, B1C8 and H1B2 reacted relatively weakly with the purified SR proteins.

The high molecular weight proteins recognized by the three matrix mAbs have epitopes in common with SR proteins and might share the common property of precipitation by Mg^{2+} . The presence of these antigens were assayed in preparations of HeLa nuclear extract incubated under conditions which resulted in efficient Mg^{2+} precipitation of SR proteins (see Materials and Methods for details). The levels of SR proteins in the depleted nuclear extract were assayed by Western blotting with mAb 104 (see Fig. 8). Significantly, all of the high molecular weight antigens recognized by the B1C8, B4A11, and H1B2 mAbs were also efficiently depleted from the centrifuged Mg^{2+} treated nuclear extract (*SP + NE*, Fig. 5, D–F, lanes 2). The levels of most proteins in the treated nuclear extract, as detected by protein staining and probing with control mAbs, were not reduced by precipitation with Mg^{2+} , and none of the antigens were depleted from extracts prepared without Mg^{2+} incubation (data not shown). Furthermore, the antigens were not destroyed by the high Mg^{2+} conditions as the levels of both mAb 104 and anti-NM mAb-reactive proteins were not altered in a treated extract that had not been centrifuged (*SP – NE*, Fig. 5, D–F, lanes 1; see also Fig. 8). All of the major reactive antigens precipitated by Mg^{2+} were recovered intact in the pellet fraction following centrifugation (*SP + Pel*, Fig. 5, D–F, lanes 3), demonstrating that depletion in the centrifuged nuclear extract was due to precipitation and not nonspecific losses.

These results demonstrate that the high molecular weight antigens recognized by the three anti-NM mAbs belong to a class of nuclear proteins which are selectively precipitated by Mg^{2+} . This class of proteins include all of the mAb 104 antigens (see Fig. 8), and may therefore extend to other proteins which contain an SR domain. Consistent with a relationship between the high molecular weight NM antigens and SR proteins, the B1C8 and H1B2 mAbs also recognized at a much lower efficiency individual members of the SR family. These two mAbs may therefore detect shared epitopes on different subsets of related SR proteins. The multiplicity of antigens detected by the anti-NM mAbs however complicates the interpretation of the role of the antigens that are: (a) specifically localized in nuclear matrix speckles; (b) functioning in splicing as indicated by antibody inhibition; and (c) associated with splicing complexes as indicated by preferential immunoprecipitation. It is possible that the anti-NM mAbs specifically interact with one or more SR-related proteins as antigens in all of these roles in splicing. To address this, different biochemical assays were used to inves-

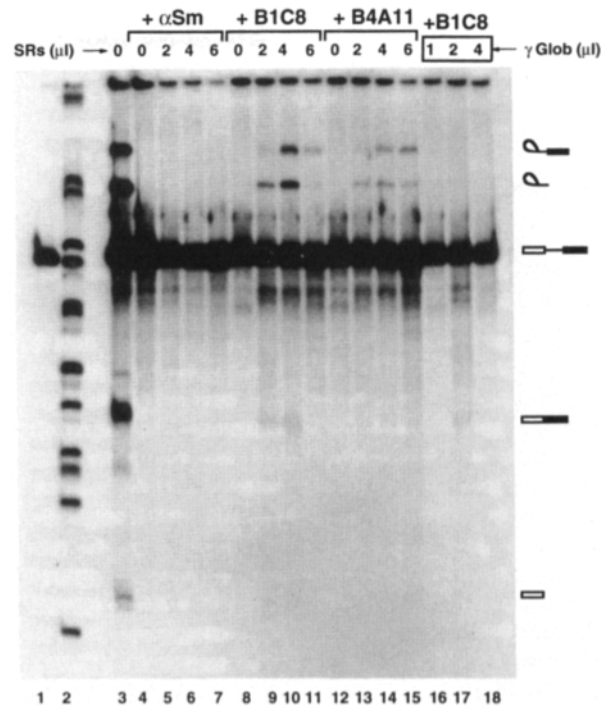


Figure 6. SR proteins restore splicing activity to anti-NM mAb-inhibited nuclear extracts. Splicing reactions were incubated with minimal inhibitory concentrations of different mAbs, together with increasing concentrations of purified SR proteins. mAbs used (indicated at top of panel) are: anti-Sm (Y12) (lanes 4–7), B1C8 (lanes 8–11, and 16–18), and B4A11 (lanes 12–15). A control splicing reaction, without added antibody, is shown in lane 3. Purified SR proteins (1.6 mg/ml) were added to antibody-inhibited reactions in a range from 0–6 μ l, as indicated. γ -globulin, (2 mg/ml) was added to a B1C8-inhibited extract as a negative control for splicing recovery (lanes 16–18).

tigate the role of epitopes shared with SR proteins in the recognition of splicing complexes by the anti-NM mAbs.

Restoration of Splicing Activity in B1C8 mAb-inhibited Nuclear Extracts by the Addition of Purified SR Proteins

Anti-Sm (Y12), B1C8, or B4A11 mAbs were added to the minimum level necessary to suppress splicing activity in nuclear extract. Increasing concentrations of purified SR proteins were also added to these mAb-inhibited reactions (Fig. 6). This addition restored splicing activity to the B1C8-inhibited reaction (Fig. 6, lanes 8–11) and, to a significantly lesser extent, the B4A11 inhibited reaction (lanes 12–15). By contrast, adding purified SR proteins to the Y12-inhibited reaction did not result in any detectable restoration of splicing activity. Furthermore, addition of equivalent levels of a control protein (γ -globulin) to a B1C8-inhibited reaction did not result in any increase in activity. In a repeat experiment using instead a β -globin pre-mRNA substrate, a very similar pattern of splicing activity was observed, indicating that the effects were not dependent on the particular sequence of the pre-mRNA substrate used (data not shown). Addition of purified SR proteins to splicing reactions in the absence of inhibitory antibody resulted only in a minor increase in

splicing activity. This indicates that one or more of the purified SR proteins can specifically compete inhibition of splicing by the BIC8 mAb. The low level of splicing restoration in the B4A11 inhibited extract could be due to the low level of splicing stimulation upon addition of SR proteins. This would be consistent with the observation that B4A11 did not show detectable reactivity with one of the defined SR proteins.

Preferential Immunoprecipitation of Exon-containing Splicing Complexes by the Anti-SC35 mAb

Inhibition of splicing by BIC8 and possibly B4A11, may be due to the binding of these mAbs to one or more SR proteins in nuclear extracts. It was therefore of interest to determine whether the specific pattern of immunoprecipitation obtained with the anti-NM mAbs (Fig. 2) was due to the mAbs binding to SR proteins in splicing complexes. If this was correct, then the SC35 mAb (Fu and Maniatis, 1990) might display the same pattern of immunoprecipitation as the anti-NM mAbs. To test this, immunoprecipitation of splicing complexes was carried out with the SC35 mAb in parallel with the anti-NM mAbs; Fig. 7 shows a comparison with the B4A11 mAb. The SC35 mAb shows an essentially identical pattern of immunoprecipitation as the B4A11 mAb, displaying a distinct preference for splicing intermediates over the lariat-product species in a splicing reaction that has been incubated for 40 min (compare lanes 5–7). A quantitatively similar immunoprecipitation pattern was also observed when both antibodies were also used to immunoprecipitate from splicing reactions that had been pre-incubated for 2 h. Immunoprecipitation with the SC35 mAb also specifically enriched for the exon-product RNA, but not for the lariat product of the splicing reaction (data not shown). These data suggest that the preferential immunoprecipitation of exon-containing pre-mRNA splicing complexes by the anti-NM mAbs and anti-SC35 mAb may be due to the specific recognition of SR or related proteins by these mAbs.

Reconstitution of Pre-mRNA Immunoprecipitation in SR-depleted Nuclear Extracts

Immunoprecipitation of pre-mRNA from HeLa cell nuclear extracts by the anti-NM mAbs specifically requires SR proteins. A reconstitution experiment was performed (Fig. 8) using the SR depleted nuclear extract described in Fig. 5 (see Materials and Methods for details). Similar to the high molecular weight antigens detected by the anti-NM mAbs, immunoblot analysis using mAb104 (Fig. 8A) showed that proteins corresponding to the purified SR proteins (lane 6) were efficiently depleted from the centrifuged Mg^{2+} nuclear extract (*spin* + NE, lane 3). The levels of these proteins were also not changed in a treated extract that had not been centrifuged (*spin* – NE; compare lanes 2 and 4) and could be recovered intact in the pellet fraction following centrifugation of the *spin* + NE (lane 5).

Analysis of splicing activity in the *Spin* + and *Spin* – extracts showed that the *Spin* – NE was active for processing of exogenous pre-mRNA, whereas the *Spin* + NE was inactive, even in the presence of added SR proteins (Fig. 8B; data not shown). The same preparation of SR proteins could however restore activity to an S100 cytoplasmic extract which is deficient in these proteins (Zahler et al., 1992; data not shown). This indicates that the *Spin* + NE lacks one or

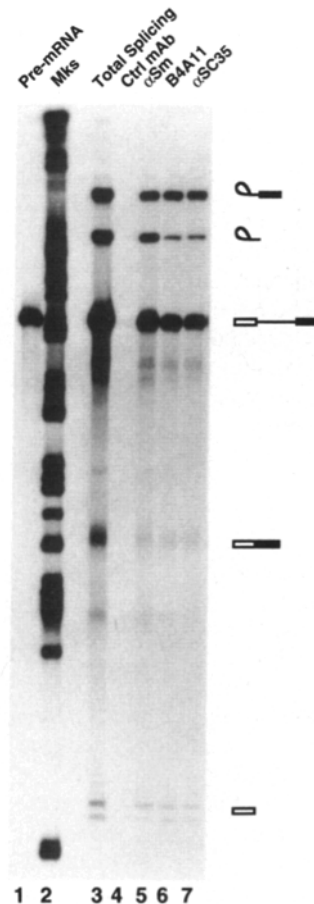


Figure 7. Immunoprecipitation of pre-mRNA splicing complexes with anti-SC35 mAb. Immunoprecipitations were carried out as in Fig. 3. mAbs used are: anti-NuMA (lane 3), anti-Sm (Y12) (lane 5), B4A11 (lane 6), and anti-SC35 (lane 7). Total splicing (lane 3) and markers (lanes 1 and 2) are as in Fig. 3A.

more essential splicing factors, in addition to the purified SR proteins. This hypothesis was confirmed by adding back the Mg^{2+} pellet fraction to the *Spin* + NE; the reconstituted reaction yielded splicing levels comparable to those found in the *Spin* – NE (data not shown).

To address whether the immunoprecipitation of splicing complexes by the anti-NM mAbs was dependent on SR proteins, a reconstitution experiment was performed. The efficiency of specific immunoprecipitation of a pre-mRNA substrate (PIP85A) was compared between the anti-NM, SC35, 104, and Y12 mAbs from reactions containing either the *Spin* – NE (lanes 1–7), *Spin* + NE (lanes 8–14) or *Spin* + NE to which purified SR proteins had been added (lanes 15–21) (Fig. 8B). Significantly, the levels of pre-mRNA immunoprecipitated by all three of the anti-NM mAbs and the SC35 and 104 mAbs, was greatly reduced in the *Spin* + NE (lanes 10–14) as compared to levels in the *Spin* – NE (lanes 3–7). The decrease in pre-mRNA immunoprecipitation in the *Spin* + NE by these mAbs was not due to nonspecific losses, since the nonimmunoprecipitated RNA was recovered intact in the supernatant fractions (Fig. 8C). In contrast to the anti-NM mAbs to the SR proteins, the Y12 mAb immunoprecipitated a similar level of pre-mRNA from the *Spin* + and *Spin* – extracts (compare lanes 2 and 9). Immunoprecipitation of pre-mRNA by the BIC8 and HIB2 mAbs was fully restored by adding purified SR proteins to the *Spin* + NE extract (lanes 17 and 18). Similar results were observed for both the SC35 and 104 mAbs (lanes 20 and 21). Addition of SR proteins to the *Spin* + NE did not

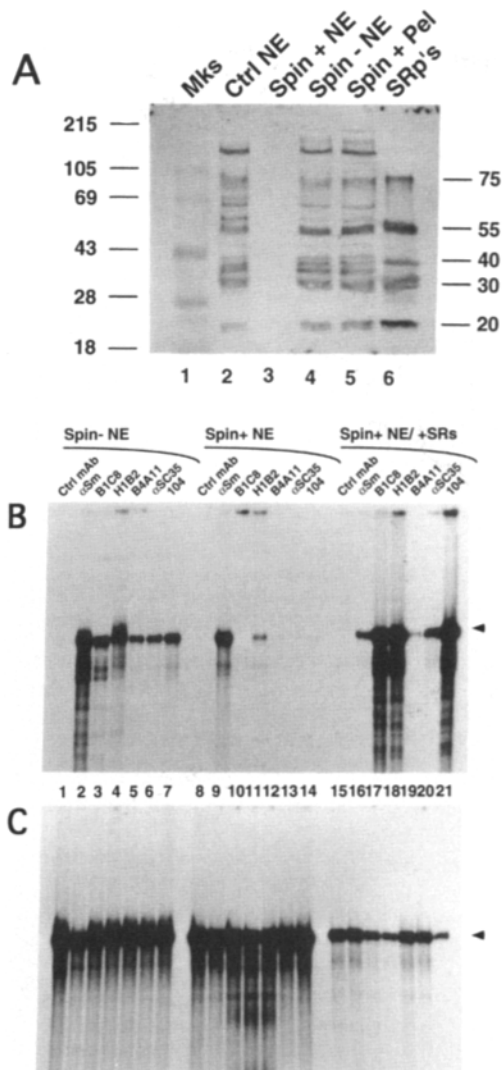


Figure 8. Reconstitution of pre-mRNA immunoprecipitation in SR-depleted nuclear extracts. SR proteins were depleted from HeLa cell nuclear extracts by high-speed centrifugation in the presence of 20 mM Mg. (A) Levels of SR proteins in different nuclear extracts assayed by Western blotting using mAb 104 (lanes 1–4). Lanes 3 and 4 show depleted and mock-depleted extracts respectively. Lane 5 shows the pellet fraction from the depleted extract in lane 3. Lane 2 shows a control, untreated nuclear extract. Lane 6 shows purified SR proteins (sizes indicated on panel right). Lane 1 shows pre-stained markers (sizes indicated on panel left). Approximately 150 μ g of each nuclear extract was loaded in lanes 2, 4, 5, and 6 μ g purified SR proteins was loaded in lane 6. (B and C) Immunoprecipitations with different mAbs were carried out from splicing reactions containing the nuclear extracts shown in A: RNA recovered from the pellet (B) and corresponding supernatant fractions (C) are shown. Each antibody was used to immunoprecipitate from a splicing reaction containing either the mock-depleted “spin – NE” (lanes 1–7), depleted “spin + NE” (lanes 8–14) or the “spin + NE” to which SR proteins had been added (lanes 15–21). mAbs used are: Ctrl mAb (anti-NuMA) (lanes 1, 8, and 15), anti-Sm (Y12) (lanes 2, 9, and 16), BIC8 (lanes 3, 10, and 17), HIB2 (lanes 4, 11, and 18), B4A11 (lanes 5, 12, and 19), anti-SC35 (lanes 6, 13, and 20) and mAb 104 (lanes 7, 14, and 21). The ratio of supernatant:pellet fraction loaded is 1:4; exposure times for (B) and (C) are approximately the same.

however result in an increase in the level of pre-mRNA immunoprecipitation by the Y12 mAb (lane 16), nor was a significant increase observed for the B4A11 mAb (lane 19). This indicates that the ability of the purified SR proteins to promote immunoprecipitation by the BIC8 and HIB2 mAbs in the Spin + NE was a specific effect. These results, together with the immunoblotting data in Fig. 5, provide the strongest argument for suggesting that the antigens recognized by BIC8 and HIB2 anti-NM mAbs are related to one or more SR proteins. By contrast, immunoprecipitation by the B4A11 mAb was not restored by addition of the purified SR proteins to the Spin + NE. This is consistent with the data in Fig. 5, which demonstrate that the B4A11 mAb recognizes an antigen that is depleted from the Spin + NE but is not a constituent of one of the purified SR proteins.

Competition of Speckled Immunostaining by SR Proteins

The immunofluorescence data presented in Fig. 1 shows that the BIC8, HIB2, and B4A11 antigens are localized to nuclear matrix speckles which are enriched in pre-mRNA splicing factors. We performed another competition experiment to address whether this specific staining pattern on the nuclear matrix involves the recognition of determinants on SR proteins (Fig. 9). Each anti-NM mAb, and also the anti-SC35 and Y12 mAbs, were pre-incubated with SR proteins prior to immunostaining. This effectively blocked nuclear staining by the BIC8, HIB2, and SC35 mAbs, whereas the B4A11 mAb was only weakly competed and the Y12 mAb was not competed at all. In the presence of an equivalent concentration of a nonspecific control protein, no competition of staining by the BIC8, HIB2 and SC35 mAbs was observed (data not shown). These data are consistent with the notion that both the BIC8 and HIB2 mAbs specifically recognize one or more SR-related antigens concentrated in the speckled pattern of the nuclear matrix. Moreover, the lack of significant competition by SR proteins of B4A11 mAb staining is further consistent with this mAb recognizing an epitope that is not present on one of the defined SR proteins.

Discussion

Pre-mRNA splicing factors are concentrated in dense nuclear matrix structures which correspond to speckled domains observed by immunofluorescence. In this report, the function of antigens concentrated in these structures was investigated using three different murine mAbs raised against the human nuclear matrix (anti-NM mAbs) BIC8, HIB2, and B4A11. The experiments suggest that antigens recognized by these three mAbs participate in pre-mRNA splicing. The data are summarized in Table I.

A striking feature of the mAbs is that they all preferentially immunoprecipitate splicing complexes that contain exon sequences. Complexes containing unspliced pre-mRNA, RNA intermediates of splicing, and the ligated exon product RNA were all efficiently immunoprecipitated from *in vitro* splicing reactions by the anti-NM mAbs. By contrast, the anti-NM mAbs immunoprecipitated relatively little of the complex containing the excised intron-lariat RNA. Each of the RNA species immunoprecipitated contains exon sequences whereas the intron-lariat RNA does not, but instead is bound to snRNPs in a stable postslicing complex (Konarska

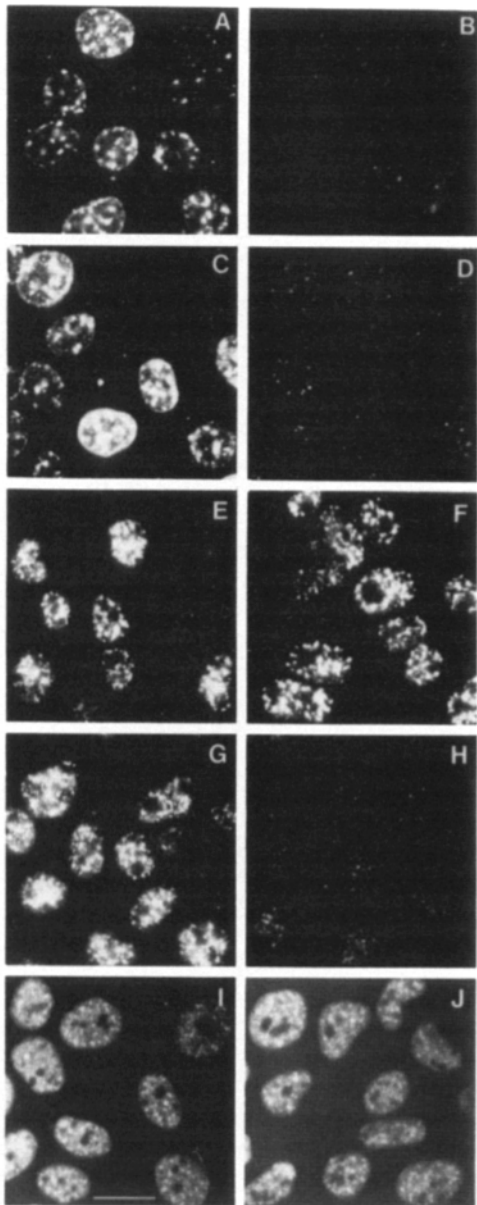


Figure 9. Competition of nuclear staining by SR proteins. CaSki cells were stained with mAbs pre-incubated with (right column, *B, D, F, H, and J*) or without (left column, *A, C, E, G, and I*) purified SR proteins (see Materials and Methods). mAbs used are: H1B2 (*A and B*); B1C8 (*C and D*); B4A11 (*E and F*); SC35 (*G and H*), and Y12 (*I and J*). Each panel shows several cells representative of the entire field. Each pair of images in the left and right columns were recorded using identical exposures.

and Sharp, 1987). Consistent with the NM antigens having an important role in splicing, both steps of pre-mRNA splicing were completely inhibited when either B1C8 or B4A11 was added to a reaction. The addition of H1B2 mAb had no effect on splicing although the antigen was bound to the spliceosome. None of the antigens recognized by the three anti-NM mAbs correspond to components of the abundant U1, U2, U4/6, or U5 snRNPs. Immunoblotting and biochemical assays suggest that the B1C8 and H1B2 antigens may be related to different members of the SR family of protein splicing factors.

Table I. Summary of Results

Property	Anti-NM mAbs		
	B1C8	H1B2	B4A11
Nuclear localization	speckles	speckles	speckles
Immunoprecipitation of splicing complexes	+	+	+
Splicing inhibition	+	-	+
Recognition of defined SR proteins	+ (30 kD)	+ (55 kD)	-
Major antigen probably SR related	180/160 kD*	~240 kD*	~300 kD*

* Sizes refer to the most prominent reactive species, all of which are precipitable with high Mg^{2+} . The B1C8 antigen migrates as a doublet consisting of 180 kD and ~160-kD proteins. The predominant H1B2 antigen is ~240 kD. B4A11 mAb reacts primarily with a ~300-kD antigen and weakly with a 80-kD doublet which is not Mg^{2+} precipitable.

It is intriguing that the three anti-NM mAbs, which specifically react in a speckled pattern with the nuclear matrix, share the ability to preferentially bind to RNA containing exon sequences. In fact, the anti-NM mAbs preferentially immunoprecipitated exon-containing RNAs from three distinct splicing substrates. The preferential immunoprecipitation of exon-containing RNA sequences by the B1C8, H1B2 and B4A11 mAbs was specific. Other mAbs against nuclear antigens did not show this pattern of immunoprecipitation. As expected, antibodies against snRNP Sm antigens preferentially immunoprecipitated complexes containing snRNPs. In contrast to the recent results of Zeng et al. (1994), several different antibodies to the nuclear matrix protein NuMA (Nuclear mitotic apparatus protein) (Yang et al., 1992; Compton et al., 1991), immunoprecipitated neither splicing complexes, nor RNA. These anti-NuMA antibodies do not show a speckled pattern but instead give essentially a uniform nuclear staining.

The B1C8, H1B2, and B4A11 anti-NM mAbs recognize different antigens. These antigens have separate and distinct behaviors at mitosis and also have distinct properties in assays of nuclear extracts. Immunoblotting of proteins from HeLa nuclear extract with the anti-NM mAbs revealed distinct patterns of reactivity. All three anti-NM mAbs reacted predominantly with different high molecular weight proteins. These proteins may be related to members of the SR family of proteins, which are concentrated in speckles and are associated with splicing complexes. Similar to SR proteins, all of these high molecular weight proteins were selectively precipitated from nuclear extract by high Mg^{2+} concentrations. Significantly, the B1C8 and H1B2 anti-NM mAbs also reacted at much lower efficiency with specific SR proteins in purified preparations. Depleting nuclear extracts of SR proteins prevented immunoprecipitation of pre-mRNA by all three mAbs. Addition of purified SR proteins restored immunoprecipitation of pre-mRNA by the B1C8 and H1B2 mAbs but not the B4A11 mAb. Unlike the B1C8 and H1B2 mAbs, the B4A11 mAb did not react with any of the purified SR proteins. Furthermore, purified SR proteins did not significantly compete inhibition of splicing or immunofluorescent staining of nuclei by the B4A11 mAb. The data suggest that the B1C8 and H1B2 mAbs immunoprecipitate splicing complexes by binding to one or more proteins which may be

related to SR proteins and which are associated with exon sequences. In contrast, the B4A11 mAb recognizes an antigen with similar properties, but which may not contain an epitope common to the purified SR proteins.

The apparent relationship between the high molecular weight proteins detected by the anti-NM mAbs and SR proteins is intriguing and raises the possibility that these proteins belong to an extended class of Mg^{2+} precipitable proteins which are related to the SR family. The experiments with the SC35 mAb strengthen the apparent link between SR proteins and the cognate proteins of the anti-NM mAbs. The SC35 mAb recognizes several members of the SR protein family (Fu and Maniatis, 1990; Fu et al., 1992). This antibody gives essentially the same speckled immunofluorescence as the anti-NM mAbs and also an identical pattern of immunoprecipitation from splicing reactions. We note however that since none of the mAbs are uniquely specific for individual proteins in nuclear extracts, it is not possible to distinguish which of the cognate antigens are responsible for the different activities observed. This may not be surprising as a notable feature of SR proteins is their overlapping functional activities in certain *in vitro* assays. For example, any one of the purified SR proteins can restore activity to a splicing deficient S100 cytoplasmic extract which is depleted of SR proteins (Zahler et al., 1992, 1993). Likewise, it is possible that the high molecular weight BIC8 and HIB2 antigens correspond to large SR proteins which overlap in function with the purified SR proteins.

The nuclear matrix consists of a complex lattice of interconnecting filaments composed of protein and RNA (reviewed in Nickerson and Penman, 1992). This substructure retains spliceosome components including snRNPs and also non-snRNP splicing factors (Vogelstein and Hunt, 1982; Spector et al., 1983; Verheijen et al., 1986; Smith et al., 1986, 1989). Evidence that splicing occurs in association with the nuclear matrix was first provided by Zeitlin and co-workers, who reported that a β -globin pre-mRNA retained on the nuclear matrix was rapidly processed following the addition of a soluble nuclear fraction (Zeitlin et al., 1987). More recently, fluorescent *in situ* hybridization using gene-specific exon and intron probes have detected RNA tracks in association with the nuclear matrix (Lawrence et al., 1989; Huang and Spector, 1991; Xing et al., 1993). Significantly, both the quantitative and qualitative appearance of individual RNA tracks was preserved during a matrix preparation consisting of removal of 95% of chromatin and also bulk protein and phospholipid material (Xing and Lawrence, 1991). Based on these observations, it has been proposed that the nuclear matrix may provide a solid support on which both RNA splicing and transport takes place. This view of pre-mRNA processing on the nuclear matrix may also explain the observation of nonrandom paths followed by transcripts in *Drosophila* polytene nuclei (Zachar et al., 1993).

The present study supports a role for the nuclear matrix in RNA processing. In this model, nuclear matrix proteins which include SR proteins and SR-associated factors, function in the recognition and sequestration of exon sequences during the processing of pre-mRNA. The SR protein sub-components of the matrix may be responsible for the exon recognition. This is consistent with recent experiments showing that the activities of SR proteins on splicing *in vitro* are dependent upon purine-rich sequences in exons (Lavign

neur et al., 1993; Sun et al., 1993). Also sequestered in the matrix are the snRNPs and non-snRNP splicing factors which promote the formation of spliceosomes. Many of these splicing factors, such as: SC35 (Fu and Maniatis, 1991), ASF/SF2 (Ge et al., 1991; Krainer et al., 1991), U2AF (Zamore et al., 1992; Zhang et al., 1992), and the U1 snRNP 70-kD protein (Theissen et al., 1986), possess SR domains. The association of SR domains appears to be critical for the activities of these proteins in splicing and could also be the point of their attachment to the matrix (Li and Bingham, 1991). This model extends to cases of alternative splicing, in which the selection and utilization of specific splice sites could be determined by their proximity to binding to the matrix. For example, several of the proteins responsible for regulation of the alternative splicing patterns in sex determination in *Drosophila* contain SR repeats (McKeown et al., 1987; Amrein et al., 1988; Goralski et al., 1989). These proteins bind to RNA in a sequence-specific manner and promote the utilization of nearby splice sites (Hedley and Maniatis, 1991; Inoue et al., 1992; Tian and Maniatis, 1993). Perhaps these proteins regulate alternative splicing patterns by association of the nearby splice site with other SR proteins and splicing factors in the nuclear matrix.

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References

- Amrein, H., M. Gorman, and R. Nöthiger. 1988. The sex-determining gene *Tra2* of *Drosophila* encodes a putative RNA binding protein. *Cell*. 55:1025-1035.
- Ascoli, C. A., and G. G. Maul. 1991. Identification of a novel nuclear domain. *J. Cell Biol.* 112:785-795.
- Beyer, A. L., and Y. N. Osheim. 1988. Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev.* 2:754-765.
- Blencowe, B. J., M. Carmo-Fonseca, S.-E. Behrens, R. Lührmann, and A. I. Lamond. 1993. Interaction of the human autoantigen p150 with splicing snRNPs. *J. Cell Sci.* 105:685-697.
- Blencowe, B. J., B. S. Sproat, U. Ryder, S. Barabino, and A. I. Lamond. 1989. Antisense probing of the human U4/U6 snRNP with biotinylated 2'-OME RNA oligonucleotides. *Cell*. 59:531-539.
- Carter, K. C., D. Bowman, W. Carrington, K. Fogarty, A. McNeil, F. S. Fay, and J. B. Lawrence. 1993. A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. *Science (Wash. DC)*. 259:1330-1335.
- Carter, K. C., K. L. Taneja, and J. B. Lawrence. 1991. Discrete nuclear domains of poly(A) RNA and their relationship to the functional organization of the nucleus. *J. Cell Biol.* 115:1191-1202.
- Compton, D., and D. W. Cleveland. 1993. NuMA is required for the proper completion of mitosis. *J. Cell Biol.* 120:947-957.
- Dignam, J. D., R. M. Lebowitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids. Res.* 11:1475-1489.
- Fakan, S., E. Puvion, and G. Spohr. 1976. Localization and characterization of newly synthesized nuclear RNA in isolated rat hepatocytes. *Exp. Cell Res.*

- 99:155-164.
- Fey, E. G., G. Krochmalnic, and S. Penman. 1986. The non-chromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *Proc. Natl. Acad. Sci. USA* 102:1654-1665.
- Fu, X.-D., and T. Maniatis. 1990. Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature (Lond.)* 343:437-441.
- Fu, X.-D., and T. Maniatis. 1992. Isolation of a complementary DNA that encodes the mammalian splicing factor SC35. *Science (Wash. DC)* 256:535-538.
- Fu, X.-D., A. Mayeda, T. Maniatis, and A. R. Krainer. 1992. General splicing factors SF2 and SC35 have equivalent activities in vitro, and both affect alternative 5' and 3' splice site selection. *Proc. Natl. Acad. Sci. USA* 89:11224-11228.
- Ge, H., P. Zuo, and J. Manley. 1991. Primary structure of the human splicing factor ASF reveals similarities with Drosophila regulators. *Cell* 66:373-382.
- Goralski, T. J., J. E. Edström, and B. S. Baker. 1989. The sex-determination locus *transformer-2* of Drosophila encodes a polypeptide with similarity to RNA binding proteins. *Cell* 56:1011-1018.
- Grabowski, P. J., R. A. Padgett, and P. A. Sharp. 1984. Messenger RNA splicing in vitro: an excised intervening sequence and a potential intermediate. *Cell* 37:415-427.
- Guthrie, C. 1991. Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. *Science (Wash. DC)* 253:157-163.
- Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- He, D., J. A. Nickerson, and S. Penman. 1990. Core filaments of the nuclear matrix. *J. Cell Biol.* 110:569-580.
- Hedley, M. L., and T. Maniatis. 1991. Sex-specific splicing and polyadenylation of *dax* pre-mRNA requires a sequence that binds specifically to tra-2 protein in vitro. *Cell* 65:579-586.
- Herman, R., L. Weymouth, and S. Penman. 1978. Heterogeneous nuclear RNA-protein fibers in chromatin depleted nuclei. *J. Cell Biol.* 78:633-674.
- Hozák, P., A. B. Hassan, D. A. Jackson, and P. R. Cook. 1993. Visualization of replication factories attached to a nucleoskeleton. *Cell* 73:361-373.
- Huang, S., and D. L. Spector. 1991. Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes & Dev.* 5:2288-2302.
- Inoue, K., K. Hoshijima, I. Higuchi, H. Sakamoto, and Y. Shimura. 1992. Binding of the Drosophila transformer and transformer-2 proteins to the regulatory elements of doublesex primary transcript for sex-specific RNA processing. *Proc. Natl. Acad. Sci. USA* 89:8092-8096.
- Jackson, D. A., and P. R. Cook. 1985. Transcription occurs at the nucleoskeleton. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:919-925.
- Jackson, D. A., A. B. Hassan, R. J. Errington, and P. R. Cook. 1993. Visualization of focal sites of transcription within human nuclei. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1059-1065.
- Krainer, A. R., A. Mayeda, D. Kozak, and G. Binns. 1991. Functional expression of cloned human splicing factor SF2: homology to RNA binding proteins, U1 70K, and Drosophila splicing regulators. *Cell* 66:383-394.
- Lamond, A. I., and M. Carmo-Fonseca. 1993. The coiled body. *Trends Cell Biol.* 3:198-204.
- Lamond, A. I., M. M. Konarska, and P. A. Sharp. 1987. A mutational analysis of spliceosome assembly: evidence for splice site collaboration during spliceosome formation. *Genes & Dev.* 1:532-543.
- Lawrence, J. B., R. H. Singer, and L. M. Marselle. 1989. Highly localized tracks of specific transcripts within the nuclei visualized by in situ hybridization. *Cell* 57:493-502.
- Lavigne, A., H. La Branche, A. Kornblihtt, and B. Chabot. 1993. A splicing enhancer in the human fibronectin alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding. *Genes & Dev.* 7:2405-2417.
- Lerner, E. A., M. R. Lerner, L. A. Janeway, and J. A. Steitz. 1981. Monoclonal antibodies to nucleic acid containing cellular constituents: probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA* 78:2737-2741.
- Li, H.L., and P.M. Bingham. 1991. Arginine/Serine-rich domains of the *su(w⁺)* and *tra* RNA processing regulators target proteins to a subnuclear component implicated in splicing. *Cell* 67:335-342.
- Mariman, E. C. M., C. A. G. van-Eekelen, R. J. Reinders, A. J. M. Berns, and W. J. van-Venrooij. 1982. Adenoviral heterogeneous nuclear RNA is associated with the host nuclear matrix during splicing. *J. Mol. Biol.* 154:103-119.
- McKeown, M., J. M. Belote, and B. S. Baker. 1987. A molecular analysis of transformer, a gene in Drosophila melanogaster that controls female sexual differentiation. *Cell* 48:489-499.
- Miller, T. E., C. Y. Huang, and A. O. Pogo. 1978. Rat liver nuclear skeleton and ribonucleoprotein complexes containing hnRNA. *J. Cell Biol.* 76:675-691.
- Moore, M. J., C. C. Query, and P. A. Sharp. 1993. Splicing of precursors to mRNA by the spliceosome. In *The RNA World*. R. Gesteland, and J. Atkins, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 303-357.
- Moore, M. J., and P. A. Sharp. 1992. Site-specific modification of pre-mRNA: the 2'-hydroxyl groups at the splice sites. *Science (Wash. DC)* 256:992-997.
- Nakamura, H., T. Morita, and C. Sato. 1986. Structural organization of replication domains during DNA synthetic phase in the mammalian nucleus. *Exp. Cell Res.* 165:291-297.
- Nakayasu, H., and R. Berezney. 1989. Mapping replication sites in the eukaryotic cell nucleus. *J. Cell Biol.* 108:1-11.
- Nickerson, J. A., G. Krochmalnic, K. M. Wan, C. D. Turner, and S. Penman. 1992. A normally masked nuclear matrix antigen that appears at mitosis on cytoskeleton filaments adjoining chromosomes, centrioles and midbodies. *J. Cell Biol.* 116:977-987.
- Nickerson, J. A., and S. Penman. 1992. The nuclear matrix: structure and involvement in gene expression. In *Molecular and Cellular Approaches to the Control of Proliferation and Differentiation*. G. Stein, and J. Lian, editors. Academic Press, New York. 343-380.
- Saunders, W. S., C. A. Cooke, and W. C. Earnshaw. 1991. Compartmentalization within the nucleus: discovery of a novel subnuclear region. *J. Cell Biol.* 115:919-931.
- Schröder, H. C., D. Trölltsch, U. Friese, M. Bachman, and W. E. G. Müller. 1987. Mature mRNA is selectively released from the nuclear matrix by an ATP/dATP-dependent mechanism sensitive to topoisomerase inhibitors. *J. Biol. Chem.* 262:8917-8925.
- Schröder, H. C., D. Trölltsch, R. Wenger, M. Bachman, B. Diehl-Seifert, and W. E. G. Müller. 1987. Cytochalasin B selectively releases ovalbumin mRNA precursors but not the mature ovalbumin mRNA from hen oviduct nuclear matrix. *Eur. J. Biochem.* 167:239-245.
- Smith, H. C., S. G. Harris, M. Zillmann, and S. M. Berget. 1989. Evidence that a nuclear matrix protein participates in pre-mRNA splicing. *Exp. Cell Res.* 182:521-533.
- Smith, H. C., R. L. Ochs, E. A. Fernandez, and D. L. Spector. 1986. Macromolecular domains containing matrix protein p107 and U-snRNP protein p28: further evidence for an in situ nuclear matrix. *Mol. Cell. Biochem.* 70:151-168.
- Spector, D. L. 1993. Nuclear Organization of pre-mRNA processing. *Curr. Opin. Cell Biol.* 5:442-448.
- Spector, D. L., X.-D. Fu, and T. Maniatis. 1991. Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3467-3481.
- Spector, D. L., W. H. Schrier, and H. Busch. 1983. Immunoelectron microscopic localization of snRNPs. *Biol. Cell* 49:1-10.
- Stuurman, N., A. D. Graaf, A. Floore, A. Josso, B. Humbel, L. de Jong, and R. van Driel. 1992. A monoclonal antibody recognizing matrix-associated nuclear bodies. *J. Cell Sci.* 101:773-784.
- Sun, Q., A. Mayeda, R. K. Hampson, A. R. Krainer, and F. M. Rottman. 1993. General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. *Genes & Dev.* 7:2598-2608.
- Thiessen, H., M. Etzerodt, R. Reuter, C. Schneider, R. Lottspeich, P. Argos, R. Lührmann, and L. Philipson. 1986. Cloning of the human cDNA for the U1 RNA-associated 70K protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3209-3217.
- Tian, M., and T. Maniatis. 1993. A splicing enhancer complex controls alternative splicing of *doublesex* pre-mRNA. *Cell* 74:105-114.
- Van-Eekelen, C. A. G., and W. J. van Venrooij. 1981. HnRNA and its attachment to a nuclear protein matrix. *J. Cell Biol.* 88:554-563.
- Verheijen, R., H. Kuipers, P. Vooijs, W. van Venrooij, and F. Ramaekers. 1986. Distribution of the 70k U1 RNA-associated protein during interphase and mitosis: correlation with other U RNP particles and proteins of the nuclear matrix. *J. Cell Sci.* 86:173-190.
- Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight. 1988. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes & Dev.* 2:801-806.
- Vogelstein, B., and B. F. Hunt. 1982. A subset of small nuclear ribonucleoprotein particle antigens is a component of the nuclear matrix. *Biochem. Biophys. Res. Commun.* 105:1224-1232.
- Wan, K., J. A. Nickerson, G. Krochmalnic, and S. Penman. 1994. The B1C8 protein is in the dense assemblies of the nuclear matrix and relocates to the spindle and pericentriolar filaments at mitosis. *Proc. Natl. Acad. Sci. USA* 91:594-598.
- Wansink, D. G., W. Schul, I. van der Kraan, B. van Steensel, R. van Driel, and L. de Jong. 1993. Fluorescent labeling of Nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J. Cell Biol.* 122:283-293.
- Xie, K., E. Lambie, and M. Snyder. 1993. Nuclear dot antigens may specify transcriptional domains in the nucleus. *Mol. Cell. Biol.* 13:6170-6179.
- Xing, Y., C. V. Johnson, P. R. Dobner, and J. B. Lawrence. 1993. Higher level organization of individual gene transcription and splicing. *Science (Wash. DC)* 259:1326-1330.
- Xing, Y., and J.-B. Lawrence. 1991. Preservation of specific RNA distribution within the chromatin-depleted nuclear substructure demonstrated by in situ hybridization coupled with biochemical fractionation. *J. Cell Biol.* 112:1055-1063.
- Yang, C. H., E. J. Lambie, and M. Snyder. 1992. NuMA: An unusually long coiled-coil related protein in the mammalian nucleus. *J. Cell Biol.* 116:1303-1317.
- Zachar, Z., J. Kramer, I. Mims, and P. M. Bingham. 1993. Evidence for chan-

- neled diffusion of pre-mRNAs during nuclear RNA transport in metazoans. *J. Cell Biol.* 121:729-742.
- Zahler, A. M., W. S. Lane, J. A. Stolk, and M. B. Roth. 1992. SR proteins: a conserved family of pre-mRNA splicing factors. *Genes & Dev.* 6: 837-847.
- Zahler, A. M., K. M. Neugebauer, J. A. Stolk, and M. B. Roth. 1993. Human SR proteins and isolation of a cDNA encoding SRp75. *Mol. Cell. Biol.* 13:4023-4028.
- Zamore, P. D., J. G. Patton, and M. R. Green. 1992. Cloning and domain structure of the mammalian splicing factor U2AF. *Nature (Lond.)*. 355: 609-614.
- Zeitlin, S., A. Parent, S. Silverstein, and A. Efstratiadis. 1987. Pre-mRNA splicing and the nuclear matrix. *Mol. Cell. Biol.* 7:111-120.
- Zeitlin, S., R. C. Wilson, and A. Efstratiadis. 1989. Autonomous splicing and complementation of in vivo assembled spliceosomes. *J. Cell Biol.* 108:765-777.
- Zeng, C., D. He, S. M. Berget, and B. R. Brinkley. 1994. Nuclear-mitotic apparatus protein: A structural protein interface between the nucleoskeleton and RNA splicing. *Proc. Natl. Acad. Sci. USA.* 91:1505-1509.
- Zhang, M., P. D. Zamore, M. Carmo-Fonseca, A. I. Lamond, and M. R. Green. 1992. Cloning and intracellular localization of the U2 small nuclear ribonucleoprotein auxiliary factor small subunit. *Proc. Natl. Acad. Sci. USA.* 89:8769-8773.