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A MECHANISM FOR GALECTIN-3 INCORPORATION INTO THE SPLICEOSOME THROUGH ITS ASSOCIATION WITH U1 SNRNP

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Abstract

Previously, we showed that galectin-1 and galectin-3 are redundant pre-mRNA splicing factors associated with the spliceosome throughout the splicing pathway. Here we present evidence for the association of galectin-3 with snRNPs outside of the spliceosome (i.e., in the absence of pre-mRNA splicing substrate). Immunoprecipitation of HeLa nuclear extract with anti-galectin-3 resulted in the co-precipitation of the five spliceosomal snRNAs, core Sm polypeptides as well as the U1-specific protein, U1-70K. When nuclear extract was fractionated on glycerol gradients, some galectin-3 molecules co-sedimented with snRNP complexes. This co-sedimentation represents *bona fide* galectin-3-snRNP complexes as (i) immunoprecipitation of gradient fractions with anti-galectin-3 yielded several complexes with varying ratios of snRNAs and associated proteins and (ii) the distribution of galectin-3-snRNP complexes was altered when the glycerol gradient was sedimented in the presence of lactose, a galectin ligand. A complex at approximately 10S showed an association of galectin-3 with U1 snRNP that was sensitive to treatment with ribonuclease A. We tested the ability of this U1 snRNP to recognize an exogenous pre-mRNA substrate. Under conditions that assemble early splicing complexes, we found this isolated galectin-3-U1 snRNP particle was sufficient to load galectin-3 onto a pre-mRNA substrate, but not onto a control RNA lacking splice sites. Pretreatment of the U1 snRNP with micrococcal nuclease abolished the assembly of galectin-3 onto this early complex. These data identify galectin-3 as a polypeptide associated with snRNPs in the absence of splicing substrate and describe a mechanism for the assembly of galectin-3 onto the forming spliceosome.

Pre-mRNA splicing involves nearly 300 proteins and five snRNAs[1] (1–5), assembled into a spliceosome that carries out the chemistry of intron removal and exon ligation. The canonical model for spliceosomal assembly involves the stepwise addition of the snRNPs into early, commitment and active complexes. U1 snRNP assembles onto the pre-mRNA at the 5' splice site in the absence of ATP. Addition of ATP allows U2 snRNP to recognize U2AF at the branch point and form a stable commitment complex. Finally the U4/U6.U5 tri-snRNP particle binds at the 3' splice site resulting in the active spliceosome (5,6). In addition, various protein cofactors are differentially incorporated into and disassemble from these complexes.

The galectin family of proteins consists of 15 members, characterized by binding affinity for β-galactosides (7). Using a cell free splicing assay, we have shown previously that galectin-1

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(Gal1) and galectin-3 (Gal3) are required and redundant splicing factors. The key findings include: (a) depletion of both galectins from HeLa nuclear extracts (NE) abolished splicing activity and blocked spliceosome formation at an early complex; (b) both splicing activity and spliceosome formation were restored by addition of recombinant Gal1 or Gal3; and (c) each galectin was a component of early and active splicing complexes as determined by galectin-specific co-immunoprecipitation of splicing substrate RNAs (8–10).

A key question is how galectins are assembled into early splicing complexes. We now report that in the absence of splicing substrate, Gal3 is associated with several snRNP particles and, in particular, the U1 snRNP under conditions of the *in vitro* splicing assay. We present evidence that one mechanism of Gal3 incorporation into the splicing pathway is mediated by its association with the U1 snRNP particle.

EXPERIMENTAL PROCEDURES

Antibodies

All experiments documented in the present manuscript were carried out with polyclonal antibodies against Gal3 derived from rabbit #49. When NE (prepared as described by Dignam et al, (11)) from HeLa cells was subjected to immunoblotting with this antiserum, a single polypeptide corresponding to Gal3 (~30 kD) was obtained. In some critical experiments (e.g. co-immunoprecipitation of U1 snRNA and U1 70K protein by anti-Gal3), the results obtained with rabbit #49 were checked using antisera from two other rabbits (#24 and #33). These antisera, whose characterization had been previously reported (12), yielded the same results as the antiserum from rabbit #49. For immunoprecipitation experiments, the antibodies were covalently cross-linked to protein A-Sepharose CL-4B beads (Amersham Biosciences) as previously described (9) using a 2:1 ratio of antiserum to protein A beads. A mouse monoclonal antibody against TMG was purchased as an agarose bead conjugate (Calbiochem; clone K121).

The following antibodies were used for immunoblotting. Rabbit anti-Gal3 (#49), rat monoclonal anti-Mac2 (13), or mouse monoclonal NCL-GAL3 (Vector Laboratories) were used for blotting Gal3 protein. Polyclonal rabbit antibodies directed against Gal1 had been previously characterized and described (10). The sources of commercially available antibodies were: affinity purified rabbit anti-PSF and goat anti-Slu7 from Santa Cruz Biotechnology; rabbit anti-TFII-I and rabbit anti-Gemin4 from Bethyl Laboratories; mouse monoclonal anti-SMN and anti-Ran from BD Transduction Laboratories; mouse monoclonal anti-U1 70K protein from Synaptic Systems; and human autoimmune sera ENA anti-Sm from The Binding Site.

[1]Abbreviations used:
snRNA: small nuclear RNA
snRNP: small nuclear ribonucleoprotein
Gal1: Galectin-1
Gal3: Galectin-3
NE: nuclear extract
TMG: tri-methyl guanosine
PI: pre-immune serum
rRNA: ribosomal RNA
SMN: survival of motor neurons
PSF: PTB-associated splicing factor
Lac: lactose
Cel: cellobiose
MN: micrococcal nuclease

Polyacrylamide gel electrophoresis, blotting analysis and immunoprecipitation

These were performed as described in ref. 10. For RNA analysis, the sample was extracted and precipitated. The precipitated RNA was subjected to electrophoresis through 13% polyacrylamide - 8.3 M urea gels. The radioactive RNA species were revealed by autoradiography. For northern analysis the RNA was transferred via wicking in 20X SSC (3 M NaCl, 0.3 M sodium citrate) overnight onto a nylon membrane (Hybond-N, Amersham Biosciences) and cross-linked by exposure to UV light (120 mJoules/cm^2). Glycerol gradient fractions 3 and 4, containing predominantly U1 snRNP, were incubated with antisera (anti-Gal3 or PI) and then were subjected to native gel electrophoresis as described by Lamond et al, (14). Following transfer to membranes, northern blotting for U1 snRNA was carried out as described.

The following oligonucleotides were synthesized at the Research Technology Support Facility at Michigan State University:

U1 (nucleotides 1–20): TCCCCTGCCAGGTAAGTATC,

U2 (109–134): TTAGCCAAAAGGCCGAGAAGCGAT,

U4 (65–85): GGGGTATTGGGAAAAGTTTTC,

U5 (8–32): GATTTATGCGATCTGAAGAGAAACC

U6 (30–48): TTCTCTGTATCGTTCCAAT,

5S (1–23): TTCAGGGTGGTATGGCCGTAGAC.

The nucleotide residue numbers to which the probes are complementary on the respective RNAs are indicated in parenthesis. The oligonucleotide probes were labeled with [γ-^{32}P] ATP (Amersham, 3000 Ci/mmol) using T4 polynucleotide kinase (Invitrogen). The cross-linked nylon membrane was pre-hybridized in hybridization solution (20% deionized formamide, 3X SSC, 5X Denhardt's solution, 50 mM Na_2HPO_4 / NaH_2PO_4, pH 6.8, 0.1% SDS and 0.1 mg/ml heat denatured herring sperm DNA) for about 2 hours at 25 °C. After pre-hybridization, the appropriate ^{32}P-labeled probes (~2 pmol) were added to the hybridization solution and the membrane was hybridized overnight (~16 hours) at 25 °C. The membrane was then washed once with 2X SSC + 0.1% SDS at 25 °C for 20 minutes. In these hybridizations all oligonucleotide probes were in excess and the same specific activity. Northern hybridizations were quantitated by phosphorimage analysis (Molecular Dynamics). The levels of U2 in the NE are low, but consistently obtained in multiple preparations of NE from HeLa cells obtained from the National Cell Culture Center (Minneapolis, MN).

Glycerol gradient

For glycerol gradient sedimentation, a 250 μl reaction containing 150 μl HeLa NE, 3 mM $MgCl_2$, 0.5 mM ATP and 20 mM creatine phosphate was incubated for 30 minutes at 30°C and loaded onto a 5 ml 12–32% glycerol gradient in 60% buffer D (minus glycerol). Buffer D is 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol. The gradient was centrifuged in a Beckman SW50.1 rotor at 44,000 rpm at 4°C for 3.5 to 4 hours. Gradient fractions were collected manually as 250 μl aliquots from the top. For RNA and protein analysis, 10% of each fraction was subjected to gel electrophoresis and analyzed for RNA or protein as described above. Size markers separated on parallel gradients include immunoglobulin G (7S), thyroglobulin (19S) and post-nuclear supernatant for ribosomal subunits (40S and 60S). Similar reactions incubated with 100 mM Lactose (Lac0 or 100 mM Cellobiose (Cel) were loaded onto gradients containing either 100 mM Lac or 100 mM Cel in 60% buffer D (minus glycerol). Recombinant Gal3 (rGal3; ~400 ng), purified from a bacterial expression system (12), was also subjected to glycerol gradient centrifugation as a control.

Gradient complexes

For immunoprecipitation experiments from gradient fractions, 200 µl of the indicated fractions were pooled and the total volume brought to 600 µl with 60% buffer D. The total reaction was incubated with antibody coupled beads, washed and eluted with SDS sample buffer. Bound material was analyzed for protein and RNA as described above.

To test RNase sensitivity of gradient complexes, 50 µl of the indicated pooled fractions were treated with 2 µg RNase A for 30 minutes at 30°C, and then immunoprecipitated with anti-Gal3.

To test the nuclease sensitivity of the association of Gal3 with pre-mRNA substrate, the indicated pooled fractions were subjected to digestion by micrococcal nuclease (MN). Each reaction included 45 µl of pooled fractions 3 and 4, 3 mM $MgCl_2$, 4 mM $CaCl_2$ and 4000 gel units of MN (New England Biolabs). Control reactions contained EGTA-inactivated MN. Reactions were incubated at 37°C for 30 minutes. MN activity was stopped by adding EGTA to a final concentration of 8 mM. After stopping the digestion reaction, ^{32}P-labeled MINX pre-mRNA was added to each tube, incubated for 15 minutes at 30°C followed by addition of 15 µl antibody-coupled beads. Bound MINX pre-mRNA was eluted with SDS sample buffer and detected by subjecting an aliquot of the bound material to electrophoresis and quantitated by phosphorimage analysis. An identical protocol was used for SP65 immunoprecipitation. MINX pre-mRNA has been described previously and the plasmid containing this construct was a gift from Dr. Susan Berget (Baylor College of Medicine, Houston, TX) (15). The plasmid containing SP65 has been described previously (16,17). The MINX pre-mRNA and SP65 RNA were labeled with [α-^{32}P] GTP (Amersham; 3000 Ci/mmol) and a monomethyl cap added during *in vitro* transcription by SP6 RNA polymerase (18).

RESULTS

Galectin-3 is associated with snRNPs in the absence of splicing substrate

NE was incubated *without* splicing substrate at 30 °C with ATP for 30 minutes to disassemble endogenous splicing complexes (19) and then subjected to immunoprecipitation with anti-Gal3. Northern hybridization revealed that anti-Gal3 co-precipitated a significant proportion of U1, U2, U4, U5 and U6 snRNAs whereas pre-immune serum did not (Figure 1A). In contrast, 5S rRNA, a prominent RNA species in NE, was not observed in the anti-Gal3 precipitate (i.e., less than 0.1% of nuclear 5S rRNA). These results suggest that the association of Gal3 with a fraction of nuclear snRNAs was specific.

A similar conclusion regarding the specificity of the association of Gal3 with snRNPs was obtained through analysis of protein components (Fig. 1B). In addition to its cognate antigen, anti-Gal3 co-precipitated the Sm core polypeptides B/B' and D of snRNPs and the 70K protein specific to U1. Furthermore, the precipitate contained several proteins identified in snRNP complexes; the survival of motor neurons (SMN) protein and two factors that have recently been found in complexes containing Gal3: (a) the polypyrimidine tract binding protein associated splicing factor PSF (20) and (b) the general transcription factor TFII-I (21). In contrast, both Ran (a nuclear transport factor) and Slu7 (a second-step splicing factor) were not detected in the anti-Gal3 precipitate. Finally, the anti-Gal3 precipitate did not contain Gal1, a finding consistent with the mutually exclusive association of either galectin with spliceosomes (10).

Galectin-3 is associated with multiple snRNP complexes

Multiple nuclear snRNP complexes exist including the U4/U6 di-snRNP and the U4/U6.U5 tri-snRNP as well as the mono-snRNPs U1 and U2. Recently, complexes containing all five

snRNPs in the absence of splicing substrate have been described (20,22–24). We wanted to determine whether Gal3 was associated with multi-snRNP complexes as well as mono-snRNP complexes. To test this, pre-incubated NE was fractionated by glycerol gradient sedimentation and the distribution of snRNAs and several RNA processing proteins was determined. Blotting for U1 snRNA (Fig. 2A) the U1–70K protein (Fig. 2B) and the snRNP core polypeptides Sm B/B' and D (Fig. 2B) indicates that fractions 3–5 contain the mono U1 snRNP predominantly, consistent with previous findings that U1 snRNP sediments at about 10S (25). Fractions at higher molecular weights (~19S and greater) contained various combinations of the snRNAs (Fig. 2A).

Individual or pooled fractions throughout the gradient were immunoprecipitated with anti-Gal3 to determine the distribution and snRNA and protein composition of Gal3-containing complexes (Fig. 3). The anti-Gal3 precipitate of pooled fractions 3 and 4 yielded a single snRNA, U1, and associated proteins Sm B/B' and U1–70K (Fig. 3A and B, lane 2). This suggests that some of the Gal3 in fractions 3 and 4 was associated with the mono U1 snRNP. On the other hand, the absence of U1–70K protein (Fig. 2B) and the miniscule amount of U1 snRNA (Fig. 2A) in fraction 1 both indicate that a portion of Gal3 is not associated with a snRNP complex. Consistent with this notion, fraction 1 yields no snRNAs or other proteins in the anti-Gal3 precipitate (Fig. 3A and B, lane 1). The amount of Gal3 in fractions 1 and 2 accounted for 33% of the total Gal3 in NE sedimented on glycerol gradients (Fig. 2B). On this basis, we estimate that about one third of the Gal3 in NE fractionates as free protein. In contrast, when purified rGal3 was sedimented on a parallel gradient, ~90% of the protein was recovered in fractions 1 and 2 (Fig. 2C), consistent with the predicted hydrodynamic behavior of a ~30 kD polypeptide. It appears, therefore, that approximately 70% of the Gal3 in NE is complexed in high molecular weight particles.

The anti-Gal3 precipitate of fractions 6 and 7 (~19S), fractions 9–11, fractions 13 and 14 (>40S) and fractions 16–18 (~60S) yielded multiple snRNAs in various proportions along with Sm B/B', U1–70K and Gal3, the cognate antigen (Fig. 3A and B). In a subsequent northern blot, the 5S rRNA was not detected in the anti-Gal3 precipitated material from any fraction (data not shown). In high molecular weight fractions (fractions 10 and beyond), we found the SMN protein was co-precipitated by anti-Gal3 (Fig. 3B). Although these precipitates also contain various snRNAs, we do not know whether all three components are in the same complex or whether these exist in separate complexes.

Based on three independent immunoprecipitations in which Gal3 was quantitatively precipitated from pooled fractions 3 and 4, 12.8 ± 3.5% of U1 in these fractions were associated with Gal3. In contrast, pre-immune serum precipitated only 2.6 ± 0.7 % of U1. This determination was made by calculating the quantity of U1 precipitated by anti-Gal3 antibody (total radioactive U1 oligonucleotide hybridized to the antibody bound fraction) divided by the total quantity of U1 in fractions 3 and 4 (total radioactive U1 oligonucleotide hybridized to the amount of fractions 3 and 4 used for the antibody precipitation). Using similar calculations, we estimate that 40 – 50% of U2, U4, U5 and U6 were precipitated by Gal3 antiserum from large complexes (40–60S).

Gal3 is associated with the mono U1 snRNP

The finding that anti-Gal3 immunoprecipitated the U1 snRNP from fractions 3 and 4 prompted us to investigate further this interaction. Four observations indicate the Gal3-U1 snRNP association is specific. First, comparison between immunoprecipitations of pooled fractions 3 and 4 with anti-Gal3 and pre-immune serum showed that none of the U1-associated proteins were precipitated by the control antibodies (Fig. 4A, lanes 2 and 3). Second, the association of Gal3 with U1 snRNP is dependent on the integrity of the U1 snRNA, as co-precipitation of the U1–70K protein by anti-Gal3 is abolished following RNase A digestion of U1 snRNA (Fig.

4B, compare lane 3 to lane 2). As expected, the immunoprecipitation of Gal3 itself was unaffected by RNase A treatment (data not shown). A possible explanation for the association of Gal3 with RNPs is that Gal3 binds RNA directly. We previously demonstrated using a gel mobility shift assay that Gal3 does not bind a pre-mRNA substrate directly (10). Using the same assay, addition of rGal3 to *in vitro* transcribed U1 snRNA did not shift the mobility of U1 snRNA (data not shown) suggesting that protein-protein interactions are responsible for the association of Gal3 with U1 snRNP. When fractions 3 and 4 were incubated with anti-Gal3, a small portion of the U1 snRNP complexes was super-shifted (Fig. 4D, lane 1, arrow). Pre-incubation of the anti-Gal3 serum with purified rGal3 abolished the super-shifted band. Incubation of fractions 3 and 4 with PI had no effect on the mobility of U1 snRNP complexes (Fig. 4D, lane 3).

Third, a reciprocal immunoselection of snRNPs using antiserum specific for the tri-methyl guanosine (TMG) cap of snRNAs co-precipitated Gal3 (Fig. 4C, lane 2). As expected, this procedure precipitated U1 snRNA (Figure 4C, lane 2.). The bound material from control beads contained neither Gal3, Sm proteins nor U1 snRNA (Figs. 4C and 4D, lane 1). Finally, the co-immunoprecipitation of Gal3 in fractions 3 and 4 by anti-TMG is decreased in the presence of 0.1 M Lac while cellobiose (Cel) failed to yield this effect (Fig. 4C, compare lane 2 to lane 3 for the Gal3 blot). These results indicate that some portion of U1 snRNP is specifically associated with Gal3 which can be disrupted when Gal3 binds Lac. Consistent with this notion, a striking difference was noted when the distribution of selected proteins and snRNAs for a gradient sedimented in the presence of Lac was compared to the corresponding data for a gradient run in Cel. U1 snRNA and some proteins showed a shift toward the top or lower molecular weight in the Lac gradient relative to the Cel gradient (Fig. 5). For example, in the short exposure for Gal3 (Fig. 5A), the band for Gal3 is clearly seen through fraction 8 in the Cel gradient; in the Lac gradient, however, the Gal3 band becomes quite weak in fractions 6 and 7 and there is an apparent accumulation of Gal3 in fraction 1 (corresponding to free Gal3). The effect of Lac on the shift of Gal3 distribution toward the top (lower molecular weight) and accumulation in fraction 1 was also apparent in the longer exposure of the Gal3 western blots (Fig. 5B). These data suggest that binding of the saccharide ligand Lac to Gal3 dissociates it from the Gal3-snRNP complexes. Supporting this suggestion, U1–70K, Sm B/B', and U1 snRNA (Fig. 5C, 5D, 5E, respectively) show a similar shift to a lower sedimentation value.

U1 snRNP mediates the loading of Gal3 onto pre-mRNA

The binding of U1 snRNP to the pre-mRNA substrate at the 5' splice site results in the formation of the early (E) complex in spliceosome assembly (15,26,27). We had previously documented that Gal3 is associated with E-complexes (10). Thus, our present findings that at least some Gal3 is bound to mono-U1 snRNP prompted the questions whether (i) Gal3-containing U1 snRNPs in fractions 3 and 4 could bind to ^{32}P-labeled pre-mRNA substrate under conditions that lead to E-complex formation and (ii) this interaction was U1 snRNA dependent. To test for U1 snRNA dependence, micrococcal nuclease (MN) was used to degrade RNA in fractions 3 and 4 and then subsequently inhibited by the addition of EGTA. However, a previous report detailed the possibility of "substrate masking" (e.g., binding of EGTA-inactivated MN to the target RNA) during MN-mediated degradation of RNA (28). To control for this "masking" possibility, EGTA-inactivated MN was incubated with fractions 3 and 4. After incubation of the fractions with MN, addition of MINX substrate followed by anti-Gal3 precipitation showed that the MINX substrate was significantly co-precipitated (Fig. 6A, lane 6) compared to the amount precipitated by pre-immune serum (Fig. 6A, lane 4). That this interaction was U1 snRNA dependent is shown in lane 5, in which fractions 3 and 4 were treated with MN prior to incubation with MINX. After nuclease digestion, the level of MINX precipitated by anti-Gal3 was reduced to levels bound to pre-immune serum. Residual MN activity did not degrade the substrate to account for the decreased precipitation of the MINX substrate as approximately

equal amounts of MINX were used for the anti-Gal3 precipitation (Fig. 6A, lanes 2 and 3). U1 snRNA in fractions 3 and 4 was indeed degraded by the MN treatment (Fig. 6B, compare lanes 1 and 2). U1 snRNA was detected in the anti-Gal3 precipitate from the EGTA-inactivated MN reaction (Fig. 6B, lane 6), but not in the anti-Gal3 precipitate from the MN-digested sample or the pre-immune control precipitate (Fig. 6B, lane 5). The U1–70K and core Sm B/B' proteins also were detected in the anti-Gal3 precipitate of the EGTA-inactivated MN treated sample (Fig. 6C, lane 6) but were absent in the U1 snRNA degraded reaction (Fig. 6C, lane 5) and the PI precipitate (Fig. 6C, lane 4). Finally, the bound material showed no significant change in precipitation of Gal3 after nuclease treatment (Fig. 6C, lanes 5 and 6).

We next tested for a requirement for splice site recognition in the association of pre-mRNA with the isolated U1-Gal3 complex. Zillman et al. (1987) used SP65 RNA, which lacks consensus splice sites, to document the initial recognition of the pre-mRNA 5' splice site by U1 snRNP. In the presence of fractions 3 and 4, antibodies against Gal3 immunoprecipitated the MINX pre-mRNA (Fig. 6D, lane 4), but not the SP65 substrate (Fig. 6D, lane 5). These results can not be explained by differences in the immunoprecipitation reactions as anti-Gal3 co-precipitated similar amounts of U1 snRNA from samples containing either substrate (Fig. 6E, compare lanes 4 and 5). We suggest that Gal3 is first assembled onto the pre-mRNA substrate through its interaction with the U1 snRNP particle.

DISCUSSION

The most important conclusion derived from the present series of studies is that the U1 snRNP can mediate the loading of Gal3 onto the pre-mRNA substrate during spliceosome assembly. Previous experiments had documented that Gal1 and Gal3 are factors involved in pre-mRNA splicing assayed in a cell-free system (8,9). Depletion of the galectins resulted in an arrest of spliceosome assembly at an early step, corresponding to the H-/E-complex. Addition of saccharide ligands specific to galections also inhibited the *in vitro* splicing assay. Given that neither Gal1 nor Gal3 interacts directly with pre-mRNA (10), it was important to define how and at what step either protein is brought into the spliceosome. Here we have demonstrated that U1 is responsible for loading Gal3 onto a pre-mRNA at an early stage in the splicing pathway. This association of U1 snRNP with Gal3 also exists outside of the spliceosome, i.e. in HeLa NE without a pre-mRNA scaffold. In addition to its association with U1 snRNP, we have found that Gal3 exists in larger complexes containing multiple snRNAs and other RNA processing factors. Thus, it appears that Gal3 is a member of a novel class of snRNP-associated proteins (see 20 and later discussion).

The conclusion that U1 snRNP facilitates the loading of Gal3 onto the pre-mRNA scaffold is based on several key considerations. First, Gal3 is associated with multiple snRNP complexes in the absence of splicing substrate as deduced from immunoprecipitation analysis of glycerol gradient fractions derived from NE. In particular, the position of sedimentation (~10S) and the RNA and protein composition of fractions 3 and 4 indicate these fractions contain, predominantly, the mono U1 snRNP. Importantly, the anti-Gal3 precipitate of fractions 3 and 4 contained U1 snRNA and the U1-specific polypeptide U1–70K, suggesting that some of Gal3 in these fractions was associated with the mono U1 snRNP. Conversely, a significant fraction of Gal3 remains free in the nucleus. It will be of interest to determine what regulates the proportion of Gal3 associated with the U1 snRNP.

Second, we have taken advantage of the initial ATP-independent recognition of the 5' splice site by U1 snRNP (6,13) to devise a functional test of the Gal3-containing U1 snRNP complex in fractions 3 and 4. Under conditions of E-complex formation (30° C in the absence of ATP), incubation of splicing substrate with fractions 3 and 4 should result in the association of Gal3 with the pre-mRNA. Indeed, we were able to demonstrate this by immunoprecipitating

fractions 3 and 4 with antibodies against Gal3 and detecting radioactive MINX pre-mRNA as well as U1 snRNA. Degradation of U1 snRNA in fractions 3 and 4 prior to substrate addition prevented the assembly of Gal3 onto the splicing substrate as did using an RNA species lacking consensus splice sites. Thus, incorporation of Gal3 from fractions 3 and 4 onto a forming spliceosome required intact U1 snRNA and a 5' splice site.

A key caveat to our conclusion is that previous studies on the protein composition of purified U1 snRNP all describe the same core Sm proteins and the U1-specific polypeptides. The SDS gels of these studies (29–31) did not reveal any band (~30 kD) that might correspond to Gal3. We note that U1 snRNP purification protocols used buffers of high ionic strength (150 mM NaCl or 175 mM NH_4Cl) which release galectins from spliceosomal complexes (10). In the context of the present study, we also have observed that the co-precipitation of snRNAs by anti-Gal3 was sensitive to disruption by high ionic strength (K.C. Haudek and R.J. Patterson, unpublished observations).

Peng et al. (20) recently reported the isolation of a macromolecular complex that assembles in the absence of pre-mRNA substrate and contains all five snRNAs. Mass spectrometry analysis of this complex, designated as the PCC (PSF-containing complex), revealed a protein composition similar to, but distinct from, the composition of fully assembled active spliceosomes. Gal3 was found as a component of the PCC (20). Co-precipitation of all five snRNAs by anti-Gal3, either from complete NE or from selected gradient fractions, raises the possibility that we also have isolated a mammalian penta-snRNP (24), possibly similar if not identical to the PCC. Multiple components (PSF, TFII-I, SMN) identified in the PCC were detected with Gal3 in the anti-Gal3 precipitate. Together with the discovery of a functional, preassembled penta-snRNP in yeast (22), these results suggest that, under certain conditions, spliceosome assembly may be facilitated through the association of large ribonucleoprotein complexes that are already preformed in the absence of a pre-mRNA scaffold. In addition, the identification of novel proteins complexed with snRNAs under splicing conditions, characterized in this report and previously (20,22), raises the possibility that the network of snRNA-interacting proteins outside of the spliceosome contains more members than originally described. That this set of new snRNA-interacting proteins is functionally significant can be inferred from our observation that at least one such protein (Gal3) can be specifically dissociated from U1 snRNP by a ligand that binds to Gal3 (Lac) but not by Cel. Further, including Lac in glycerol gradients alters the mobility of U1 snRNP and several of the proteins associated with snRNPs.

U1 snRNP-Gal3 complexes are the focus of our present study because: (a) in the canonical model of spliceosome assembly, the first event is recognition of the 5'splice site via base-pairing of U1 snRNA with the pre-mRNA substrate; and (b) spliceosome assembly can be arrested after U1 binds the substrate as the next step is ATP-dependent; thus, we can assay whether Gal3 is loaded onto the pre-mRNA an early step. This is important because we had previously documented that spliceosome assembly is arrested at this initial step in nuclear extracts depleted of galectins (8). The results documented in the present report are consistent with our earlier studies. Finally, we have found that a significant portion of the snRNAs in large complexes is associated with Gal3. Although we have been able to demonstrate a function for the Gal3-U1 snRNP complex in terms of 5' splice site recognition and E-complex formation, this does not preclude the entry of Gal3 into the spliceosome through its association with other snRNPs. The role(s) of the higher molecular weight multi-snRNP complexes containing Gal3 remains as a challenge for future studies.

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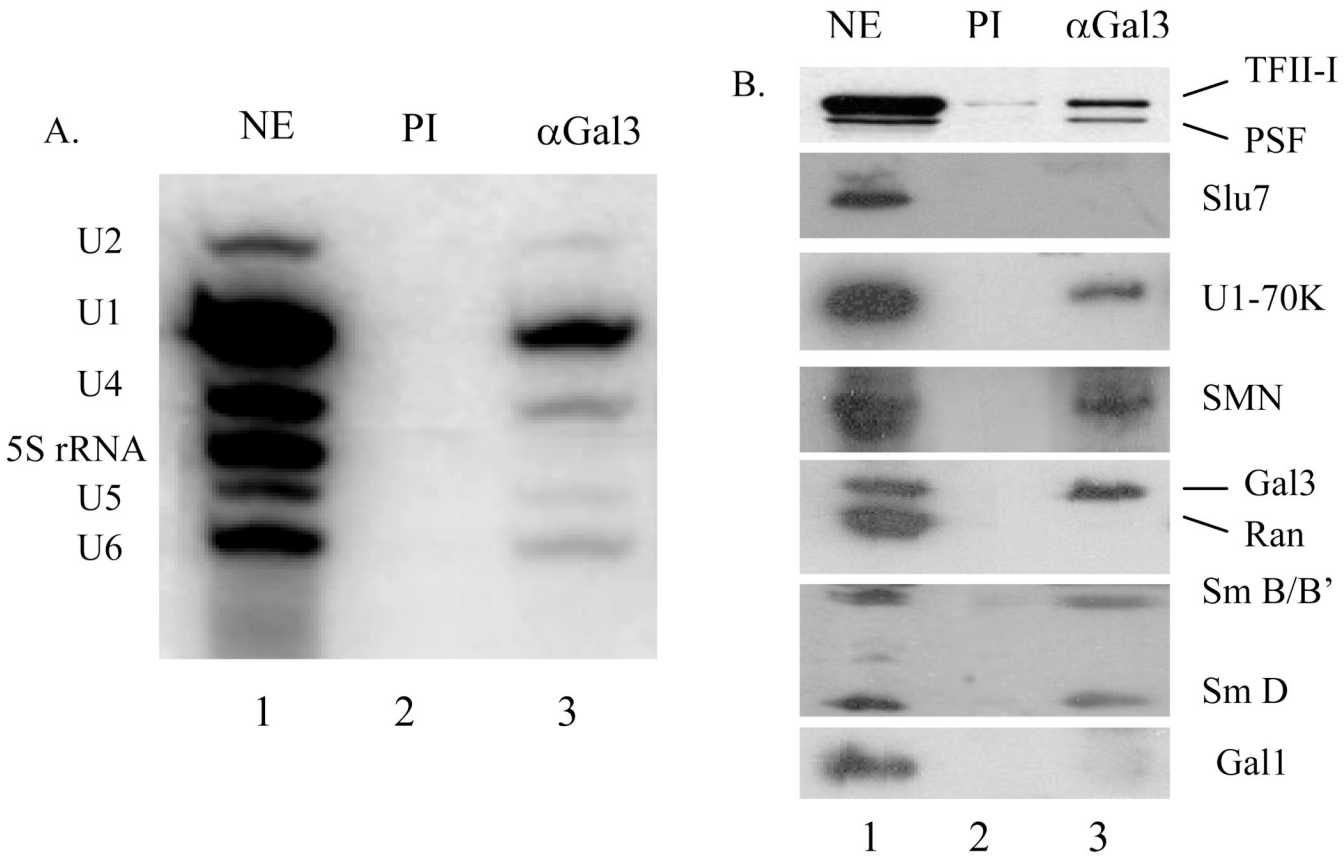

FIGURE 1.

Analysis of RNA and proteins present in NE immunoprecipitated by anti-Gal3. NE was pre-incubated as described in Experimental Procedures. The reaction was then incubated with beads coupled with anti-Gal3 (αGal3) or PI serum (PI). Panel A shows a northern blot of 25% of the bound material probed with ^{32}P-labeled oligonucleotides complementary to the RNA species indicated on the left. Lane 1: NE represents 12% of the amount subjected to immunoprecipitation; lane 2: RNA species bound by PI; lane 3: RNA species bound by anti-Gal3. Panel B shows western blots of the bound material. Antibodies were used to detect the proteins indicated on the right.

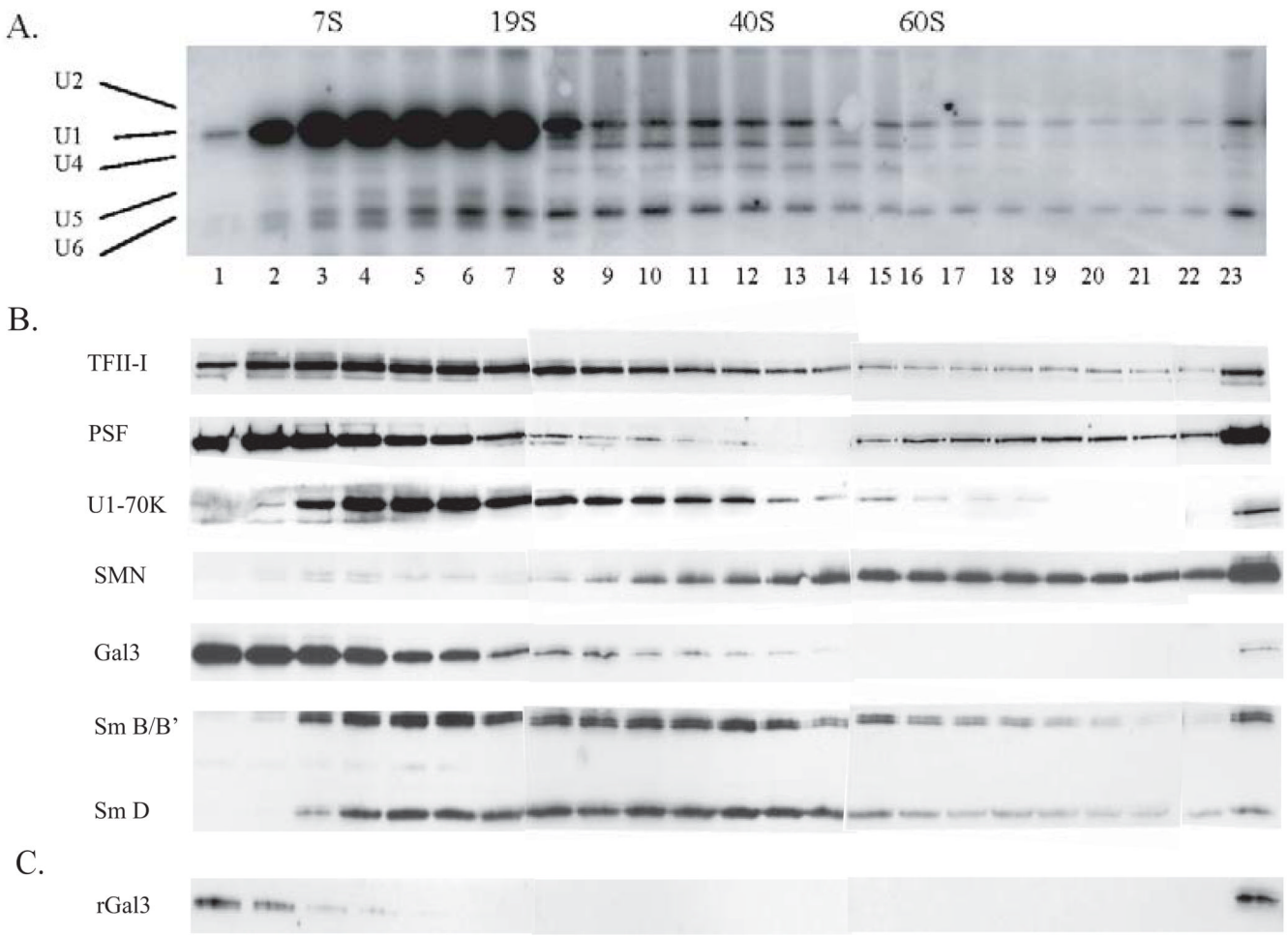

FIGURE 2.

Analysis of nuclear RNA and proteins separated on a 12–32% glycerol gradient. In panels A and B, NE was treated and then loaded onto gradients as described in Experimental Procedures. Panel A shows a northern blot of each fraction using ^{32}P-labeled oligonucleotides specific for each snRNA. Size markers run in parallel are indicated at the top. Panel B shows western blots of each fraction for the proteins indicated at left. In panel C, recombinant Gal3 (rGal3) was sedimented on a parallel glycerol gradient and analyzed by western blotting.

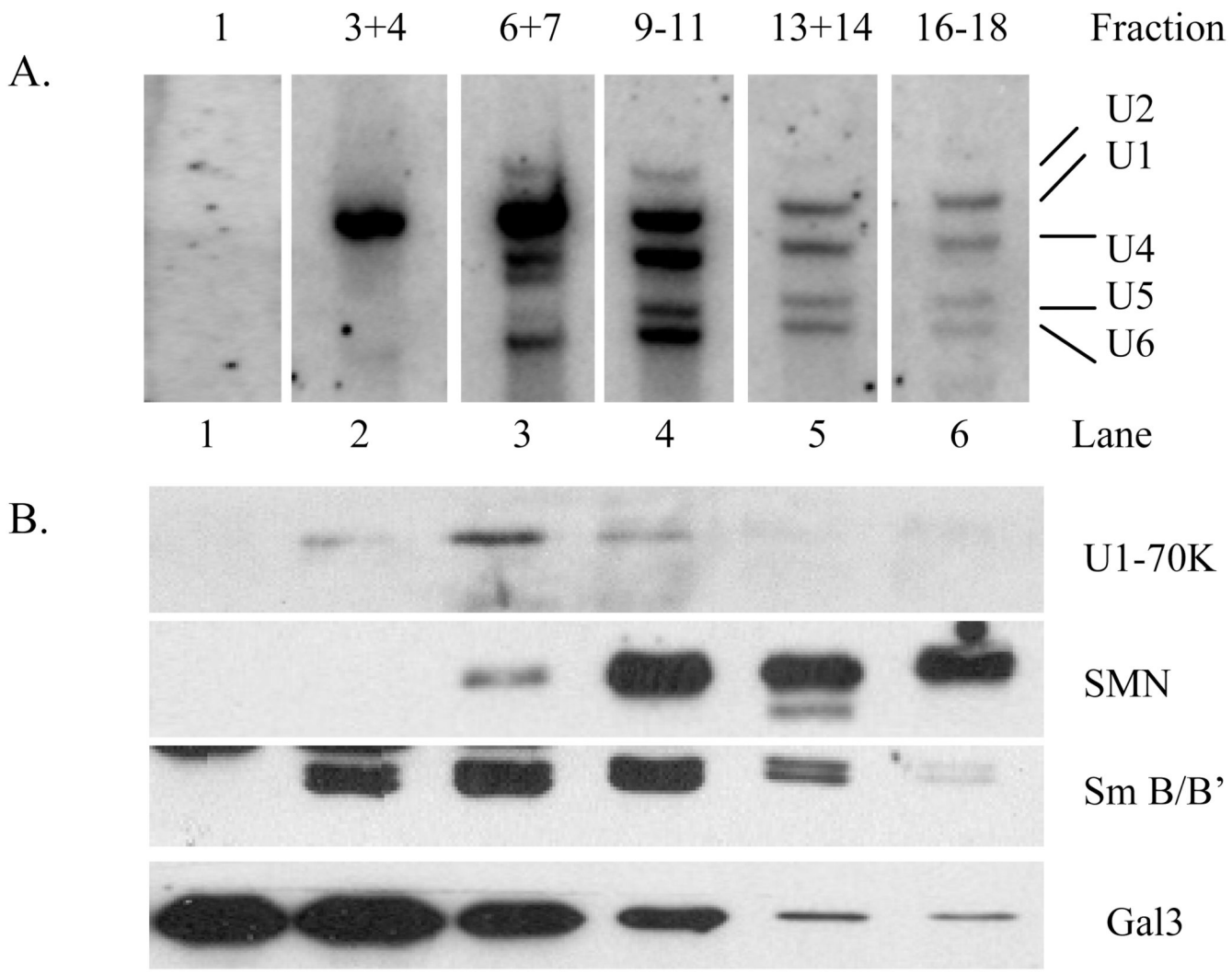

FIGURE 3.

Analysis of RNA and proteins immunoprecipitated by anti-Gal3 from glycerol gradient fractions. Indicated pooled fractions from glycerol gradient fractionation of NE (see Fig. 2) were subjected to immunoprecipitation by anti-Gal3 coupled beads. Panel A shows a northern blot of the bound material (~50%) of the anti-Gal3 beads from each immunoprecipitation. ^{32}P-labeled oligonucleotides specific for each snRNA were used to detect the RNA species indicated at right. Panel B shows a western blot for proteins immunoprecipitated from the indicated fractions by anti-Gal3 beads. Material subjected to western blotting represents 50% of the bound fraction and each lane corresponds to the fraction (s) indicated in Panel A. Respective antibodies against the proteins are indicated at right.

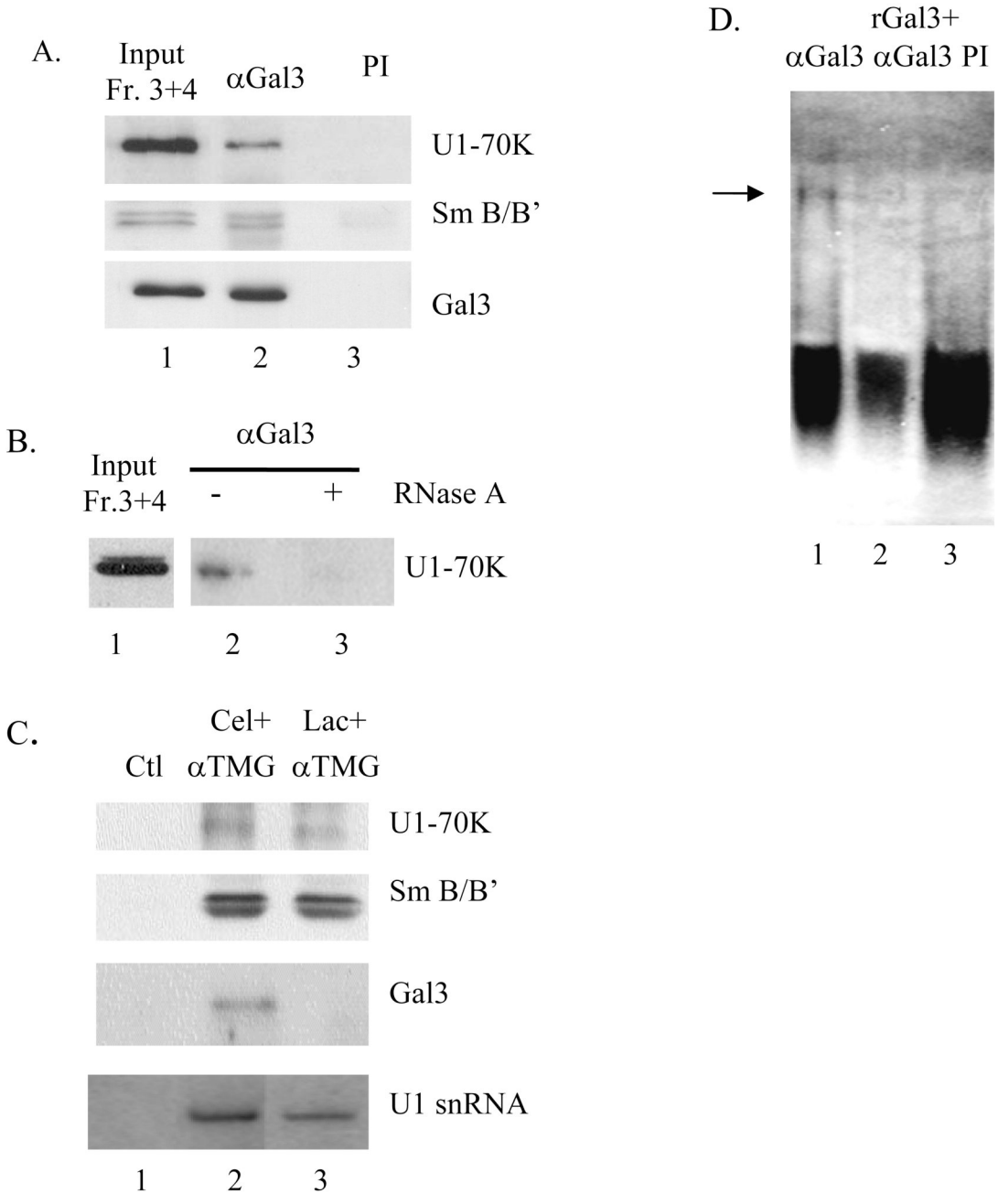

FIGURE 4.

Characterization of the association of Gal3 with U1 snRNP. Panel A shows fractions 3 and 4 of the glycerol gradient (see Fig. 2) subjected to immunoprecipitation by anti-Gal3 (αGal3, lane 2) or by PI serum (PI, lane 3). The bound material (~25%) was blotted for the proteins indicated at right. Input represents 10% of the material subjected to immunoprecipitation. Panel B shows the results of fractions 3 and 4 treated with RNase A or a mock control. Anti-Gal3-bound material from the mock treated (lane 2) or RNAse treated (lane 3) fractions was western blotted for the U1-70K protein. Panel C shows western blots of the bound material from anti-TMG beads incubated with fractions 3 and 4 in the presence of Lac (lane 3) or Cel (lane 2). Bound material from control beads lacking antibody (Ctl, lane 1) or anti-TMG beads (αTMG,

lanes 2 and 3) was blotted for the proteins indicated at right as well as for U1 snRNA. Panel D is a northern blot of a native gel of the U1 snRNP found in fractions 3 and 4 following incubation with αGal3 or PI serum. Lane 1 shows incubation of fractions 3 and 4 with αGal3 while lane 2 shows pre-treatment of the αGal3 with rGal3. Incubation of fractions 3 and 4 with PI is shown in lane 3. Arrow at left indicates anti-Gal3 super-shifted band.

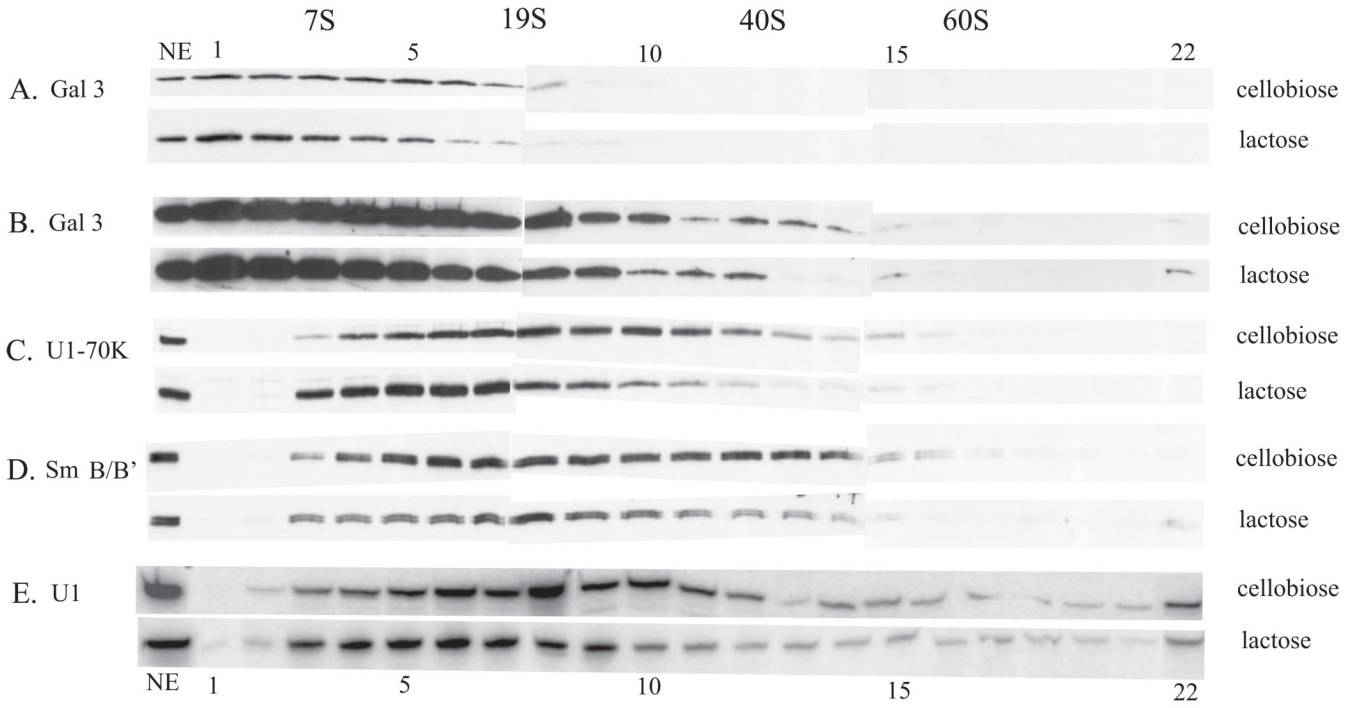

FIGURE 5.

Analysis of nuclear RNA and proteins separated on a 12–32% glycerol gradient containing Lac or Cel (indicated at right). NE was treated and then loaded onto gradients containing either 100 mM Lac or 100 mM Cel as described in Experimental Procedures. Panels A – D show western blots of 10% of each fraction for the proteins indicated at left. For Gal3, panel A is a short exposure and panel B is a longer exposure. Panel E shows a northern blot of 10% of each fraction using ^{32}P-labeled oligonucleotides specific for U1 snRNA. Size markers run in parallel are indicated at the top. The first lane of each panel is a NE sample.

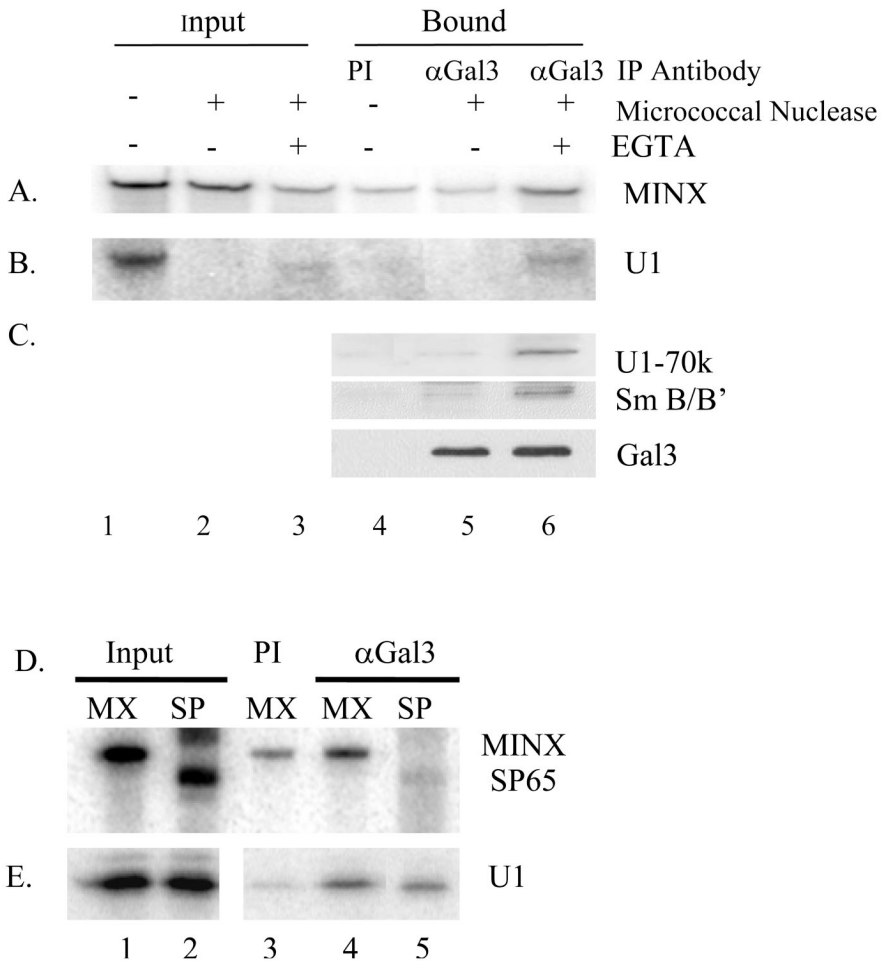

FIGURE 6.

Immunoprecipitation by anti-Gal3 of ^{32}P-labeled pre-mRNA after incubation with glycerol gradient fractions 3 and 4. The pooled fractions were treated with micrococcal nuclease (αGal3) or mock treated (PI). Nuclease activity was stopped by addition of EGTA either prior to incubation with fractions 3 and 4 (+EGTA) or after incubation with fractions 3 and 4 (-EGTA). ^{32}P-labeled MINX pre-mRNA was then added for 15 minutes at 30°C. The reactions were incubated with beads coupled with either αGal3 or PI serum. Panel A shows MINX pre-mRNA detected by autoradiography. Panel B shows a northern blot for U1 snRNA. Panel C shows western blots for the indicated proteins. Input represents 10% of the fraction material and ^{32}P-MINX subjected to immunoprecipitation in reactions either mock treated (lane 1) or treated with micrococcal nuclease (lanes 2 and 3). Bound material (50% panels A and B; 50% panel C) from immunoprecipitation of mock-treated fractions by PI serum (lane 4) and by micrococcal nuclease treated fractions by anti-Gal3 (lanes 5 and 6) is shown. Panels D and E. Immunoprecipitation by anti-Gal3 of ^{32}P-labeled pre-mRNAs containing either consensus splice sites (MINX/MX) or no splice sites (SP65/SP). Pooled fractions 3 and 4 were incubated with ^{32}P-labeled RNA and then with beads coupled to either anti-Gal3 (αGal3) or PI serum (PI). Panel D shows substrate RNAs by autoradiography. Panel E shows a northern blot for U1 snRNA. Inputs for panel D represent 6% of the reaction subjected to immunoprecipitation and the bound material represents 12% of the recovered material. For panel E, inputs represent 12% of the material subjected to immunoprecipitation and 25% of the bound material.