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13. ABSTRACT (Maximum 200) Deregulation of splicing has been linked to malignant transformation in breast cancers. Therefore, to fully understand breast cancer, it will be important to identify and characterize factors that regulate the splicing process. Nuclear matrix proteins related to the serine/arginine-rich (SR) family of constitutive and regulatory splicing factors were isolated and characterized. B1C8 is a novel 820 amino acid SR phosphoprotein that, unlike previously defined SR family proteins, lacks an RNA Recognition Motif (RRM). B1C8 and a second novel SR phosphoprotein, B4A11 (300kDa), associate with each other and with splicing complexes through both steps of the splicing reaction. However, B1C8/B4A11 is required for splicing of only specific introns. Purified B1C8/B4A11 promotes splicing synergistically when added in combination with SR family proteins. B1C8/B4A11 may therefore function as a splicing "coactivator" by augmenting the activity of SR family proteins. The absence of an RRM in B1C8 further suggests that it may define a new class of SR protein factors that promotes splicing primarily through protein-protein interactions. These results provide new insights into mechanisms by which pre-mRNA splicing may be regulated.				
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CONTENTS

PAGE

Introduction	1
Body	3
Conclusions	8
References	9
Appendix 1	10

Introduction

The majority of mammalian genes contains one or more intervening sequences that must be precisely excised by pre-mRNA splicing to ensure functional expression of the gene product (reviewed in Moore et al., 1993). Often splicing is regulated to generate alternative transcripts encoding functionally distinct products from a single pre-mRNA molecule (reviewed in Chabot et al., 1996). Importantly, deregulation of alternative splicing in transcripts encoding the CD44 cell surface adhesion protein and hormone receptor molecules has been linked to malignant progression and the formation of metastases in breast cancers (reviewed in Cooper, 1995; Terp, 1996). The investigation of mechanisms underlying the regulation of splicing is therefore important for the understanding, and potentially for the treatment, of breast cancer.

Proteins containing domains rich in alternating serine and arginine residues (SR proteins) perform important roles both in constitutive and regulated pre-mRNA splicing (reviewed in Valcarcel and Green, 1996; Manley and Tacke, 1996, Chabot et al., 1996). Accumulating evidence suggests that these proteins are key factors in the regulation of many alternative splicing events. In previous studies, a family of seven SR proteins (SRp's: 20, 30a-c, 40 55 and 75) have been identified which contain one or two N-terminal domains involved in sequence specific RNA recognition and also a C-terminal SR domain. These proteins function by establishing networks of interactions that promote the recognition of splice sites and stabilize the assembly of splicing components on the pre-mRNA. Important questions to be addressed include the nature of the specificity of these interactions and also how these interactions are modulated in alternative splicing.

A significant development during the fellowship research has been the isolation of two new proteins that contain SR domains and that interact and function in conjunction with the SR family proteins. These proteins were initially identified as nuclear matrix antigens concentrated in splicing factor-rich "speckle" structures in the nucleus (see Year 1 report; Blencowe et al., 1994, 1995). In this report, evidence will be presented that these new SR

domain proteins belong a new class of splicing factors that appear to function as "coactivators" of the SR family (Blencowe et al., in preparation). Experiments described in this report contribute to objectives outlined in the Statement of Work for Years 1 and 2.

An unexpected development during the past year has been the identification of interactions between the largest subunit of RNA polymerase II and splicing components. In collaboration with the groups of Professors Stephen Warren (Yale) and Ronald Berezney (SUNY, Buffalo), we have demonstrated that a hyperphosphorylated form of the largest subunit of RNA polymerase II (pol Ilo) is associated with SR and snRNP splicing factors and also assembled splicing complexes (Mortillaro/Blencowe et al., 1996). Similar to the new SR protein splicing factors, this form of pol Ilo is highly concentrated in splicing-factor rich (speckle) structures in the nucleus, suggesting that may also be associated with RNA processing in vivo. The implications of these results in terms of the possible influence of RNA transcription on splicing will be discussed.

Body

In the Report for Year 1, the purification and preliminary characterization of nuclear matrix antigens B1C8 (160kDa) and B4A11 (300kDa) was described. These were initially identified as candidate phosphoproteins associated with nuclear matrix speckle structures and exon-containing splicing complexes (Blencowe et al., 1994). Furthermore, we reported that these antigens share biochemical and immunological properties with the SR family of splicing factors (Blencowe et al., 1995). To investigate these antigens and their possible functional significance in splicing, we obtained peptide microsequences from both proteins to facilitate the isolation of corresponding cDNA clones.

Microsequences from both proteins were compared with available sequences in the public data bases. These microsequences did not match to any known proteins but did match to sequences in the data base of expressed sequence tags (dbest). The corresponding est cDNA clones were used to probe phage cDNA libraries to obtain full length cDNA clones. A cDNA clone for the B1C8 protein was isolated which corresponds in size to the major B1C8 transcript detected in Northern blots (approximately 3.8 kb). Isolation of cDNA clones spanning the full length of the B4A11 transcript (approx. 12kb) is in progress. So far, this protein also contains SR repeats and a remarkably long, uninterrupted polyserine domain (unpublished observations).

Sequencing of the B1C8 cDNA has identified an open reading frame encoding a protein of 840 amino acids. The B1C8 protein has a remarkably high content of serine (S=15%), arginine (R=15%) and proline (P=13%) residues arranged in several types of motifs. The protein contains SR repeats, two S-rich domains and also a domain consisting of several tandem repeats a novel R and P-rich motif. All of the repetitive regions of B1C8 are located in the C-terminal three quarters of the protein, whereas the N-terminal quarter is devoid of repeat sequences. To determine if the B1C8 cDNA encodes the B1C8 antigen associated with exon containing splicing complexes and nuclear speckle structures, a polyclonal antibody was raised against the N-terminal, repeatless region of the protein fused to GST. The

affinity purified polyclonal antibody (B1C8 rAb) was tested in immunoblotting, immunoprecipitation and immunofluorescence staining experiments.

B1C8 rAb specifically recognized a 160kDa protein in immunoblots of total nuclear extract proteins and also recognized B1C8 protein purified by column chromatography and Mg^{2+} precipitation. These immunoblotting experiments confirmed that the B1C8 cDNA encodes the 160 kDa antigen recognized by the original B1C8 mAb.

The B1C8 rAb specifically immunoprecipitated exon-containing splicing complexes, in an identical fashion to B1C8 mAb. Furthermore, B1C8 rAb specifically stained nuclear speckle structures, as did B1C8 mAb. Speckle structures were recognized in total fixed nuclei as well as in nuclei in which over 95% of chromatin and soluble nuclear material had been removed to expose the underlying nuclear matrix. These experiments confirmed that the cloned protein corresponds to the B1C8 nuclear matrix antigen associated with exon-containing splicing complexes *in vitro*, as well as speckle structures *in vivo*.

Experiments were performed to investigate the mode of interaction of B1C8 protein with splicing complexes. Proteins co-immunoprecipitated with the B1C8 rAb were screened by western blotting with mAbs to different splicing components. Interestingly, it was found that two of the seven SR family proteins (SRp 40 and SRp 75) co-immunoprecipitated with B1C8 rAb. In addition, the 300kDa B4A11 protein, which co-purified with B1C8, was also detected in the B1C8 rAb immunoprecipitates. Analysis of the supernatant fractions of the immunoprecipitates demonstrated that virtually all of the B4A11 protein was removed from nuclear extracts with the B1C8 protein, whereas only a subfraction of SRp 40 and SRp 75 were removed. These experiments demonstrated that essentially all of the B4A11 protein in nuclear extracts forms a complex with B1C8 and that this complex associates less stably and/or with substoichiometric levels with SRp 40 and SRp75.

The function of the B1C8/B4A11 complex and the significance of the association of this complex with SR family proteins was investigated using an S100 splicing assay (Krainer et al., 1990). S100 cytoplasmic extracts contain all of the factors required for splicing except SR proteins. Individual SR family proteins can restore splicing to an S100 assay incubated with a beta-globin pre-mRNA substrate but not an Adenovirus-derived pre-mRNA substrate, referred to as PIP85A. Unlike SR family proteins, the highly purified B1C8/B4A11 complex did not restore splicing to an S100 extract incubated with the globin pre-mRNA. This preparation of B1C8/B4A11 was however active in reconstitution of B1C8 depleted extracts (see below). Significantly, the addition of B1C8/B4A11 to an S100 extract, together with SR proteins, stimulated splicing to levels higher than that obtained when SR proteins were added alone. Thus B1C8/B4A11 synergizes with SR family proteins in the S100 assay.

Neither SR proteins, nor B1C8/B4A11, restored splicing to an S100 assay incubated with PIP85 pre-mRNA. However, when added together, these proteins restored splicing to levels equivalent to that obtained in a regular splicing extract. These results demonstrated that the S100 is missing an additional component required for splicing of PIP85 pre-mRNA, besides the SR family proteins. The results also demonstrated that B1C8/B4A11 and SR family proteins function synergistically in splicing distinct pre-mRNA substrates.

Is B1C8/B4A11 complex required for pre-mRNA splicing? Splicing of PIP85A pre-mRNA was assayed in nuclear extracts depleted of the B1C8/B4A11 complex. In these reactions, the first step of splicing was blocked. However, splicing could be specifically restored by adding back the preparation of highly purified B1C8/B4A11 proteins. Remarkably, depletion of B1C8/B4A11 did not affect splicing of globin pre-mRNA, even though addition B1C8/B4A11 to a non-depleted extract stimulated globin splicing. It is possible that the level of B1C8/B4A11 remaining after depletion is sufficient to promote efficient splicing of globin pre-mRNA, or, more likely, B1C8/B4A11 is dispensable for globin pre-mRNA splicing.

To investigate these differences further and to identify sequences in globin pre-mRNA which facilitate splicing in the B1C8/B4A11 depleted reactions, chimeric pre-mRNAs in which different globin sequences were substituted for PIP85A sequences were tested for their ability to splice in non-depleted vs. B1C8/B4A11 depleted extracts. These experiments demonstrated that sequences including the 3' splice site and 3' exon of globin, when substituted for the equivalent region of PIP85A, fully restore splicing activity in the B1C8/B4A11 depleted reaction. These data indicate that B1C8/B4A11 dependent and independent splicing pathways exist and that in the absence of B1C8/B4A11 proteins, other factors promote splicing. Preliminary studies indicate that these alternative factor are the SR family proteins.

These results have interesting implications for the role of the B1C8/B4A11 complex in splicing. These proteins may function in the splicing of a subset of pre-mRNAs. Modulating the levels of these proteins in vivo may therefore affect the levels of splicing of some introns but not others. This could provide a mechanism for the regulation of alternative splicing in different tissue types. Consistent with this proposal, the levels of expression of B1C8 and B4A11 transcripts vary in different human tissues. Future investigations will be required to understand the significance of this variation in relation to the possible functions of B1C8/B4A11 in regulated pre-mRNA splicing.

The work described above addresses the objectives outlined in the Statement of Work (SoW) for Years 1 and 2 of the fellowship research. New SR domain proteins associated with the nuclear matrix were isolated and characterized. These studies have expanded our knowledge of the factors and mechanisms underlying pre-mRNA splicing. In particular, the identification of the B1C8/B4A11 "coactivator" complex has led to a new conceptual framework for our understanding of the interactions between SR family proteins and other components of the splicing machinery. These studies also set the stage for investigating nuclear structure-function relationships in pre-mRNA processing since B1C8/B4A11 is tightly complexed to non-chromatin subnuclear speckle structures and may function in association with these structures.

It should be noted that many of the experiments outlined in the SoW for the defined SR family proteins have been investigated in connection with the new SR domain proteins B1C8/B4A11. Sequence specificities of individual SR family proteins were defined by other groups using Selex procedures shortly after the fellowship research was initiated. These experiments were therefore not pursued. However, cell lines expressing epitope tagged SR proteins, SRp30a and SRp30b, were produced (in collaboration with Qiang Zhou in the lab), to address objectives 1 and 2 in the SoW. However, expression levels were low and the integration of the tagged cDNAs in the host cell genome were unstable in the absence of drug selection. Experiments on the characterization of the B1C8/B4A11 matrix antigens (objectives 3,4 and 5; Years 1-2) were therefore prioritized.

Additional experiments not included in the original statement of work have led to the important observation that the largest subunit of RNA pol II is associated with the splicing process. This work is summarized in Appendix 1 and a corresponding publication is attached.

Conclusions

Complementary DNAs encoding the nuclear matrix antigens B1C8 and B4A11 were isolated and the function of the corresponding phosphoproteins in pre-mRNA processing were characterized. B1C8 and B4A11 are novel SR proteins that form a complex with each other and with the SR family proteins SRp40 and 75. Consistent with a functional role for these interactions, the B1C8/B4A11 complex synergistically activates splicing with SR family proteins. However, the complex appears to be required only for the splicing of specific introns, indicating the existence of B1C8/B4A11 dependent and independent splicing pathways. These studies provide new insights into the pre-mRNA splicing process and provide the ground work for future investigations of new mechanisms underlying the regulation of splicing. This information will contribute to our understanding of complex alternative splicing events including those implicated in breast cancer.

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A hyperphosphorylated form of the large subunit of RNA polymerase II is associated with splicing complexes and the nuclear matrix

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ABSTRACT A hyperphosphorylated form of the largest subunit of RNA polymerase II (pol I_o) is associated with the pre-mRNA splicing process. Pol I_o was detected in association with a subset of small nuclear ribonucleoprotein particle and Ser-Arg protein splicing factors and also with pre-mRNA splicing complexes assembled *in vitro*. A subpopulation of pol I_o was localized to nuclear "speckle" domains enriched in splicing factors, indicating that it may also be associated with RNA processing *in vivo*. Moreover, pol I_o was retained in a similar pattern following *in situ* extraction of cells and was quantitatively recovered in the nuclear matrix fraction. The results implicate nuclear matrix-associated hyperphosphorylated pol I_o as a possible link in the coordination of transcription and splicing processes.

Increasing evidence suggests that transcription and processing of RNA polymerase II (pol II) transcripts are temporally and spatially linked. Visualization of chromatin spreads by electron microscopy has revealed that the majority of introns are removed cotranscriptionally from pre-mRNA (1, 2). These studies are supported by recent fluorescent *in situ* hybridization experiments, indicating that the synthesis and splicing of specific pol II transcripts are coincident at discrete foci (3–5). In several cases, transcript foci appear to be localized in association with specific nuclear domains that are highly enriched in splicing factors, referred to as "speckles" (3, 5–7). Although not mutually exclusive with evidence implicating speckle domains in splicing factor storage and/or assembly (8, 9), these transcript localization experiments indicate a possible direct role of speckle domains in the processing of pre-mRNAs (10, 11).

Mammalian nuclei typically contain 20–50 speckle domains, which, in addition to the four spliceosomal small nuclear ribonucleoprotein particles (snRNPs; U1, U2, U4/6, and U5), are also enriched for non-snRNP splicing factors and poly(A)⁺ RNA (8, 9, 11). Many of the non-snRNP splicing factors in speckles are related to the Ser-Arg (SR) family of proteins, all of which contain one or more domains rich in alternating serine and arginine residues (12). Besides splicing components, speckle structures also contain elevated concentrations of proteins involved in transcription and cellular transformation (13–15). Since these structures are retained in the nuclear matrix (NM) following the removal of chromatin and soluble nuclear components (11, 16), the identification of matrix proteins in speckles may provide insights into their structure and function.

In previous studies, mAbs raised against the NM have been identified which stain nuclei in the speckle pattern (17, 18). Many of these anti-NM mAbs recognize antigens associated

with pre-mRNA processing that are related to the SR family. In the present study, a new anti-NM mAb, B3, is characterized that recognizes a 250-kDa NM protein concentrated in speckles. Similar to anti-NM mAbs which recognize SR proteins, B3 preferentially binds *in vitro* to a subset of splicing complexes containing exon sequences. Surprisingly, the B3 antigen corresponds to a hyperphosphorylated form of the large subunit of pol II (pol I_o). In addition to splicing complexes, pol I_o is associated with a subset of snRNP and SR protein splicing factors. The possible implications of these findings in relation to the regulation of RNA processing are discussed.

MATERIALS AND METHODS

Production of the B3 Monoclonal Antibody. Livers were extracted from adult male Sprague–Dawley rats and processed for rat liver matrices as described by Belgrader *et al.* (19). Immunization was carried out using 200 μ g of matrix protein per BALB/C mouse with multiple boosters. IgM-producing hybridomas were screened for recognition of specific matrix antigens using standard procedures (20).

Indirect Immunofluorescence. Rat kangaroo kidney (PtK-1) cells were fixed in 3% paraformaldehyde for 3 min at 20°C or extracted *in situ* for NMs (19). Cells were processed for immunofluorescence as described (21). Cells double-labeled with B3 and an anti-speckle antibody (B4A11; ref. 17) were imaged on an Olympus GB200 Laser Scanning Confocal microscope (Olympus, New Hyde Park, NY).

Preparation of Cellular Extracts and Protein Fractionation. HeLa nuclear extracts were prepared essentially as described by Dignam *et al.* (22). Defined SR proteins were purified from HeLa whole cell extracts as described by Zahler *et al.* (23). The "total" SR protein ("HS Sup") fraction was prepared as described by Blencowe *et al.* (18). Purified pol II was prepared as described by Carthew *et al.* (24). Potato acid phosphatase treatment of HeLa nuclear extracts was performed as detailed by Blencowe *et al.* (18).

Western Blot Analysis. SDS gel electrophoresis, semi-dry transfer of proteins, and immunoblotting was performed essentially as described by Harlow and Lane (20).

Abbreviations: pol II, RNA polymerase II; CTD, carboxyl-terminal domain; pol II LS, pol II large subunit; pol I_a, hypophosphorylated form of pol II LS; pol I_o, hyperphosphorylated form of pol II LS; snRNP, small nuclear ribonucleoprotein particle; SR, serine-arginine; NM, nuclear matrix.

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Pre-mRNA Splicing Assays. Splicing assays and immunoprecipitation of splicing complexes was performed as described (17).

Immunoprecipitation of Splicing Factors and Pol II. HeLa nuclear extracts were incubated for 10 min at 30°C in the presence of 1.5 mM ATP and 5 mM creatine phosphate, centrifuged, and then added to mAbs prebound to rabbit anti-mouse IgG/IgM (Pierce) linked to protein A-Sepharose beads, essentially as described (25).

RESULTS

The B3 Antigen Is Localized to Nuclear Speckles and Associated with the NM. A mAb raised against rat liver NM proteins, designated B3, stains interphase nuclei in a complex pattern consisting of 20–50 discrete nonnucleolar domains and additional smaller foci (Fig. 1*A*). B3 stains in essentially the same pattern after *in situ* extraction resulting in a typical NM preparation, which removes >98% of chromatin and soluble components from the nucleus (Fig. 1*B*).

The large foci recognized by B3 appeared similar in size and number to splicing factor-rich domains referred to as “speckles.” To test this possibility, cells were double-labeled with B3 in combination with an antibody to the B4A11 protein, which has been localized to speckle structures (17). Confocal analyses of cells labeled with these two antibodies demonstrated that the large B3 foci were also stained with the B4A11 antibody (Fig. 1*C–E*). Similar results were obtained when comparing the staining pattern of B3 with mAbs specific for snRNP Sm antigens (Y12; ref. 26) and the U1 snRNP-70 kDa protein (results not shown). These data demonstrate that the B3 antigen is localized to speckle domains enriched in pre-mRNA splicing factors.

The B3 Antigen Is Associated with Splicing Complexes. Many proteins concentrated within speckles are involved in pre-mRNA processing (8, 9, 11). To test whether the B3 antigen is also associated with pre-mRNA processing, its interaction with splicing complexes was assayed in an immunoprecipitation experiment. B3 immunoprecipitates splicing complexes assembled on a pre-mRNA derived from adenovirus sequences (PIP85A; Fig. 2*A*, lane 5). Comparison of the B3 immunoprecipitation pattern with that of a mAb specific for snRNP Sm proteins (Y12) demonstrates that B3 preferentially recognizes splicing complexes containing exon sequences. In contrast to the Y12 mAb, which immunoprecipitated a higher ratio of lariat product to splicing intermediates, B3 enriches for the intermediates and exon product RNA (compare lanes 3 and 5). This preference is similar to that observed for antibodies to proteins related to the SR family of splicing factors, including the NM protein B1C8 (compare lanes 4 and 5; refs. 17 and 27). The B3 immunoprecipitation pattern was general for splicing substrates, since a similar exon-RNA enrichment was observed for a pre-mRNA derived from a β -globin gene (Fig. 2*B*, compare lanes 3 and 4 with lane 1). Moreover, B3 did not result in significant immunoprecipitation of a nonsplicing substrate (data not shown).

To characterize the antigen recognized by B3, Western analyses were performed on subcellular fractions. B3 is specific for a single antigen of 250 kDa (Figs. 3–5). Consistent with the immunofluorescence localization experiments in Fig. 1, this 250-kDa antigen was detected exclusively in a fraction containing total nuclear proteins (Fig. 3*A*; NE, lane 2) and was quantitatively enriched in the NM fraction (NM, lane 3). It was not detected in other subcellular fractions assayed, including cytoplasmic, chromatin, and soluble nuclear proteins (data not shown).

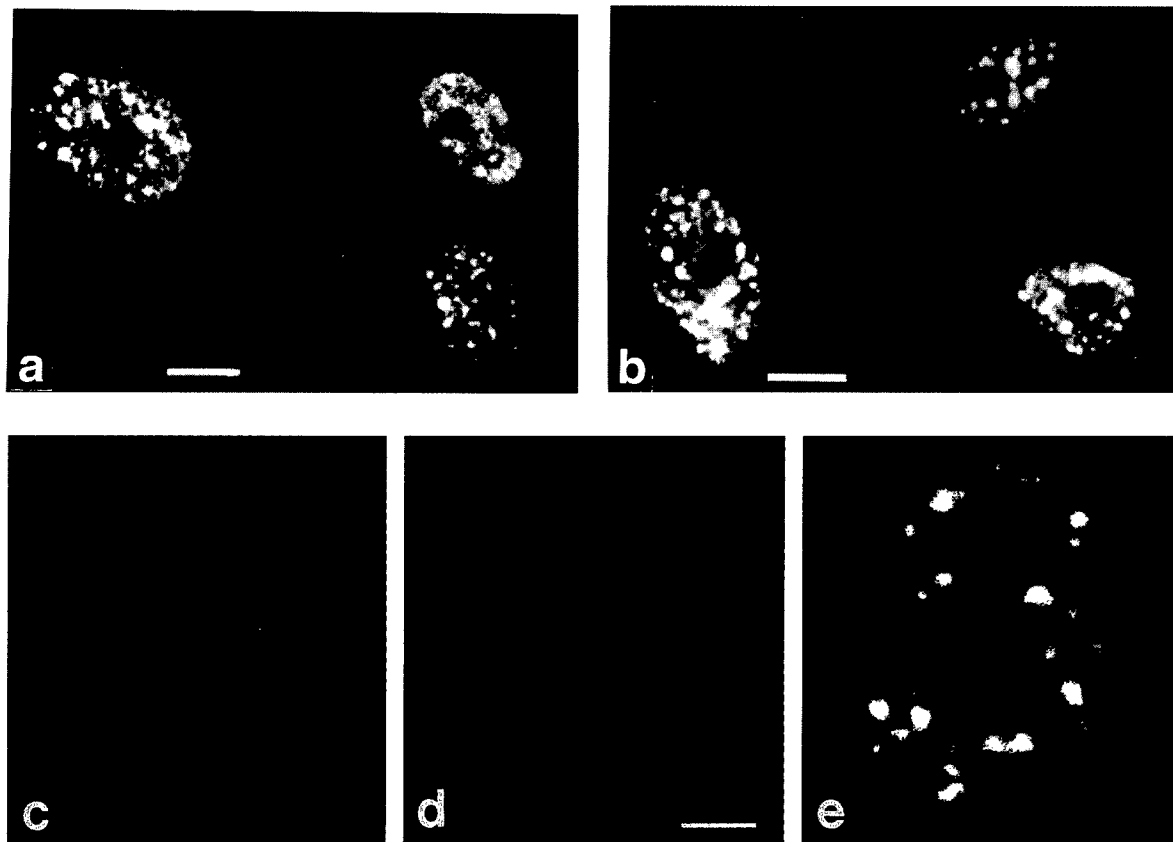


FIG. 1. Immunolocalization of the B3 NM antigen. PtK-1 cells were labeled with mAb B3 before (*A*) or after (*B*) extraction of chromatin and soluble nuclear components to reveal the underlying NM. B3 localization was detected with a fluorescein isothiocyanate-conjugated second antibody and visualized by immunofluorescence microscopy. (*C–E*). Confocal imaging of cells double-labeled with mAb B3 (*C*) and B4A11 (*D*). Colocalization of the two antigens is indicated by yellow coloration in *E*. (Bars = 10 μ m.)

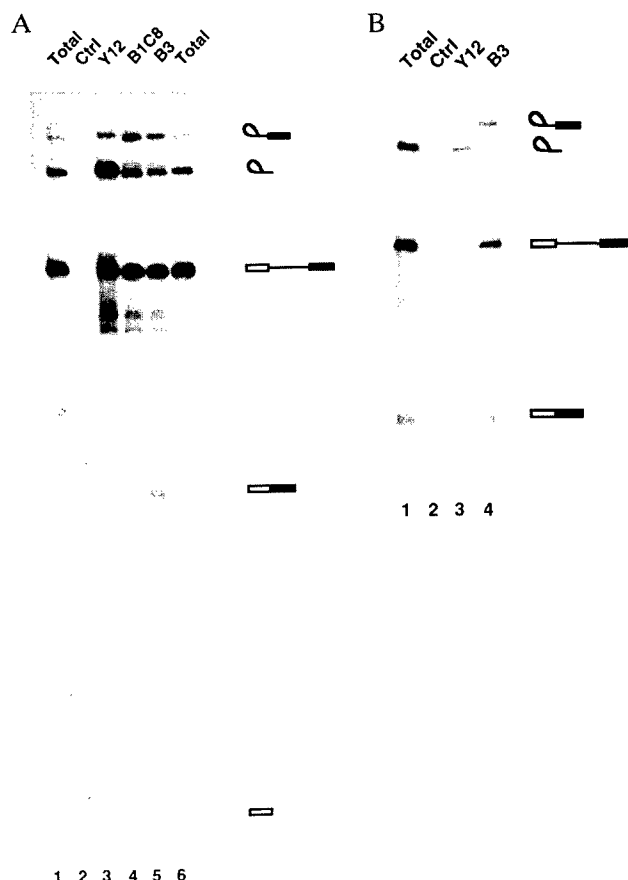


FIG. 2. mAb B3 preferentially immunoprecipitates exon-containing splicing complexes. Immunoprecipitation was performed from splicing reactions preincubated for 40 min containing PIP85A pre-mRNA (*A*) or from reactions preincubated for 2 hr containing β -globin pre-mRNA (*B*). RNA recovered following immunoprecipitation was separated on a 15% (PIP85A) or 8% (β -globin) denaturing polyacrylamide urea gel. Immunoprecipitations in *A* were performed with a nonspecific control mAb (lane 2), Y12 (lane 3), B1C8 (lane 4), and B3 (lane 5). RNA recovered directly from a parallel splicing reaction was loaded for comparison in lanes 1 and 6, representing 50% of the total RNA; all of the immunoprecipitated RNA was loaded. Immunoprecipitations in *B* were performed using a nonspecific control mAb (lane 2), Y12 (lane 3), and B3 (lane 4). Of the total RNA recovered directly from a parallel splicing reaction, 25% was loaded for comparison in *B* (lane 1), whereas all of the immunoprecipitated RNA was loaded in lanes 2–4.

Since B3 resulted in a very similar pattern of splicing complex immunoprecipitation and immunolocalization compared with mAbs reactive with SR proteins, it was next tested whether the B3 antigen may correspond to an SR-related protein and/or recognize an epitope on one of the defined SR proteins. B3 did not crossreact with any of the six defined SR proteins present in a highly purified fraction (Fig. 3*B*, lane 3; ref. 22), nor did it detect any of the additional proteins present in a fraction enriched for SR-related proteins, HS Sup (lane 2; ref. 23). Although the 250-kDa B3 antigen was detected in the HS Sup, it was only 2- to 4-fold enriched compared with the 30- to 40-fold enrichment for SR-related proteins in this fraction (ref. 18; data not shown). This low level of enrichment could indicate that B3-250 kDa is distinct from SR proteins and/or is an SR-associated antigen that is partially dissociated by high salt during the fractionation procedure.

B3 Recognizes a Hyperphosphorylated Form of Pol II Large Subunit (Pol II LS). Previously identified nuclear antigens in the 240–250 kDa molecular weight range were screened for recognition by B3. A candidate antigen was the hyperphosphorylated form of pol II LS (pol Ilo), which migrates on

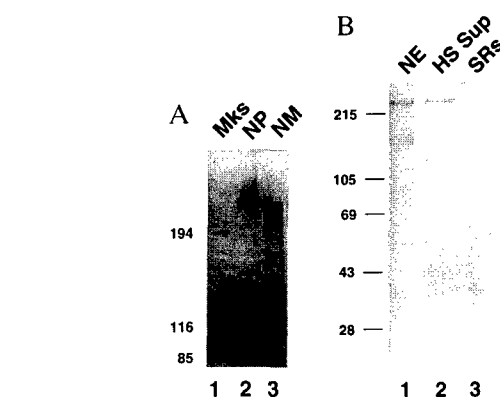


FIG. 3. B3 specifically recognizes a 250-kDa NM antigen. (*A*) Immunoblot stained with B3. Rat liver nuclear protein (10 μ g; lane 2) and 10 μ g of rat liver NM proteins (lane 3) were separated in a SDS/5% polyacrylamide gel, blotted, and probed with mAb B3. Sizes of molecular mass markers (lane 1) are in kDa. (*B*) The B3-250 kDa antigen is detected in HeLa nuclear extract and is enriched in an SR protein fraction (HS Sup). HeLa nuclear extract (100 μ g; lane 1), 24 μ g of HS Sup fraction (lane 2), and 5 μ g of defined SR proteins (lane 3) were separated in a SDS/12.5% polyacrylamide gel, transferred, and probed with B3. Transfer of proteins was confirmed by Ponceau staining (data not shown). Molecular mass markers are indicated in kDa.

SDS-polyacrylamide gels at 250 kDa and was recently localized to nuclear speckles (14). To test this possibility, HeLa nuclear extract was probed with B3 alongside two mAbs specific for different forms of pol II LS: H5, which reacts with pol Ilo (14), and 8WG16 (28), which primarily reacts with the hypophosphorylated form of pol II (pol Ila; ref. 14). B3-250 kDa precisely comigrates with pol Ilo as recognized by H5 (Fig. 4*A*, compare lanes 2 and 3) and not with the faster migrating pol Ila form recognized by 8WG16 (compare lanes 1 and 3).

To further address whether the B3 antigen corresponded to pol Ilo, immunoprecipitates prepared with the pol II-specific mAbs H5 and 8WG16 were probed with B3. Significantly, H5 and, to a lesser extent, 8WG16 immunoprecipitated the 250-kDa B3 antigen (data not shown). A highly purified preparation of biochemically active pol II was next probed with B3. Consistent with the identity of the B3 antigen as pol Ilo, both B3 and H5 showed prominent reactivity for the 250-kDa component enriched in this fraction (Fig. 4*B*, compare lanes 2 and 3). These data provide strong evidence that the 250-kDa B3 antigen corresponds to pol Ilo.

B3 Recognizes a Phosphopeptide within the Carboxyl-Terminal Domain (CTD) of Pol II LS. Since B3 is specific for pol Ilo, it was next determined whether it recognizes a phosphopeptide present in pol Ilo but not in pol Ila. Pretreatment of HeLa nuclear extracts with phosphatase essentially eliminated recognition of pol Ilo by B3 (Fig. 4*C*, compare lane 3 with lanes 1 and 2). To address whether the B3 phosphopeptide is located within the highly phosphorylated CTD of pol Ilo, nuclear lysates from cells transfected with a plasmid encoding the intact CTD domain were probed with B3 (Fig. 4*D*). Cells transfected with the CTD plasmid (pCTD-52, lane 3), but not cells transfected with a control plasmid (pCTD-less, lane 2), contained a new B3-reactive polypeptide of 140–150 kDa, consistent with the size of a hyperphosphorylated CTD domain. Similarly, cells transfected with a construct containing a β -galactosidase-CTD fusion expressed a second B3-reactive species of 220 kDa, consistent with the size expected for the chimeric protein (lane 5); the 220-kDa band was not detected in cells transfected with β -galactosidase alone (lane 4). Blots containing the same samples were probed in parallel with the pol Ilo-reactive mAbs H5 and H14 with similar results (data not shown). These data strongly suggest that B3 recognizes a phosphopeptide located within the CTD of pol II LS.

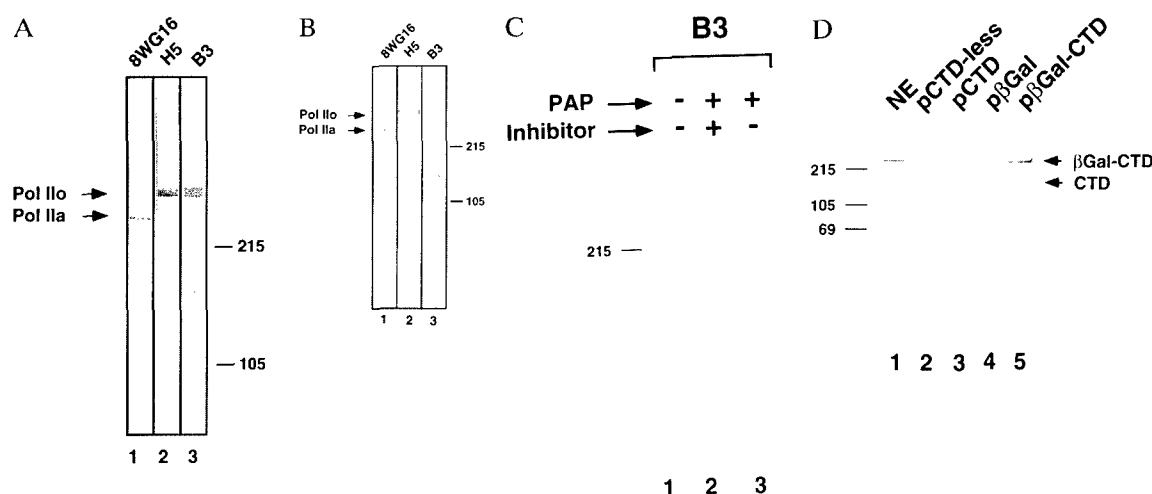


FIG. 4. B3 recognizes a phosphopeptide within the CTD of pol IiO. (A) B3-250 kDa comigrates with pol IiO, recognized by mAb H5, but not with pol IiA, recognized by mAb 8WG16. HeLa nuclear extract proteins (30 µg/lane) were separated in a SDS/6% polyacrylamide gel, transferred, and probed in separate strips with mAbs 8WG16 (lane 1), H5 (lane 2), and B3 (lane 3). (B) Recognition of purified pol IiO by B3. A fraction highly enriched for transcriptionally active polymerase II was separated (≈ 5 µg/lane) in a SDS/7% polyacrylamide gel, transferred, and probed with 8WG16 (lane 1), H5 (lane 2), and B3 (lane 3). (C) B3 recognizes a phosphopeptide on pol IiO. HeLa nuclear extract (100 µg) was pretreated with (lane 3) or without (lane 1) potato acid phosphatase (PAP), separated on a SDS/6% polyacrylamide gel, transferred, and probed with B3. Lane 2 shows 100 µg of a control HeLa extract pretreated with PAP in the presence of phosphatase inhibitors. (D) B3 phosphopeptide(s) are located within the CTD of pol IiO. CV-1 cells were transiently transfected with a control vector (pCTD-less, lane 2) or with vectors expressing intact human CTD (pCTD, lane 2), β -galactosidase (lane 4), and a β -galactosidase-CTD fusion (lane 5). Nuclei were purified from transfected cells and solubilized in SDS loading buffer. Protein from transfected cells was separated in a SDS/12.5% polyacrylamide gel in parallel with 100 µg of HeLa nuclear extract as a marker (lane 1), transferred, and probed with B3. β -Galactosidase-CTD and CTD polypeptides are indicated. Size markers in A-D are indicated in kDa.

Hyperphosphorylated Pol II-LS Is Associated with a Subset of Splicing Factors. The immunoprecipitation of splicing complexes by B3 suggests that pol IiO could be bound to splicing factors before complex formation. To address this possibility, immunoprecipitates prepared with mAbs specific for snRNP and non-snRNP splicing factors were probed with B3. Significantly, mAb Y12, which efficiently enriches the four splicing snRNPs, coimmunoprecipitates pol IiO (Fig. 5, lane 3). This was also observed in Y12 immunoprecipitates probed with H5 and H14 (data not shown). Similar results were obtained using a mAb specific for the snRNP-associated trimethyl-guanosine cap structure, although this mAb was not as efficient in snRNP immunoprecipitation and also showed a correspondingly lower signal for pol IiO (data not shown). Neither of these mAbs immunoprecipitated the pol IiA form recognized by 8WG16, suggesting that only the pol IiO form is associated with snRNPs (data not shown). A comparison of the level of pol IiO in total nuclear extract and the Y12 immunoprecipitate indicated that ≈ 25 -30% of B3-reactive pol IiO was bound to snRNPs. In a reciprocal experiment, B3 immunoprecipitated Sm antigens, as detected by mAb Y12 (data not shown). The enrichment of pol IiO by mAbs Y12 and trimethyl-

guanosine appeared specific, as essentially no pol IiO could be detected in immunoprecipitates prepared with a nonspecific IgM (lane 2).

Two monoclonal antibodies previously shown to recognize SR proteins, NM4 and NM22 (18), also immunoprecipitated pol IiO (lanes 6 and 7). NM4 and NM22 are highly specific for different phosphopeptides present in the defined set of six SR proteins (23) and additional SR-related proteins (18). NM22 is significantly more efficient in immunoprecipitation of SR proteins than NM4 (data not shown). Consistent with this, NM22 coimmunoprecipitated a higher level of B3-reactive pol IiO compared with NM4 (compare lanes 6 and 7), corresponding to ≈ 40 -50% of that present in the total extract (compare lanes 4 and 7). Immunoprecipitation of B3-reactive pol IiO by these mAbs appeared to occur through the recognition of SR proteins and not by direct binding to pol IiO, as neither NM22 nor NM4 were reactive with pol IiO in the preparation of purified polymerase (data not shown). These results indicate that a fraction of pol IiO is stably associated with snRNP and SR protein splicing factors, in the absence of exogenous pre-mRNA.

DISCUSSION

A pol IiO-reactive monoclonal antibody, B3, specifically immunoprecipitated splicing complexes assembled *in vitro* on pre-mRNA substrates. Several observations support B3 recognizing splicing complexes through binding to pol IiO. First, B3 is highly specific for a phosphopeptide within the CTD of pol IiO and does not recognize other nuclear antigens. Second, detection of pol IiO in immunoprecipitates of snRNP and SR protein splicing factors provides independent evidence for an association of pol IiO with splicing components and also suggests that its binding to splicing factors is not dependent on spliceosome formation. Experiments to further address the specificity of the pol II LS-splicing factor complex interactions demonstrated that only the pol IiO form recognized by B3, and not the pol IiA form recognized by 8WG16, binds to splicing complexes (data not shown).

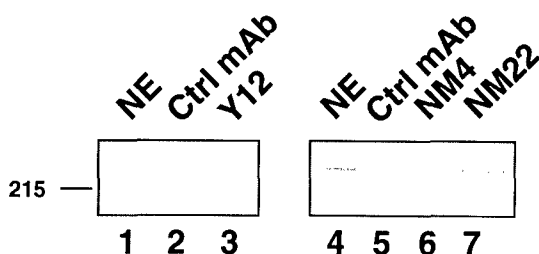


FIG. 5. Pol IiO is associated with snRNP and SR protein splicing factors. Immunoprecipitations were performed from 100 µg of HeLa nuclear extract with a control Ab (anti-actin, lanes 2 and 5), Y12 (lane 3), and SR-reactive mAbs NM4 (lane 6) and NM22 (lane 7). Total HeLa nuclear extract (25 µg) was loaded in lanes 1 and 4. Proteins were separated in a SDS/12% polyacrylamide gel and probed with B3 as in Fig. 4.

The interaction of pol IIO with splicing components is intriguing and may have implications for a functional connection between transcription and RNA processing. For example, it is tempting to speculate that pol IIO, which is correlated with transcription elongation (29, 30), may recruit splicing factors and influence the assembly of splicing complexes on nascent transcripts. This may also relate to previous observations indicating that changes in promoter structure and context can influence RNA processing (31, 32). This model is similar to a previous proposal (33) and is also supported by the recent identification of four novel proteins containing SR domains that interact with the CTD of pol II (34). These latter proteins could mediate an interaction between the CTD and splicing complexes. Consistent with this proposed interaction is the detection of pol IIO in immunoprecipitates of the SR-reactive mAbs NM22 and NM4, and also the partial enrichment of Pol IIO in the HS Sup fraction in this study. Moreover, the preferential binding of B3 to exon-containing splicing complexes is remarkably similar to that observed for mAbs to SR proteins, which could also reflect binding of pol IIO to splicing complexes through SR proteins.

The observation that a significant fraction of pol IIO is associated with snRNPs indicates that pol IIO could also bind to snRNPs in splicing complexes. However, unlike mAb Y12, B3 did not enrich for the lariat product complex of splicing, and therefore the interaction between pol IIO and snRNPs may be transient and/or occur early or before splicing complex formation. Consistent with this possibility, S.L.W. and colleagues have demonstrated that pol IIO is associated with snRNPs and SR proteins at stages of mitosis when transcription is not occurring (E. Kim, L.D., D. Bregman, and S.L.W., unpublished data). It is possible that a subset of SR proteins may mediate an interaction between pol IIO and snRNPs in nuclear extracts, as it has also been observed that mAbs Y12 and anti-trimethyl-guanosine coimmunoprecipitate a set of SR-related antigens from HeLa nuclear extracts (E. Kim, L.D., D. Bregman, and S.L.W., unpublished data; B.J.B. and P.A.S., unpublished observations).

The identification of the B3 NM antigen as pol IIO indicates that this form of polymerase is bound to the substructure of the nucleus. This observation is consistent with previous studies indicating that pol IIO LS and pol II transcriptional activity are associated with the NM, *in vitro* and *in situ* (refs. 35–38; R.B., unpublished observations). Accumulating evidence suggests that the majority of nuclear processes occur in association with the NM. Moreover, many of these functions occur within specialized domains that are maintained in the NM following *in situ* extraction of cells (reviewed in refs. 11, 16, and 39). This has led to the suggestion that specific matrix proteins may bridge different nuclear processes as a potential mechanism for the regulation of gene expression. The present study supports this notion and provides an example of how the identification of a matrix protein may lead to insights into the coupling of nuclear processes. Further investigation of the interaction between pol IIO and splicing factors may contribute insights into possible mechanisms by which transcription may influence pre-mRNA processing.

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Deputy Chief of Staff for
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