A Conserved Epitope on a Subset of SR Proteins Defines a Larger Family of Pre-mRNA Splicing Factors

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Abstract. The removal of introns from eukaryotic pre-mRNA occurs in a large ribonucleoprotein complex called the spliceosome. We have generated a monoclonal antibody (mAb 16H3) against four of the family of six SR proteins, known regulators of splice site selection and spliceosome assembly. In addition to the reactive SR proteins, SRp20, SRp40, SRp55, and SRp75, mAb 16H3 also binds ~20 distinct nuclear proteins in human, frog, and *Drosophila* extracts, whereas yeast do not detectably express the epitope. The antigens are shown to be nuclear, nonnucleolar, and concentrated at active sites of RNA polymerase II transcription which suggests their involvement in pre-mRNA processing. Indeed, most of the reactive proteins observed in nuclear extract are detected in spliceosomes (E and/or B complex) assembled in vitro, including the U1 70K component of the U1 small nuclear ribonucleoprotein particle and both subunits of U2AF. Interestingly, the 16H3 epitope was mapped to a 40-amino acid polypeptide composed almost exclusively of arginine alternating with glutamate and aspartate. All of the identified antigens, including the human homolog of yeast Prp22 (HRH1), contain a similar structural element characterized by arginine alternating with serine, glutamate, and/or aspartate. These results indicate that many more spliceosomal components contain such arginine-rich domains. Because it is conserved among metazoans, we propose that the "alternating arginine" domain recognized by mAb 16H3 may represent a common functional element of pre-mRNA splicing factors.

PRECURSOR-mRNA splicing takes place in a large ribonucleoprotein (RNP)[1] complex designated the spliceosome (Guthrie, 1991; Moore et al., 1993). This complex of RNA and protein is assembled at intron/exon boundaries in the pre-mRNA such that two exons are brought together and, subsequently, the intron is removed by two sequential transesterification reactions. Because spliceosome assembly is the ultimate determinant of exon definition, splice site selection, and the fidelity of splicing, efforts to characterize the RNA and protein components of the spliceosome are ongoing. Small nuclear ribonucleoprotein particles (snRNPs) have been described biochemically as complexes containing specific sets of proteins associated with U1, U2, and U4/U5/U6 snRNAs, and these are known to interact with the pre-mRNA during spliceosome assembly (reviewed in Moore et al., 1993). In addition, a growing number of non-snRNP proteins have been described by genetic or biochemical means, bringing the number of proteins in active mammalian spliceosomes to ~50 (Gozani et al., 1994).

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1. *Abbreviations used in this paper*: GST, glutathione S-transferase; RNP, ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein; snRNP, small nuclear RNP particle; RRM, RNA recognition motif.

Genetic dissection of the splicing reaction in the yeast *Saccharomyces cerevisiae* has yielded functional information about 27 genes essential for pre-mRNA processing (cf. Moore et al., 1993; Guthrie, 1991). A number of these PRP gene products appear to be either snRNP components or snRNP-associated factors. The sequences of many provide clues to how they function in the spliceosome: some have RNA recognition motifs (RRMs) or zinc-finger motifs, and a number have homology with ATPases or RNA helicases (Kenan et al., 1991; Guthrie, 1991; Moore et al., 1993). Recently, a number of mammalian homologs of PRP genes have been cloned, and relationships have been drawn between factors identified in human cells biochemically and in yeast genetically. For example, three protein components of human SF3a, an activity required for the assembly of the mature 17S U2 snRNP at the branchpoint, have been shown to be the functional homologues of the yeast genes PRP9, PRP11, and PRP21 (Bennett and Reed, 1993; Brosi et al., 1993; Legrain and Chapon, 1993; Chiara et al., 1994). While many of the yeast PRP gene homologues have not yet been identified in metazoans, it appears that the functions carried out by the yeast proteins are also required in pre-mRNA splicing by higher eukaryotes.

It is highly likely that additional regulators of pre-mRNA splicing are expressed in metazoans where splice site selection is often modulated. In particular, a family of non-snRNP

splicing factors called SR proteins have been shown to be essential for splicing in vitro but have not been found in yeast (Zahler et al., 1992). Although SR proteins interact with the U1 snRNP and are required for recruitment of the U1 snRNP to the 5′ splice site (Eperon et al., 1993; Khotz et al., 1994; Staknis and Reed, 1994), none have been detected in the highly purified spliceosome assembled in vitro (Bennett et al., 1992*a*). Thus, essential splicing factors do exist that have not been approachable genetically in yeast or through the biochemistry of the spliceosome. The discovery of SR proteins was facilitated by an immunological tool, the mAb 104 (Roth et al., 1989). This study identifies a new mAb (16H3) with specificity for four (SRp20, SRp40, SRp55, SRp75) of the family of six SR proteins. The epitope recognized by mAb 16H3 is expressed by at least 20 distinct nuclear proteins and appears to be conserved among metazoan splicing factors, such as HRH1, U2AF, and U1 70K proteins. In addition, mAb 16H3 detects many components of the earliest spliceosomal complex, E, and of the mature complex B.

Materials and Methods

Proteins and Antibodies

SR proteins were purified from calf thymus or Hela cells as described in Zahler et al. (1992). The GEX-2T vector (Smith and Johnson, 1988) was used to generate a protein fusion between glutathione S-transferase (GST) and the open reading frame of *Drosophila* SRp55 (Roth et al., 1991). The bacterially expressed fusion protein was purified by chromatography on glutathione agarose (Smith and Johnson, 1988). U2AF^{65}-GST and U2AF^{35}-GST vectors were kindly provided by M. Green (University of Mass. Worcester, MA.) and J. Wu (Harvard University, Cambridge, MA), respectively. A fusion construct encoding maltose binding protein and amino acids 151-346 of HRH1 was generously provided by M. Ohno and Y. Shimura (Kyoto University, Kyoto, Japan). Bacterially expressed heterogeneous nuclear ribonucleoprotein (hnRNP) U and mAbs 4F4 (anti-hnRNP C), 4B10 (anti-hnRNP A), and 3G6 (anti-hnRNP U) were generous gifts of G. Dreyfuss (University of Pennsylvania, Philadelphia, PA). Y12 mAb supernatant was the generous gift of Joan Steitz (Yale University, New Haven, CT). Nuclear and S100 extracts were prepared from HeLa cells and Kc cells as described in Dignam et al. (1983). Extracts of whole HeLa cells, *S. cerevisiae*, and *Schizosaccharomyces pombe* were prepared by sonication in NEST-2 buffer as described in Zahler et al. (1993*a*). E and B spliceosomal complexes were the generous gifts of R. Reed (Harvard University); they were assembled on biotinylated AdML pre-mRNA and affinity purified according to Bennett et al. (1992*a*).

Generation of mAbs

Mice bearing the Robertsonian translocation (RBF/DnJ; Jackson Laboratory, Bar Harbor, ME) were immunized initially with purified dephosphorylated bovine SRp55 and subsequently with dSRp55-GST. Immunogen was emulsified with complete and subsequently with incomplete Freund's adjuvant (Cappell Laboratories, Cockranville, PA). Mice were boosted every 2 wk for 3 mo, and test bleeds were taken. One test bleed showed a positive reaction with calf thymus SR proteins on an immunoblot. The spleen cells of the positive mouse were fused with Fox-NY hybridoma cells, and hybrids were selected as described by Taggart and Samloff (1983). After 10 d, hybridoma supernatants were tested for reactivity with SR proteins (see above) by ELISA (Harlow and Lane, 1988). Positive clones were subcloned by serial dilution and tested by immunoblot for their specificity for SR proteins.

Immunostaining

L cells grown on acid-washed coverslips were fixed with acetone at −20°C for 2 min, rinsed twice with PBS containing 10 mM $MgCl_2$, and exposed to hybridoma supernatant at room temperature for 1 h. After three washes in PBS, coverslips were incubated for 1 h with RITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Washed coverslips were then mounted in 50% glycerol containing 0.5 μg/ml DAPI. For *Drosophila melanogaster* polytene chromosome spreads, salivary glands from third instar larvae were prepared by Dr. B. Ostrow according to the method of Silver et al. (1978).

Immunoblotting and Immunoprecipitation

For immunoblotting, SDS-polyacrylamide gels were transferred to nitrocellulose, stained with Ponceau S, blocked for 10 min at room temperature in 3%BSA in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), and incubated for 1 h at room temperature in primary antibody. Blots were then washed three times in TBST and processed for colorimetric or ECL (Amersham Corp., Arlington Heights, IL) detection using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). mAb 16H3 was used as undiluted hybridoma supernatant. 3G6 (anti-hnRNP U) ascites fluid was diluted 1:1,000 in 20% FCS (Biowhittaker, Inc., Walkersville, MD).

Heterogeneous nuclear ribonucleoprotein (hnRNP) complexes and snRNPs were immunoprecipitated from an extract of unlabeled HeLa cells as follows: 10^9 HeLa cells were harvested from a suspension culture extracted in 12 ml NET-2 buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.05% NP-40) plus 1 mM aprotinin and 1 mM PMSF and sonicated on ice for three 30-s bursts. The extract was centrifuged for 15 min at 4°C at 17,000 *g*. The supernatant was adjusted to 15 ml with NET-2, and 5 ml each was added to 100 μl Gammabind (Pharmacia Inc., Piscataway, NJ) or 10 μl magnetic beads (Dynal Inc., Great Neck, NY) previously coated with either 10 μl 4F4 (anti-hnRNP C) ascites fluid in 20% FCS, 1 ml Y12-conditioned medium (anti-Sm), 0.5 ml anti-U1 70K mAb (Billings et al., 1982), or 20% FCS alone. Beads and extracts were incubated together at 4°C on a rotator for 2 h. The beads were then washed with 5 × 10 ml ice-cold NET-2 over the course of 1 h. The final bead pellet was extracted in protein sample buffer and subjected to SDS-PAGE, after which the gel was transferred to nitrocellulose and cut into strips. For immunoblot detection, Y12 was used as undiluted hybridoma supernatant. 4F4 and 4B10 (anti-hnRNP A1) ascites fluid were diluted 1:1,000 in 3% BSA in TBST.

For immunoprecipitation of ^{32}P-labeled RNA, 5×10^7 HeLa cells were grown in suspension for 4 h in phosphate-free medium plus 5 mCi ortho-[^{32}P]phosphate. Cells were harvested by centrifugation at 1,000 *g*, washed once in PBS, and extracted in NET-2 as described above. Immune complexes were extracted from the beads with protein sample buffer for protein or with phenol/chloroform for RNA. After ethanol precipitation, RNA samples were raised in formamide dyes, loaded onto a 5% acrylamide/7 M urea gel, and run at 1,700 V/45 W for 2 h.

In Vitro Splicing Reactions

Capped, ^{32}P-labeled SP64HβΔ6 RNA containing two exons and a single intron (Ruskin et al., 1984) was prepared and used to assay pre-mRNA splicing in vitro as described (Zahler et al., 1992). For antibody inhibition studies, IgGs were purified by affinity chromatography on Gammabind, dialyzed against PBS, diluted to 250 μg/ml, and added to each splicing reaction while still on ice and immediately before the addition of the RNA substrate. After incubation at 30°C for 2 h, each reaction was subjected to Proteinase K digestion and phenol/chloroform extraction. RNAs present in the aqueous phase were precipited with ethanol, using 2 μg tRNA as a carrier. Recovered RNAs were subjected to denaturing electrophoresis on 5% acrylamide gels for 3 h at 45 W.

Expression Screening and Epitope Mapping

Approximately 1.5×10^5 plaques from a Lambda Zap *Xenopus laevis* young ovary polyA$^+$ cDNA expression library (Stratagene Inc., Burlingame, CA) were screened with mAb 16H3. Reactive plaques were detected using the Vectastain ABC kit as described above for immunoblotting. Two independent cDNAs were sequenced using the Sequenase system (USB). 3×10^5 plaques from a genomic *S. cerevisiae* library were screened with 16H3 using the same methods, and no positives were obtained.

Results

We generated a monoclonal antibody that recognizes a subset of the SR protein family of splicing factors. One mouse was immunized with a bacterially expressed fusion between GST and *Drosophila* SRp55. Supernatants from 400 clones resulting from the spleen cell fusion were screened by

ELISA for reactivity with calf thymus SR proteins. mAb 16H3 was among these positive supernatants and was subsequently characterized by immunoblotting. Fig. 1 *A* shows that while mAb 104 (lane *1*) binds all six SR proteins purified from human HeLa cells, mAb 16H3 (lane *2*) only binds SRp75, SRp55, SRp40, and SRp20, but not SRp30a or b (ASF/SF2 or SC-35). mAb 16H3 bound neither SRp30 band when they were separated from one another on a 13.3% polyacrylamide gel (data not shown).

Nuclear extract prepared from HeLa cells contains all of the factors required for pre-mRNA splicing in vitro, including all of the SR proteins (Krainer and Maniatis, 1985; Zahler et al., 1993*b*). Surprisingly, nuclear extract contains many more antigens recognized by mAb 16H3 than by mAb 104, even though mAb 16H3 binds fewer SR proteins. The SR proteins as well as two additional, higher molecular mass (190 and 125 kD) bands were bound specifically by mAb 104 (lane *3*), but 15–20 16H3 antigens appear in lane *4*. Interestingly, bands comigrating with SRp75, SRp55, and the two high molecular weight bands bound by mAb 104 were readily detected by mAb 16H3. 16H3-reactive bands comigrating with SRp40 and SRp20 were faintly detectable. In contrast to nuclear extract, cytoplasmic S100 extract is only competent for in vitro splicing upon the addition of one or more SR proteins (Krainer and Maniatis, 1985; Zahler et al., 1992). Thus, the observation that 16H3 antigens (lane *6*), like SR proteins (lane *5*; Zahler et al., 1993*b*), are much less abundant in S100 suggests that at least some of the 16H3 antigens may have an essential function in pre-mRNA splicing. Indeed, the level of only one (145 kD) of the 16H3 antigens in S100 is comparable with that detected in nuclear extract (compare lanes *4* and *6*).

The mAb 16H3 epitope appears to be conserved throughout the animal kingdom but is not detectably expressed by yeast cells. Fig. 1 *B* shows a mAb 16H3 immunoblot of whole HeLa cell extract as well as HeLa nuclear extract, *Drosophila* Kc cell nuclear extract, and *Xenopus laevis* oocyte nuclei in which a complex set of reactive polypeptides was observed. The pattern of 16H3 antigens detected in whole HeLa cell extract (lane *1*) appeared nearly identical to that found in nuclear extract (lane *2*), suggesting that most of the antigens are nuclear. The relative mobilities of some of the bands detected in *Xenopus* and *Drosophila* differed from the human antigens, but the overall number of antigens was quite similar (lanes *3* and *4*). In contrast, no reactive polypeptides could be detected in extracts of *S. cerevisiae* or *S. pombe* (lanes *5* and *6*). Moreover, although the 16H3 epitope is known to be expressed by bacterial cells and to be useful in expression screening (see below), we were unable to obtain positive plaques in an expression screen of 3×10^5 pfu from an *S. cerevisiae* genomic library. We therefore consider the possibility that 16H3 binds an epitope common to a subset of SR proteins and other nuclear proteins in metazoan but not yeast cells.

Immunostaining experiments revealed that 16H3 antigens are indeed predominantly nuclear and are localized to active sites of polymerase II transcription. mAb 16H3 stained the nuclei of cultured mouse L cells very intensely relative to the cytoplasm, while staining of nucleoli was undetectable (Fig. 2). Denser regions of mAb 16H3 staining appear indistinguishable from staining produced by antibodies against the SR protein family, such as mAb 104 (Roth et al., 1989; data not shown), but an even, grainy staining also extended throughout the nucleoplasm. On polytene chromosomes from the salivary gland of *D. melanogaster*, mAb 16H3 reacted with discreet bands of chromatin and, in particular, with developmental chromosome puffs (Fig. 3). RNA polymerase II, Sm antigens, and U1 and U2 snRNPs are also localized to chromosomal puffs, the sites of high levels of gene transcription (Sass, 1982; Sass and Pederson, 1984). Transcription also occurs in bands that are not puffed, and therefore the banded staining seen with mAb 16H3 is not in-

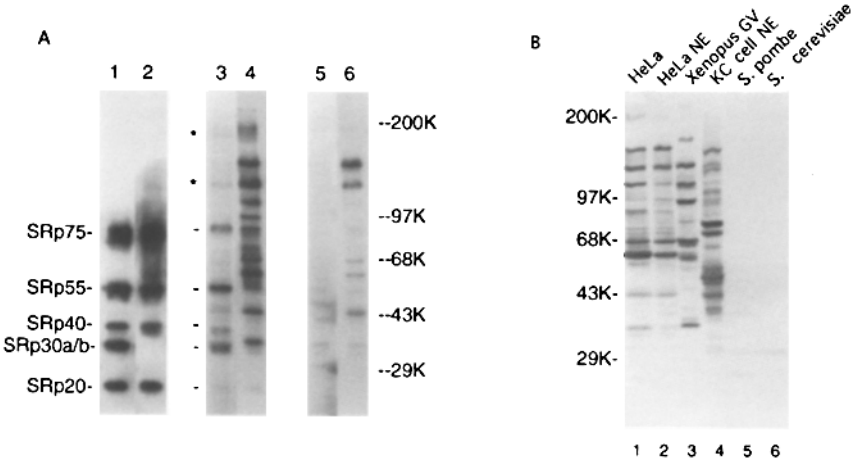

Figure 1. mAb 16H3 recognizes purified SRp75, SRp55, SRp40, SRp20, and ~20 prominent nuclear antigens in extracts from human, frog, and fly. (*A*) mAb 16H3 and mAb 104 immunoblots of SR proteins purified from HeLa cells (~0.1 μg in lanes *1* and *2*), HeLa cell nuclear extract (10 μg in lanes *3* and *4*) and S100 (10 μg in lanes *5* and *6*): SRp75, SRp55, SRp40, SRp30a/b, and SRp20 are indicated by tic marks at the left of lanes *1* and *3*. Lane *1* was probed with mAb 104, which reacts with all of the SR proteins. Lane *2* was probed with mAb 16H3, which binds SRp75, SRp55, SRp40, and SRp20, but not SRp30a/b. mAb 104 binds all of the canonical SR proteins in addition to two higher molecular mass bands (*asterisks*, 190 and 125 kD) in nuclear extract (lane *3*), whereas mAb 16H3 binds many more polypeptides (lane *4*). mAb 104 antigens are depleted in S100 extract (lane *5*), as are mAb 16H3 antigens (lane *6*), with the exception of bands at 145, 125, 68, 60, and 45 kD. (*B*) 200 μg each of whole-cell extracts of HeLa cells (lane *1*), *S. cerevisiae* (lane *6*) and *S. pombe* (lane *5*), 10 μg each of HeLa cell and *Drosophila* Kc cell nuclear extracts (*NE*; lanes *2* and *4*), and hand-dissected *X. laevis* oocyte nuclei (*GV*; lane *3*) were probed with mAb 16H3. Ponceau S staining of the nitrocellulose filter indicated numerous protein bands in the lanes containing the yeast extracts. The faint, low molecular mass bands in each of the yeast lanes were also present in blots that were processed in the absence of primary antibody and are, therefore, nonspecific. All of the samples in *A* and *B* were separated by SDS-PAGE on 5–15% polyacrylamide gradient gels, transferred to nitrocellulose, and subjected to immunoblotting.

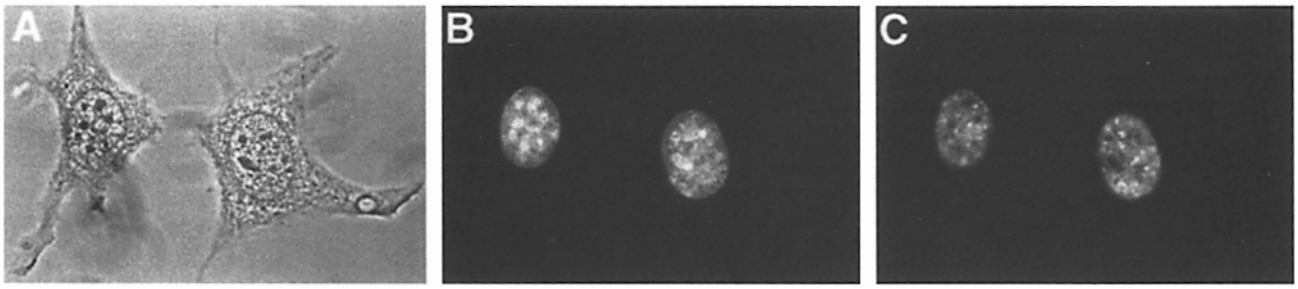

Figure 2. Localization of mAb 16H3 antigens to the nuclei of cultured L cells. (*A*) phase contrast image, (*B*) corresponding DAPI staining, and (*C*) indirect immunofluorescent detection of mAb 16H3 followed by a RITC-conjugated secondary antibody. Note the intensity of mAb 16H3 staining in the nucleus and the absence of staining in the phase-dark nucleoli.

consistent with transcription (Sass, 1982). These data suggest that the 16H3 epitope is primarily nuclear and, furthermore, concentrated at sites of active RNA polymerase II transcription.

The localization of the 16H3 epitope to nuclei and active sites of transcription indicates that the high degree of complexity among 16H3 antigens might reflect a group of pre-mRNA processing proteins larger than, but overlapping with, the SR family of essential splicing factors. To determine whether 16H3 antigens interact with RNA in vivo, orthophosphate ^{32}P-labeled RNA was immunoprecipitated from HeLa cells under conditions that preserve interactions between proteins and RNA (Fig. 4). Lane *2* shows the U snRNAs that coimmunoprecipitate with mAb Y12 antigens, integral components of most of the nonnucleolar U snRNPs (Petterson et al., 1984). mAb 16H3 immunoprecipitated the U1 snRNA very efficiently (lane *4*) compared with a mAb against the U1 70K protein (lane *3*; Billings et al., 1982) and mAb Y12. However, U2 snRNA was not detected in either anti-U1 70K or 16H3 immunoprecipitates. The fact that 5S RNA, U3 snRNA, and tRNA were absent supports the observation that 16H3 antigens are predominantly localized to the nucleoplasm and not the nucleolus or the cytoplasm. The observation that U1 snRNA immunopurifies with a 16H3 antigen(s) indicates that mAb 16H3 does recognize other pre-mRNA processing proteins, because immunoprecipitation of SR proteins alone yields only high molecular weight RNAs and none of the snRNAs (Roth et al., 1991).

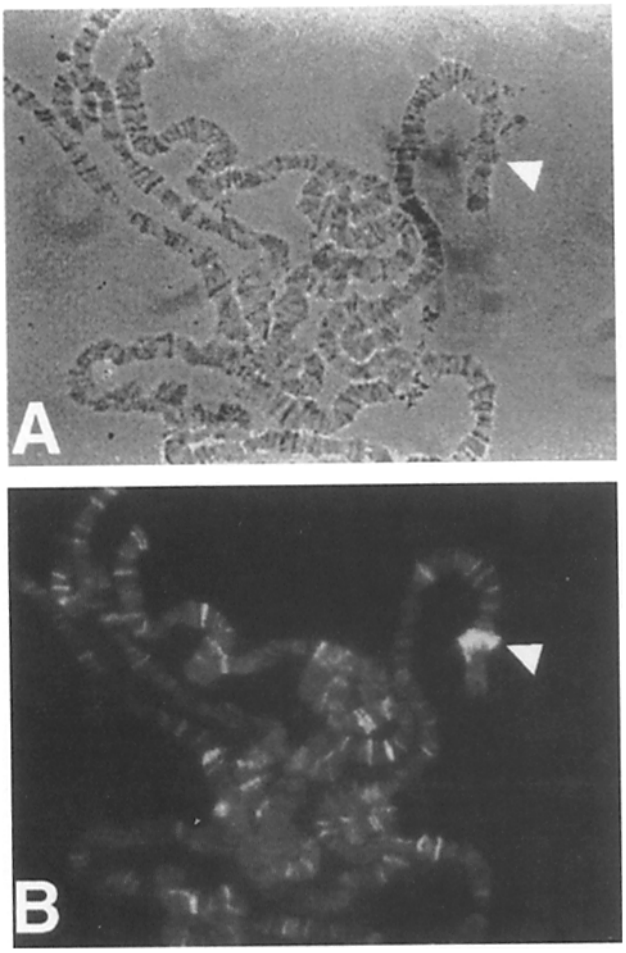

Figure 3. Localization of mAb 16H3 antigens to active sites of transcription on *Drosophila* polytene chromosomes. (*A*) phase contrast image and (*B*) corresponding fluorescent image after treatment with mAb 16H3 and a RITC-conjugated secondary antibody. A developmental puff is indicated in both panels by an arrowhead.

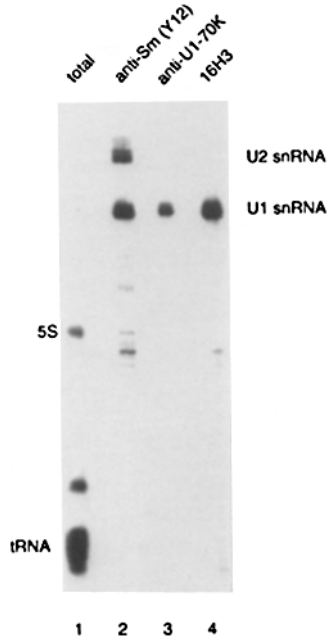

Figure 4. mAb 16H3 immunoprecipitates ^{32}P-labeled U1 snRNA from HeLa cells. HeLa cells labeled for 4 h with [^{32}P]orthophosphate were extracted and incubated with beads coated with mAb Y12 against the Sm epitope (lane *2*), a mAb against U1 70K protein (lane *3*), or mAb 16H3 (lane *4*). Extracted RNAs were separated on a 10% polyacrylamide/7M urea gel, after which the gel was dried down and exposed to film. Total RNA prepared from the same extract were run in lane *1*.

As a first step toward identifying some of the unknown 16H3 antigens, we screened a *X. laevis* ovary expression library with mAb 16H3. Several of the cDNAs isolated were shown to encode the *Xenopus* U1 70K protein when their NH_2 termini were sequenced, because the first 121 nucleotides predicted an amino acid sequence identical to amino acids 54–111 of the published sequence (Etzerodt et al., 1988). The U1 70K protein is an integral component of the U1 snRNP and binds the U1 snRNA directly (Query et al., 1989). This finding is consistent with the immunoprecipitation of U1 snRNA by mAb 16H3.

Independent confirmation that mAb 16H3 binds U1 70K is shown in Fig. 5 *A* in which the U1 snRNP was immunoprecipitated using the anti-U1 70K mAb (see above). The antigen–antibody complexes were subjected to SDS-PAGE and then reprobed by immunoblotting with either mAb 16H3 or the original anti-U1 70K mAb. A control lane shows the antibody bands (*asterisks*) present in the immune complexes and detected by the immunoblotting procedure. As expected, both the anti-U1 70K mAb and mAb 16H3 detect a single band at the appropriate molecular weight. Neither mAb binds any of the other proteins present in the UlsnRNP; the core snRNP proteins B and B′ were recognized in a parallel lane by mAb Y12 (data not shown). In a similar experiment (Fig. 5 *B*) designed to test mAb 16H3 reactivity with general U snRNP proteins, snRNPs were immunoprecipitated with the mAb Y12, which binds three polypeptides known as B, B′, and D that are shared among the U snRNPs (Pettersson et al., 1984). In the control lane, the antibody band is indicated with an asterisk. While a doublet of reactive protein (28 and 29 kD) corresponding to B and B′ was visible in the lane incubated with mAb Y12, these polypeptides were not detected by mAb 16H3. However, mAb 16H3 did recognize the U1 70K protein in the same lane, indicating that intact U snRNPs had been immunoprecipitated. Therefore, the fact that other U snRNP polypeptides were not recognized by mAb 16H3 is further evidence of mAb 16H3's selectivity for particular RNA-binding proteins.

To determine whether other classes of RNA-binding proteins are also 16H3 antigens, we tested mAb 16H3 reactivity with hnRNP proteins, which associate into large RNP complexes with pre-mRNA. In Fig. 5 *C*, intact hnRNP complexes were immunoprecipitated from HeLa cell extracts by mAb 4F4 against one of ~20 hnRNP components, hnRNP C (Choi and Dreyfuss, 1984; Dreyfuss et al., 1993). In the control lane, antibody bands are indicated by asterisks. The hnRNP C proteins were visualized by immunoblotting with mAb 4F4, and the hnRNP A proteins that coprecipitated are shown in the last lane, where they were bound by mAb 4B10 (Choi and Dreyfuss, 1984). In the lane incubated with mAb 16H3, neither hnRNP proteins A or C were detected. Finally, mAb 16H3 did not bind recombinant, bacterially expressed hnRNP U (Fig. 5 *D*), which was detected in a parallel lane by mAb 3G6. Thus, mAb 16H3 has no affinity for the hnRNP proteins A, C, or U.

These results indicate that mAb 16H3 does not bind all pre-mRNA processing factors in general, but rather has specificity for another set of nuclear proteins localized to sites of RNA polymerase II transcription (summarized in Table I). Among these, the SR proteins and U1 70K protein share two distinct structural motifs, the RRM and the SR domain, a stretch of amino acids consisting almost entirely of

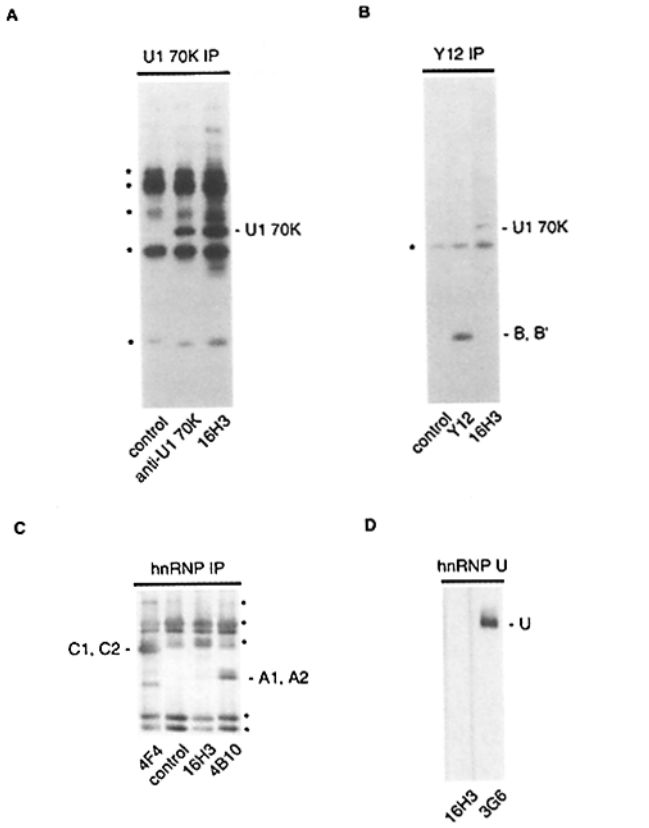

Figure 5. mAb 16H3 binds U1 70K but not core U snRNP or hnRNP components. (*A*) U1 snRNPs were immunoprecipitated from a HeLa cell extract using an anti-U1 70K mAb (Billings et al., 1982). Antibody–antigen complexes were run on 10% SDS-PAGE and blotted to nitrocellulose. Each lane was incubated with either 20% FCS (control), the anti-U1 70K mAb, or mAb 16H3 as indicated below each lane. Bands detected independent of incubation with primary antibody during the blotting procedure are indicated with asterisks. These are antibody bands present because of the immunoprecipitation procedure. The U1 70K band is indicated. (*B*) U snRNPs were immunoprecipitated from a HeLa cell extract using the anti-Sm mAb Y12 (Pettersson et al., 1984) and processed for immunoblotting as described in *A*. Sm antigens B and B′ detected in the Y12 blot are indicated. Due to the small size of Sm antigen D, it would not have been resolved in this experiment. Only U1 70K, which coprecipitated with the core snRNP proteins, is recognized by mAb 16H3. Both *A* and *B* were performed with anti-mouse IgG-conjugated magnetic beads. (*C*) hnRNP complexes were immunopurified using mAb 4F4 (bound to Gammabind) against the hnRNP A proteins and examined by immunoblotting. hnRNPs C1 and C2 are detected by mAb 4F4 in the first lane, and hnRNP A1 and A2 by mAb 4B10 in the last. mAb 16H3 bound neither of these doublets. The 12.5% polyacrylamide gels were run under nonreducing conditions so that most of the antibody molecules would migrate more slowly (i.e., in their unreduced form at approximately 180 kD). (*D*) In lanes *6* and *7*, 0.15 μg of bacterially expressed hnRNP U was run and subjected to immunoblotting by either mAb 16H3 or mAb 3G6 against hnRNP U.

alternating arginine and serine (cf. Query et al., 1989; Kenan et al., 1991; Zahler et al., 1992). mAb 16H3 reacted with bacterially expressed proteins containing intact $U2AF^{35}$ and $U2AF^{65}$ as well as the arginine-rich domain of HRH1 (Table I; data not shown). HRH1 is the putative human

Table I. Identified Protein Antigens of mAb 16H3

Antigens	Not antigens
SRp20	SRp30a
SRp40	SRp30b
SRp55	core snRNP proteins B and B′
SRp75	hnRNP A1 and A2
U1 70K	hnRNP C1 and C2
U2AF^{65}	hnRNP U
U2AF^{35}	
HRH1	

See Figs. 1 and 5 and text for details.

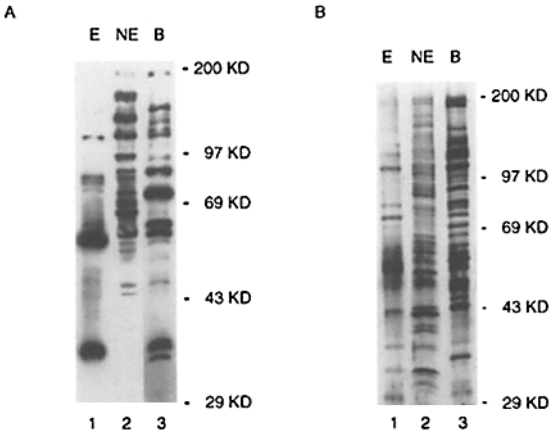

Figure 6. mAb 16H3 recognizes many polypeptide components of spliceosomes assembled in vitro. (*A*) mAb 16H3 immunoblot of E complex (lane *1*), nuclear extract (*NE*; lane *2*), and B complex (lane *3*) on a 7.5% polyacrylamide gel shows that ~20 reactive bands can be distinguished in nuclear extract. Of these, ~10 can be detected in E and 11 in B complexes at corresponding positions in the gel. (*B*) Silver stain of a parallel gel reveals the complexity of polypeptides in E complex (lane *1*), nuclear extract (lane *2*), and B complex (lane *3*).

homolog of the yeast Prp22, a member of the DEAH family of RNA helicases, which is involved in the release of spliced mRNA from the spliceosome. Although HRH1 and Prp22 have extensive homology at the amino acid level, HRH1 additionally contains a 53-amino acid sequence of arginine alternating with serine and aspartate that is absent in Prp22 (Company et al., 1991; Ono et al., 1994). U2AF^{35} and U2AF^{65} are subunits of an essential 3′ splice site binding activity that contain SR domains (Zamore et al., 1992; Zhang et al., 1992). Unlike all of the other 16H3 antigens identified thus far, U2AF^{35} does not contain an RRM. Together with the demonstration that mAb 16H3 does not bind at least four proteins that contain RRMs (SRp30a and 30b, hnRNP proteins A and C; see Figs. 1 and 5), this suggests that mAb 16H3 does not have general specificity for the RRM class of RNA binding domains (Burd et al., 1989; Kenan et al., 1991; Zahler et al., 1992). Instead, the profile of the known 16H3 antigens suggests that this set of proteins is specifically associated with pre-mRNA splicing rather than more general aspects of RNA metabolism.

Because eight of the known 16H3 antigens are pre-mRNA splicing factors, we hypothesized that the unknown antigens, which do not occur in snRNP or hnRNP complexes, would be specifically concentrated in the multicomponent splicing complex, the spliceosome. To address this possibility, polypeptide components of early (E) and mature (B) spliceosomal complexes that had been assembled on biotinylated AdML pre-mRNA and isolated by gel filtration and affinity purification (Bennett et al., 1992*a*) were examined for reactivity with mAb 16H3. For comparison with the known set of antigens, E and B complexes were separated by SDS-PAGE alongside HeLa nuclear extract. Fig. 6 shows that mAb 16H3 specifically recognized many of the components of E and B complexes. Compared with the polypeptides visualized by silver staining in Fig. 6 *B* (lane *1*), mAb 16H3 appears to react with approximately half of the detectable proteins purified in E complex (Fig. 6 *A*, lane *1*). Consistent with mAb 16H3's reactivity with both subunits of U2AF (see above), two prominent 16H3-reactive bands in E complex are certainly U2AF^{35} and U2AF^{65}, as judged by their abundance and migration in the gel (see Bennett et al., 1992*a*). U1 70K is likely to comigrate with U2AF^{65} at M_r 62,000. The other fainter bands of 200, 145, 125, 92, and 65 kD comigrate with silver-stained bands in the parallel gel (Fig. 6 *B*, lane *1*). In B complex, major bands of 200, 160, 155, 145, 135, 125, 105, 95, 82, 65, 57, 45, and 35 kD were detected in addition to the presumed U2AF and U1 70K bands (Fig. 6 *A*, lane *3*). Comparison of the B complex blot with the silver-stained preparation suggests that more than half of the polypeptides present in B complex were reactive (Fig. 6 *B*, lane *3*). mAb 16H3 reactivity with E and B complex proteins appeared to be specific, because relatively abundant proteins detected by silver stain (e.g., a 50-kD band in B complex) were not reactive. It must also be noted, however, that the strict identification of bands cannot be made based on comigration in the blot and the silver stain. Finally, it was possible to align the majority of 16H3 reactive proteins detected in nuclear extract with those found in E and/or B complex: of the ~20 major bands in nuclear extract, 14 comigrate with proteins that purify with these assembled splicing complexes. Because E and B complexes do not contain every component of the splicing machinery (e.g., SR proteins; Bennett et al., 1992*a*), it is not surprising that several of the nuclear extract antigens are absent from E and B complexes. The overwhelming conclusion of this experiment is that 16H3 antigens are prominent components of active pre-mRNA splicing but not hnRNP or snRNP complexes.

The evolutionary conservation of the 16H3 epitope and its concentrated presence in the spliceosome suggest that the epitope itself may define a functional element on pre-mRNA splicing factors. To address this possibility, we assayed the effect of purified mAb 16H3 IgG on pre-mRNA splicing in vitro (Fig. 7). H$\beta\Delta$6 pre-mRNA substrate is efficiently converted to product in a reaction containing a splicing-competent HeLa nuclear extract. In lanes *1–3*, increasing amounts of 16H3 IgG (0.25–1.25 μg) were added and resulted in a decrease in both the amount of spliced product as well as intermediates. These data can be compared to the control reactions (lanes *4–6*), which took place in the presence of equal quantities of nonimmune mouse IgG. Another control performed with a monoclonal IgG to a frog nuclear antigen yielded similar results to the control shown here (data not shown). The fact that splicing intermediates do not

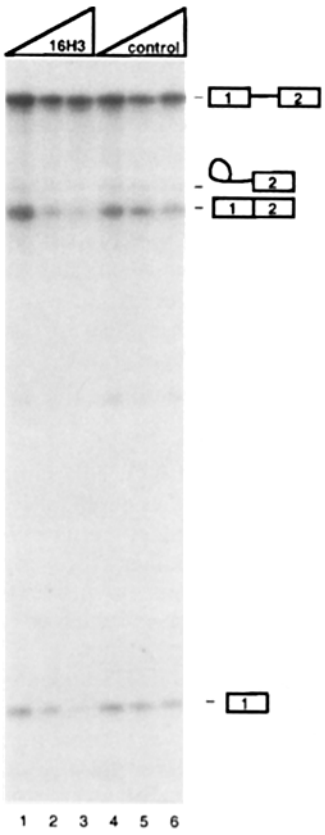

Figure 7. mAb 16H3 IgG inhibits pre-mRNA splicing in vitro. ^{32}P-labeled HβΔ6 pre-mRNA was incubated for 2 h at 30°C under splicing conditions in HeLa cell nuclear extract in the presence of either purified 16H3 IgG (0.25, 0.63, and 1.25 μg in lanes *1*, *2*, and *3*, respectively) or nonimmune mouse IgG (0.25, 0.63, and 1.25 μg in lanes *4*, *5*, and *6*, respectively). The resulting RNAs were subjected to denaturing electrophoresis in 5% acrylamide gels, dried down, and exposed to film. Pre-mRNA, intermediates, and product are indicated.

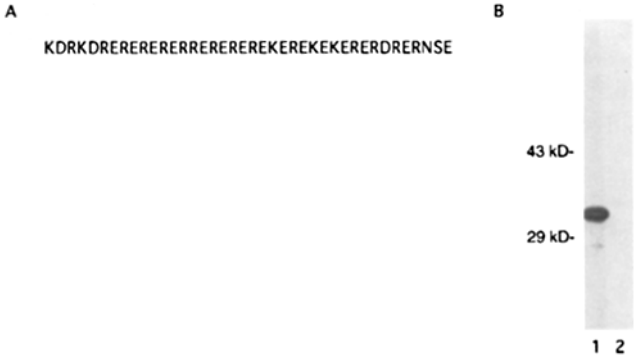

Figure 8. The 16H3 epitope is contained in a sequence of alternating glutamate and arginine. (*A*) deduced 40–amino acid sequence of the protein fragment that was fused to GST. (*B*) mAb 16H3 recognizes the GST fusion protein containing the alternating arginine domain shown in *A* by immunoblot (lane *1*), but does not recognize expressed GST alone (lane *2*).

accumulate suggests that mAb 16H3 inhibits splicing before the first transesterification reaction.

The fact that mAb 16H3 inhibits in vitro splicing suggests that the epitope itself may participate in spliceosome assembly. It is therefore of interest to define the 16H3 epitope more precisely. A partial cDNA was isolated and sequenced from the mAb 16H3 screen of the *Xenopus* ovary expression library, and we used this clone to map the 16H3 epitope. This cDNA was chosen for the analysis because, unlike many of the other 16H3 antigens, it was expressed at high levels by bacterial cells. The sequence of the 1,441-nucleotide-long clone did not reveal any identities or strong similarities within the data base. A weak similarity to the RRM of yeast poly-A polymerase was observed, suggesting that the encoded protein may bind RNA. The most remarkable feature of the predicted amino acid sequence was the presence of a region consisting almost entirely of arginine alternating with glutamate or occasionally aspartate (Fig. 8 *A*). A fusion of this sequence with GST was created, and the expressed protein was shown to react with mAb 16H3 by immunoblot (Fig. 8 *B*, lane *1*). It is striking that each of the seven known 16H3 antigens contains a domain characterized by arginine alternating with serine, glutamate, and aspartate in varying proportions (Etzerodt et al., 1988; Query et al., 1989; Zahler et al., 1992; Zamore et al., 1992; Zhang et al., 1992; Ono et al., 1994). Thus, it is possible that mAb 16H3 binds each antigen in its arginine-rich domain.

Discussion

We have developed a monoclonal antibody (16H3) with specificity for a subset of the SR protein family of essential pre-mRNA splicing factors. In addition to SRp75, SRp55, SRp40, and SRp20, this mAb recognizes ~20 prominent nuclear proteins, which can be accounted for, at least in part, by protein components of biochemically defined pre-mRNA splicing complexes. These include the U1 70K component of the U1 snRNP and both subunits of U2AF, a factor that binds to the 3′ splice site. In contrast, mAb 16H3 antigens do not appear to occur in snRNP or hnRNP complexes (see Table I for summary). Although mAb 16H3 binds this collection of proteins in humans, *Drosophila*, and frog, reactive proteins are not detectable in yeast cells, suggesting that the 16H3 epitope is conserved among pre-mRNA splicing factors but limited to metazoans. We therefore consider the possibility that the 16H3 epitope itself defines a functional element uniquely required in metazoan pre-mRNA processing.

Initially, mAb 16H3 was shown to bind SRp75, SRp55, SRp40, and SRp20, but not SRp30a or SRp30b. Surprisingly, immunoblotting experiments indicate that a minimum of 20 major 16H3-reactive bands are present in HeLa cells and nuclear extract, amphibian nuclei, and *Drosophila* nuclear extract. Two of the reactive polypeptides in HeLa cells (190 and 125 kD) appear to comigrate with two mAb 104–reactive bands, raising the intriguing possibility that these two 16H3 antigens are members of the SR protein family (Roth et al., 1989; Zahler et al., 1993*b*). However, SR proteins are defined not only as mAb 104 antigens, but by three other criteria: their quantitative recovery in a two-step purification scheme, their ability to complement a pre-mRNA splicing-deficient S100 extract in vitro, and two features of their primary structure, an RRM that is conserved within the family and an SR domain consisting almost entirely of alternating serine and arginine (Zahler et al., 1992). Because these additional criteria have not yet been satisfied for the two high molecular weight 16H3/104 antigens, they cannot yet be named SR proteins. Certainly, the presence of numerous mAb 104–reactive polypeptides in the *Xenopus* germinal vesicle indicate that additional SR proteins may well exist (Roth et al., 1989).

Consistent with the notion that 16H3 antigens represent a set of factors involved in the processing of RNA polymerase II transcripts are the observations that mAb 16H3 immuno-

staining is predominantly nuclear and nonnucleolar and occurs at active sites of transcription. The nuclear staining observed in mouse tissue culture cells has a grainy quality but is not confined to "speckles" or coiled bodies that have been observed by others using antibodies with more limited specificity (cf. Nyman et al., 1986; Moore et al., 1993). Rather, the level of staining throughout the nucleus seems to reflect the punctate staining obtained with mAbs against SR proteins (e.g., mAb 104 or SC-35) in addition to an evenly distributed pool of protein. At least some fraction of antigenic proteins can become concentrated at the sites of active RNA polymerase II transcription, such as the intensely mAb 16H3-reactive developmental puffs observed on polytene chromosomes (see Fig. 2). This pool of proteins, while diverse in apparent molecular weight, is quite limited in the types of RNAs with which it interacts. For example, mAb 16H3 does not immunoprecipitate tRNA or the nucleolar RNAs, U3 snRNA or 5S. However, the U1 snRNA is specifically immunoprecipitated, most likely reflecting the interaction between mAb 16H3 and the U1 70K protein, which is an integral component of the U1 snRNP and can bind U1 snRNA directly (Query et al., 1989; for review see Moore et al., 1993). Interestingly, the U2 snRNA was not immunoprecipitated, consistent with the demonstration that mAb 16H3 does not recognize the common components of snRNPs, like the Sm antigens (Pettersson et al., 1984).

The fact that pre-mRNA splicing factors are among the 16H3 antigens localized to active sites of transcription focused our attention on RNP complexes known to occur on nascent RNA transcripts. As transcription proceeds, pre-RNAs become coated with heterogeneous complexes called hnRNPs that contain at least 20 major proteins (cf. Dreyfuss et al., 1993). In addition, some pre-mRNA processing events, such as the removal of introns, can occur cotranscriptionally (Beyer et al., 1981). Indeed, SR proteins are localized to active transcription units on *Xenopus* lampbrush and *Drosophila* polytene chromosomes (Roth et al., 1989, 1991). To determine whether mAb 16H3 recognizes components of either of these RNPs, we probed immunopurified hnRNPs and spliceosomes assembled in vitro. mAb 16H3 did not recognize recombinant hnRNP U or hnRNP proteins A and C that were immunopurified in hnRNP complexes. In contrast, mAb 16H3 did react with many of the polypeptides present in the spliceosomal complexes E and B, suggesting that the expression of the 16H3 epitope in RNPs is limited to active splicing RNPs rather than more ubiquitous RNPs that also function in assembly and transport (e.g., snRNPs and hnRNPs; cf. Mattaj, 1988; Dreyfuss et al., 1993).

The protein components of purified complexes E and B are specifically associated with the spliceosome, because they do not accumulate on RNAs lacking functional 5′ and 3′ splice sites (Bennett et al., 1992*a*). E complex describes the collection of nuclear proteins that assembles at a splice junction in the absence of ATP and is considered, therefore, a prespliceosome (Michaud and Reed, 1991). Upon the addition of ATP, this complex can be chased into B complex, which contains additional proteins assembled during the splicing reaction. E complex contains U1 snRNP proteins and U2AF in addition to seven major spliceosome-associated proteins, while B complex contains U2 and U5 snRNP proteins and 20 spliceosome-associated proteins (Bennett et al., 1992*a*; Michaud and Reed, 1993). mAb 16H3 recognizes U1 70K, $U2AF^{65}$, and $U2AF^{35}$ in both E and B complexes, confirming the independent demonstration that all three recombinant proteins bind the mAb. In addition, mAb 16H3 binds five less abundant proteins in E complex, some of which are further enriched in B complex. B complex contains an additional nine mAb 16H3-reactive polypeptides that are not detectable in E complex. Nonreactive proteins are also present in E and B complex, consistent with our knowledge that mAb 16H3 does not bind the core snRNP components (e.g., Sm antigens B and B′), which are present in both E and B complexes (Bennett et al., 1992*a*). Significantly, most of the reactive bands in nuclear extract (~70%) can be aligned with reactive bands in E and/or B complex. It is not yet known whether the 16H3 antigen HRH1 is present in either complex, but a number of antigens, particularly in B complex, approximate the predicted molecular mass of HRH1 (150 kD; Ono et al., 1994). Naturally, failure to detect a given band in any of the lanes might reflect the relative abundance of the polypeptide rather than its absolute presence or absence. Moreover, a number of splicing factors, notably the SR proteins, do not copurify with E or B complex (Bennett et al., 1992*a*). C complex, which contains the largest number of spliceosome-associated proteins, was not tested and may contain additional 16H3 antigens (Gozani et al., 1994). The conclusion that 16H3 antigens are components of the pre-mRNA splicing pathway is supported by the observations that mAb 16H3 inhibits pre-mRNA splicing in vitro and can immunodeplete nuclear extract of splicing activity (data not shown). mAb 16H3 will be an important reagent in identifying and cloning cDNAs encoding metazoan splicing factors, as it has already been useful in expression-screening experiments.

These results indicate that the 16H3 epitope is present, to the best of our knowledge, exclusively on pre-mRNA splicing factors. This correlation and the fact that mAb 16H3 inhibits in vitro splicing suggest that the 16H3 epitope itself may be a functional element on this set of proteins. Thus, it is of interest to determine the minimally required amino acid sequence capable of binding mAb 16H3. To that end, we mapped the epitope to a 40-amino acid-long stretch of arginine alternating primarily with glutamate and sometimes aspartate (see Fig. 8 *A*). This sequence was derived from a partial cDNA isolated in a bacterial expression screen. The cDNA contains a region of weak similarity to yeast poly-A-binding protein, but otherwise contains no other known homologies. Strikingly, all of the other known 16H3 antigens also contain regions of alternating arginine. The arginine-rich domains of SRp75, SRp55, SRp40, SRp20, and the U2AF proteins contain arginine alternating with predominantly serine rather than glutamate or aspartate, although these amino acids are also present (Zahler et al., 1992; Zamore et al., 1992; Zhang et al., 1992). The analogous domains in U1 70K and HRH1 contain arginine alternating with all three amino acids—serine, glutamate, and aspartate (Etzerodt et al., 1988; Query et al., 1989; Ono et al., 1994). The fact that neither SRp30a nor SRp30b reacts suggests that glutamate provides a key component of the epitope, because neither of these SR proteins contains any glutamate-arginine (ER) combinations (Fu and Maniatis, 1992; Ge et al., 1991; Krainer et al., 1991). Similarly, nonreactive proteins—the core snRNP proteins B and B′, and hnRNP proteins A, C, and U—contain few, if any, ER (Burd et al., 1989; van Dam

et al., 1989; Kiledjian and Dreyfuss, 1992). It is interesting to note that while hnRNP U is rich in both arginine and glutamate and contains an RGG repeat domain, it contains no ER and does not react with mAb 16H3 (Kiledjian and Dreyfuss, 1992).

Because 16H3 antigens were not detected in yeast, we suggest that 16H3 antigens and possibly the epitope itself perform a regulatory function(s) uniquely required in metazoan pre-mRNA processing. The fact that mAb 16H3 inhibits in vitro splicing reactions in HeLa cell nuclear extracts supports this view. Repeated efforts to detect 16H3 antigens in extracts of *S. cerevisiae* and *S. pombe* and in a *S. cerevisiae* expression library have failed. Indeed, independent attempts to detect SR proteins in yeast with the anti-SR protein mAb 104 or by biochemical purification have also been fruitless (Zahler, A. M., and M. B. Roth, unpublished observations). Splice site definition in yeast, in contrast to higher eukaryotes, is predominantly constitutive and governed by highly conserved *cis* sequences at the 5′ and 3′ splice sites and by the yeast-specific UACUAAC box at the branch point (Guthrie, 1991), suggesting the need for additional protein–protein or protein–RNA interactions in the metazoan spliceosome. Interestingly, the putative yeast homolog of U1 70K completely lacks the arginine-rich domain characteristic of metazoan U1 70K proteins, while other structural features have been conserved (Smith and Barrell, 1991). Similarly, the human homolog of yeast Prp22, a member of the DEAH family of ATP-dependent RNA helicases, contains an SR domain that is not present in the yeast protein (Company et al., 1991; Ono et al., 1994), suggesting that further metazoan homologs of yeast splicing factors will encode added functional domains.

We speculate that this domain of arginine alternating with serine, glutamate, and/or aspartate is a structural motif of protein pre-mRNA splicing factors, of which the SR domain is a subtype. Recent studies indicate that SR domains and the SR-like domains of U1 70K and U2AF are required for splicing and may mediate both protein–protein and protein–RNA interactions in the spliceosome (Zamore et al., 1992; Caceres and Krainer, 1993; Khotz et al., 1994; Wu and Maniatis, 1993; Amrein et al., 1994; Ono et al., 1994). Because arginine is a basic amino acid, glutamate and aspartate are acidic, and serine is highly phosphorylated on SR proteins (Roth et al., 1989; Zahler et al., 1993b), it seems likely that the general structure of the domain would be determined by the pattern of alternating positive and negative charges. Indeed, theoretical considerations (Perutz, 1994) suggest that this pattern exemplified in the U1 70K protein might adopt a "polar zipper" conformation, consisting primarily of an antiparallel β-barrel in which all of the charges are compensated. It is intriguing to consider that the unknown 16H3 antigens also contain such highly charged arginine-rich domains, and that the metazoan spliceosome may contain many of these unusual structures.

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