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Evidence for a Role for Galectin-1 in Pre-mRNA Splicing

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Galectins are a family of β-galactoside-binding proteins that contain characteristic amino acid sequences in the carbohydrate recognition domain (CRD) of the polypeptide. The polypeptide of galectin-1 contains a single domain, the CRD. The polypeptide of galectin-3 has two domains, a carboxyl-terminal CRD fused onto a proline- and glycine-rich amino-terminal domain. In previous studies, we showed that galectin-3 is a required factor in the splicing of nuclear pre-mRNA, assayed in a cell-free system. We now document that (i) nuclear extracts derived from HeLa cells contain both galectins-1 and -3; (ii) depletion of both galectins from the nuclear extract either by lactose affinity adsorption or by double-antibody adsorption results in a concomitant loss of splicing activity; (iii) depletion of either galectin-1 or galectin-3 by specific antibody adsorption fails to remove all of the splicing activity, and the residual splicing activity is still saccharide inhibitable; (iv) either galectin-1 or galectin-3 alone is sufficient to reconstitute, at least partially, the splicing activity of nuclear extracts depleted of both galectins; and (v) although the carbohydrate recognition domain of galectin-3 (or galectin-1) is sufficient to restore splicing activity to a galectin-depleted nuclear extract, the concentration required for reconstitution is greater than that of the full-length galectin-3 polypeptide. Consistent with these functional results, double-immunofluorescence analyses show that within the nucleus, galectin-3 colocalizes with the speckled structures observed with splicing factor SC35. Similar results are also obtained with galectin-1, although in this case, there are areas of galectin-1 devoid of SC35 and vice versa. Thus, nuclear galectins exhibit functional redundancy in their splicing activity and partition, at least partially, in the nucleoplasm with another known splicing factor.

Galectins are a family of widely distributed proteins that (i) bind to β-galactosides and (ii) contain characteristic amino acid sequences in the carbohydrate recognition domain (CRD) of the polypeptide. At present, eight mammalian galectins have been reported and classified into three subgroups, according to the content and organization of the domains in their respective polypeptides (for reviews, see references 3 and 21). The prototype subgroup consists of polypeptides (~14 kDa) with a single domain, the CRD. Galectins-1, -2, -5, and -7 are members of this subgroup. Another subgroup is the tandem repeat type, which has three members: galectins-4, -6, and -8. These galectins have two domains, each a CRD, connected by a linker region. Finally, the chimera subgroup is, at present, represented by a single member, galectin-3. Its polypeptide contains two domains, a CRD fused onto a Pro-, Gly-rich domain.

In previous studies, we reported the localization of galectin-3 to the cell nucleus in the form of a ribonucleoprotein (RNP) complex (23, 29). We also identified it as one of the many proteins required for the splicing of pre-mRNA, assayed in a cell-free system. This conclusion was based on several key findings (12). (i) Nuclear extracts (NEs) derived from HeLa cells, capable of carrying out splicing, contain galectin-3. (ii) NEs depleted of galectin-3 by affinity adsorption on lactose (Lac)-agarose are deficient in splicing activity and spliceosome formation. (iii) The splicing activity and spliceosomal complex formation of the Lac-agarose-depleted extract are reconstituted by the addition of purified recombinant murine galectin-3 (rG3). (iv) Saccharides that bind galectin-3 with high affinity inhibit the cell-free splicing reaction. These results strongly suggest that the lectin is a required factor in splicing of pre-mRNA.

When we attempted to deplete the splicing activity by using a galectin-3-specific monoclonal antibody, the anti-Mac-2 (anti-M2) antibody (7, 17), we were surprised to find that there was some, but not complete, loss of splicing activity in the galectin-3-depleted extract. We thus performed a series of experiments to resolve this apparent dilemma. In the present communication, we report that in addition to galectin-3, galectin-1 is a component of the nucleus and plays a role in the splicing of pre-mRNA.

MATERIALS AND METHODS

Preparation of HeLa NEs. HeLa S3 cells were obtained from the American Type Culture Collection (CCL 2.2) and grown in suspension culture in minimum essential medium containing 10% defined supplemented bovine calf serum (HyClone), 100 U of penicillin per ml, and 100 U of streptomycin per ml at 37°C in a humidified atmosphere of 5% CO_2. NEs were prepared in buffer C (20 mM HEPES [pH 7.9], 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol [DTT]) as described previously (13). NEs were frozen as aliquots in a dry ice-ethanol bath and stored at −80°C. Typically, NEs with protein concentrations of 10 to 13 mg/ml were prepared. Protein concentrations were determined by the method of Bradford (6) with bovine serum albumin as the protein standard.

Antibodies and affinity columns. Anti-M2 is a rat monoclonal antibody directed against the Mac-2 antigen (17), which has been shown to be galectin-3 (7). The antibody was purified from serum-free cell culture supernatant derived from hybridoma line M3/38.1.2.8.HL.2 obtained from the American Type Culture Collection (TIB166). Anti-transferrin receptor (anti-TR) antibody is an isotype-matched rat monoclonal antibody [immunoglobulin G2a(κ)] that was used as a control for anti-M2. The hybridoma (R17 217.1.3) producing the anti-TR antibody was obtained from the American Type Culture Collection (TIB219). The polyclonal rabbit antiserum against recombinant rat galectin-1 (rG1 [anti-G1]) (10) was a gift from Sam Barondes, Hakon Leffler, and Doug Cooper (University of California, San Francisco). Human autoimmune serum reactive with the Sm antigens of small nuclear ribonucleoprotein particles (snRNPs) (anti-Sm) was

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purchased from The Binding Site (San Diego, Calif.). Anti-SC35, a mouse monoclonal antibody against the non-snRNP splicing factor SC35 (16), was obtained from Sigma (St. Louis, Mo.).

For saccharide affinity chromatography, α-Lac insolubilized on 6% beaded agarose (Lac-agarose) was purchased from Sigma. Cellobiose-agarose was used as a control for NE depletion. For immunodepletions, antibodies were immobilized on preswollen protein G-Sepharose beads (Sigma). Beads were washed with 20 mM HEPES (pH 7.9)–0.5 M NaCl. One hundred fifty microliters of washed beads was mixed with 150 μl of serum or 100 μg of monoclonal antibody. The mixture was adjusted to 20 mM HEPES (pH 7.9)–0.5 M NaCl and incubated for 1 h at room temperature with continual rocking. The beads were washed with 1 ml of 0.2 M sodium borate (pH 9.0) and resuspended in the same buffer. Dimethylpimelimidate (Pierce) was added to a final concentration of 20 mM to covalently cross-link antibodies to protein G-Sepharose. After 1 h of incubation at room temperature, the beads were washed and incubated in 1 ml of 0.2 M ethanolamine (pH 8.0) for 1 h. The coupled antibody-Sepharose mixture was washed five times with 1 ml of 20 mM HEPES (pH 7.9)–0.5 M NaCl and used for immunodepletions.

Depletion and reconstitution of NE. NEs were depleted of galectins by adsorption on either a saccharide affinity column or an antibody affinity column. For saccharide affinity depletions, 150 μl of Lac-agarose beads was washed with 20 mM HEPES (pH 7.9), 0.5 M NaCl and 30 μl of NE were added, and the mixture was incubated on ice for 20 min in disposable spin columns (Millipore). For immunodepletions, 30 μl of NE was incubated with either 60 μl of anti-M2–Sepharose or 90 μl of anti-G1–Sepharose beads for single-antibody depletions and with a mixture of both for double-antibody depletions. After incubation on ice for 20 min with the appropriate affinity matrices, the unbound fraction was removed. The beads were washed with 12 μl of buffer 1 (60% Dignam buffer D adjusted to 0.42 M NaCl; buffer D is 20 mM HEPES [pH 7.9], 20% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT), and this wash was added to the unbound fraction. The beads were washed three times with 1 ml of buffer 1, and the bound material was eluted by being boiled in 100 μl of Laemmli sample buffer (22).

Aliquots of nondepleted NE and unbound fractions of saccharide depletions or immunodepletions were dialyzed in a microdialyzer against 60% buffer D for 40 min at 4°C with a dialysis membrane with a 10-kDa cutoff. The final protein concentration of the depleted and dialyzed extracts was 6 to 8 mg/ml. The dialyzed fractions were then assayed for splicing activity. In reconstitution experiments, recombinant proteins were added to the unbound fractions prior to dialysis. rG3 was isolated from an *Escherichia coli* expression system and purified as previously described (1). The carboxyl-terminal CRD of rG3 was obtained by digesting the recombinant protein with collagenase D and purifying the CRD by affinity chromatography (1). rG1 (10) was generously provided by S. H. Barondes, H. Leffler, and D. N. W. Cooper. In experiments directly comparing the efficiency of various recombinant proteins in reconstituting splicing activity, aliquots taken from the same unbound fraction were reconstituted with different amounts of proteins. In experiments testing the effect of exogenously added carbohydrates, the extracts were incubated with thiodigalactoside (TDG) for 5 min at room temperature after dialysis but prior to the addition of pre-mRNA substrate.

In vitro splicing assay. MINX pre-mRNA (42), used as a substrate in the splicing assay, was transcribed from the plasmid provided by Susan Berget (Baylor College of Medicine, Houston, Tex.) with SP6 polymerase (Gibco). The MINX pre-mRNA was labeled with [^{32}P]GTP, and the monomethyl cap was added during transcription.

Splicing reaction mixtures (10 μl) contained dialyzed NE (4 μl) or unbound fraction (8 μl), ^{32}P-MINX pre-mRNA, 2.5 mM $MgCl_2$, 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, and 20 U of RNasin (Promega). Splicing reaction mixtures were incubated at 30°C for 45 min. Proteinase K-sodium dodecyl sulfate (SDS) solution was added to a final concentration of 4 mg of proteinase K per ml and 0.1% SDS, and the sample was incubated at 37°C for 15 min. Each splicing sample was diluted to 100 μl with 125 mM Tris (pH 8.0)–1 mM EDTA–0.3 M sodium acetate. RNA was extracted with 200 μl of phenol-chloroform (50:50 [vol/vol]) followed by 200 μl of chloroform. RNAs were precipitated with 400 μl of ethanol at −80°C. The extracted RNAs were subjected to electrophoresis through 13% polyacrylamide (bisacrylamide-acrylamide, 1.9:50 [wt/wt])–8.3 M urea gels followed by autoradiography.

The intensities of the bands on the gels were quantitated by direct β-particle counting with an AMBIS system. The percent product formation was calculated by dividing the radioactivity present in the final product (ligated exon 1-exon 2) by the total radioactivity present in the pre-mRNA substrate (exon 1-intron-exon 2), the splicing intermediates (exon 2-lariat and exon 1), and the product at the end of the incubation.

Immunoblot analysis. For immunoblot analysis, protein samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE [12.5% polyacrylamide]) as described by Laemmli (22) and electrophoretically transferred to Immobilon-P membrane (Millipore) in transfer buffer containing 25 mM Tris, 193 mM glycine, and 20% methanol. Membranes were blocked for several hours with 10% nonfat dry milk in T-TBS (10 mM Tris [pH 7.5], 0.5 M NaCl, 0.05% Tween 20). After two brief washes with T-TBS, membranes were incubated with primary antibody diluted in 1% nonfat dry milk–T-TBS for 2 h at room temperature, followed by five washes in T-TBS for 15 min each. Membranes were incubated with secondary antibodies conjugated to horseradish peroxidase for 30 min and washed in T-TBS extensively. Proteins were visualized with the enhanced chemiluminescence detection system (Amersham).

Immunofluorescence microscopy. For double-immunofluorescence microscopy, cells were seeded onto glass coverslips in six-well (8 cm^2/well) cluster dishes. Cells grown to 50 to 70% confluency were washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4 [pH 7.4]). They were first permeabilized with 0.05% Triton X-100 in permeabilization buffer {300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.2]} for 5 min at 4°C (40). Cells were rinsed twice with cold permeabilization buffer and then twice with cold PBS. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature and washed twice with T-TBS. Cells were incubated with primary antibody at an appropriate dilution (rat anti-M2 at 12.5 μg/ml, rabbit anti-G1 at 1:5 dilution of the antiserum, and mouse anti-SC35 at 1:250 dilution) in 0.2% gelatin–T-TBS for 1 h. They were washed three times (15 min each) with T-TBS and incubated for 1 h with the secondary antibody at an appropriate dilution (fluorescein isothiocyanate- [FITC]-conjugated goat anti-rat immunoglobulin [Sigma] at 1:250, FITC-conjugated goat anti-rabbit immunoglobulin preabsorbed with human serum [Sigma] at 1:500, and cy3-conjugated sheep anti-mouse immunoglobulin [Sigma] at 1:250 dilution) in 0.2% gelatin–T-TBS. Cells were washed three times in T-TBS for 15 min each and mounted in Perma Fluor (Immunon) on glass slides. Samples were analyzed with a Meridian Instruments (Okemos, Mich.) Insight confocal laser scanning microscope.

RESULTS

Saccharide affinity depletion versus immunodepletion. As reported in our previous study (12), splicing activity was depleted from NE when the NE was subjected to adsorption on Lac affinity beads under conditions of high ionic strength (0.5 M NaCl). The unbound fraction of the Lac adsorption yielded less than 5% product formation (Fig. 1A, lane 3). Parallel control experiments in which NEs were subjected to adsorption on beads containing cellobiose, which does not bind to galectins, failed to yield the same result (12). Splicing activity in the extract depleted on the Lac matrix was restored by the addition of rG3 (Fig. 1A, lane 4). In both the original NE and the reconstituted fraction, the splicing activity was sensitive to inhibition by TDG, a saccharide that binds to galectin-3 with high affinity (Fig. 1A, lanes 2 and 5). These results formed the basis for our suggestion that galectin-3 is a required factor in the splicing of pre-mRNA.

When we attempted to deplete the splicing activity by using anti-M2, a monoclonal antibody specific for galectin-3 (7, 17), there was some, but not complete, loss of splicing activity in the galectin-3-depleted extract. An isotype-matched monoclonal antibody directed against the TR was used as a control for the anti-M2 antibody. The unbound fraction of the anti-M2 column retained much of the splicing activity, compared to the corresponding unbound fraction of the anti-TR column (Fig. 2A, lane 4 [15% product] versus lane 1 [21% product]). As a positive control for immunodepletion, the snRNPs were removed from NE by an autoimmune serum reactive against the Sm antigens on the core polypeptides B and D of the snRNPs (designated as anti-Sm) (24). As expected, the Sm-depleted extract (unbound fraction of anti-Sm column) was completely deficient in splicing activity (Fig. 2A, lane 7). Western blot analyses indicated that anti-M2 removed >95% of galectin-3, while neither anti-TR nor anti-Sm removed the galectin-3 polypeptide (Fig. 2B). Thus, removal of galectin-3 from NE did not result in the simultaneous depletion of splicing activity.

Number and identity of galectins in NE. A hint at the resolution of this apparent dilemma was derived from the finding that the splicing activity of the galectin-3-depleted extract, recovered from the unbound fraction of the anti-M2 adsorption, was still sensitive to TDG inhibition (Fig. 2A, lane 6). Sucrose, a control disaccharide that does not bind to any of the galectins, failed to yield the same effect (Fig. 2A, lane 5). These results suggest that other Lac-binding proteins besides galectin-3 may be present in the splicing extract, and they are de-

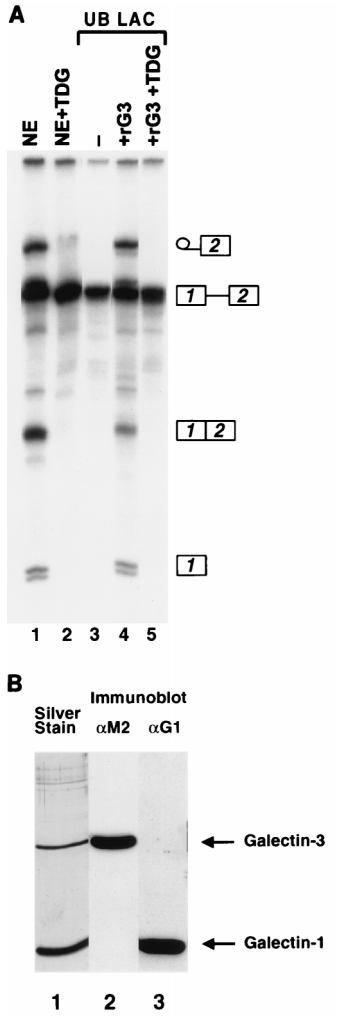

FIG. 1. (A) Comparison of the splicing activities of NE, NE depleted by Lac affinity adsorption, and depleted NE reconstituted with rG3. Lanes: 1 and 2, control NE in the absence (lane 1) and presence (lane 2) of 150 mM TDG; 3 to 5, unbound (UB) fraction from Lac affinity adsorption with no additions (lane 3), addition of 12 μM rG3 (lane 4), and addition of 12 μM rG3 plus 150 mM TDG (lane 5). Products of the splicing reactions were analyzed by electrophoresis through a 13% polyacrylamide–urea gel and autoradiography. The positions of migration of the pre-mRNA substrate, the splicing intermediates (exon 1 and lariat-exon 2), and mature RNA product are indicated on the right. (B) Analysis of the polypeptide components of the bound fraction when NE is subjected to affinity adsorption on Lac-agarose columns. The material bound to the Lac-agarose beads was solubilized by SDS-PAGE sample buffer and was electrophoresed on 12.5% polyacrylamide gels. Lanes: 1, silver stain; 2, immunoblot with anti-M2 (αM2); 3, immunoblot with anti-G1 (αG1). The positions of migration of human galectins-1 and -3 are shown on the right.

pleted, along with galectin-3, by Lac adsorption but not by anti-M2 adsorption.

This prompted us to analyze the protein components in the bound fraction of the original Lac affinity adsorption. Using silver staining, we detected two bands whose mobilities corresponded to polypeptides with M_rs of ~30,000 and ~15,000 (Fig. 1B, lane 1). The identity of the ~30-kDa polypeptide was ascertained to be galectin-3 by immunoblotting with the anti-M2 antibody (Fig. 1B, lane 2). On the basis of the apparent molecular weight of the second silver-stained band, we suspected that it might correspond to galectin-1. This notion was confirmed by immunoblotting with a rabbit antiserum raised against rG1 (anti-G1) (Fig. 1B, lane 3). Because this antibody does cross-react weakly with galectin-2, we carried out direct comparisons between galectins-1 and -2 in terms of (i) the intensities of their immunoblotted bands when probed with the anti-G1 antibody and (ii) the intensities of the silver-stained bands when known amounts of the respective recombinant proteins were electrophoresed in SDS gels. The level of immunoreactivity in the Western blot relative to the intensity of the silver-stained gel (Fig. 1B) indicated that the ~15-kDa polypeptide was galectin-1. These results suggest that galectin-1 was the other Lac-binding protein in NE and could be responsible for the residual saccharide-inhibitable splicing activity after depletion of galectin-3. Indeed, we were able to detect, by Western blotting analysis, galectin-1 in NE, as well as in the unbound fraction of the anti-M2 column (Fig. 3A, lanes 1 and 2).

Double-antibody depletion of galectins-1 and -3. The results presented above imply that when NE is subjected to Lac af-

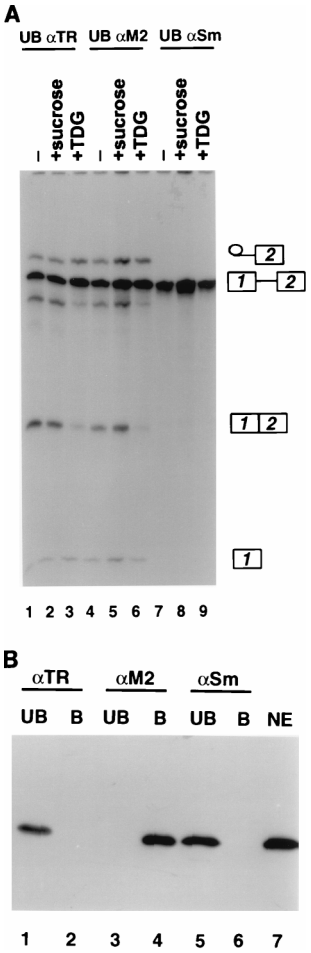

FIG. 2. (A) Comparison of the splicing activity of NE after adsorption on protein G-Sepharose beads conjugated with various antibodies. Splicing reactions were performed with the unbound (UB) fractions of the anti-TR (αTR [lanes 1 to 3]), anti-M2 (αM2 [lanes 4 to 6]), and anti-Sm (αSm [lanes 7 to 9]) adsorptions. Splicing reactions with the immunodepleted extracts were assayed in the absence of saccharide (lanes 1, 4, and 7), in the presence of 50 mM sucrose (lanes 2, 5, and 8), and in the presence of 50 mM TDG (lanes 3, 6, and 9). The positions of migration of the pre-mRNA substrate, splicing intermediates (exon 1 and lariat-exon 2), and mature RNA product are highlighted on the right. (B) Comparison of the levels of galectin-3 in NE versus the unbound (UB) and bound (B) fractions of the various immunodepletions. Samples were subjected to SDS-PAGE on 12% polyacrylamide gels and immunoblotting with anti-M2.

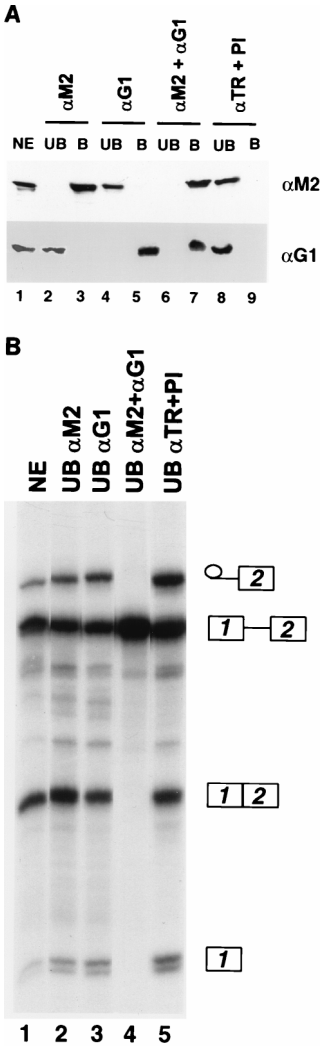

FIG. 3. (A) Comparison of the levels of galectins-1 and -3 in NE versus the unbound (UB) and bound (B) fractions from immunodepletion with antibodies directed against galectin-1 or galectin-3. Lanes: 1, NE; 2 and 3, rat monoclonal antibody against galectin-3 (αM2); 4 and 5, rabbit antibodies against rat galectin-1 (αG1); 6 and 7, αM2 and αG1 used in combination; 8 and 9, control antibodies, rat antibody against the TR (αTR) and rabbit preimmune serum (PI), used in combination. Samples were subjected to SDS-PAGE on 12.5% polyacrylamide gels and immunoblotting with anti-M2 or anti-G1. Separate panels are shown for the anti-G1 and anti-M2 immunoblots, corresponding to the regions of migration for galectin-1 and galectin-3, respectively. (B) Comparison of the splicing activity of NE versus the unbound (UB) fractions of immunodepletion with antibodies directed against galectin-1 or galectin-3. Lanes: 1, NE; 2, unbound fraction of the anti-M2 depletion. 3, unbound fraction of the anti-G1 depletion; 4, unbound fraction of immunodepletion with both antibodies, anti-M2 and anti-G1; 5, unbound fraction of immunodepletion with control antibodies, anti-TR and rabbit preimmune serum. The positions of migration of the pre-mRNA substrate, splicing intermediates (exon 1 and lariat-exon 2), and mature RNA product are highlighted on the right.

finity adsorption, both galectins-1 and -3 are bound on the Lac matrix with concomitant depletion of splicing activity. In contrast, immunodepletion with an antibody directed against any single galectin would fail to completely deplete splicing activity. Thus, a comparison was made of subjecting NE to incubation with beads derivatized with (i) anti-M2, (ii) anti-G1, (iii) anti-M2 plus anti-G1, and (iv) anti-TR plus rabbit preimmune serum. Western blotting analyses yielded the expected presence or absence of galectins-1 and -3 in the various unbound and bound fractions (Fig. 3A). For example, galectin-3 was quantitatively adsorbed to matrices containing anti-M2. Similarly, no galectin-1 could be detected in the unbound fractions of matrices containing anti-galectin-1. Finally, both galectins-1 and -3 were found exclusively in the unbound fractions of the beads containing the control antibodies.

The unbound fraction of the double-antibody column, anti-M2 plus anti-G1, showed complete loss of splicing activity (Fig. 3B, lane 4). This should be compared to the level of product formation in the unbound fraction of the control adsorption (Fig. 3B, lane 5), as well as that of the original NE (Fig. 3B, lane 1). Together, all of these results suggest that both galectins-1 and -3 contribute independently to the splicing activity of NE and that their complete removal, either by Lac affinity chromatography or by double-antibody adsorption, is necessary to deplete splicing activity.

Reconstitution of splicing in the double-antibody-depleted extracts. The unbound fraction of the anti-M2 plus anti-G1 double-antibody column, devoid of splicing activity, could be reconstituted by the addition of either rG3 or rG1 (Fig. 4, lanes 3 and 5, respectively). Moreover, the splicing activity of the double-antibody-depleted extract, reconstituted with either galectin, was again sensitive to TDG inhibition (Fig. 4, lanes 4 and 6). The reconstitution effects of galectin-1 or galectin-3 were concentration dependent. Compared on an equal molar concentration basis, rG3 was more efficient than galectin-1 in reconstitution of splicing activity (see below). Finally, neither rG1 nor rG3 had an effect on the splicing assay when added to a nondepleted NE (data not shown). These results indicate that galectin-1 and galectin-3 may serve redundant functions in the splicing activities of the NE.

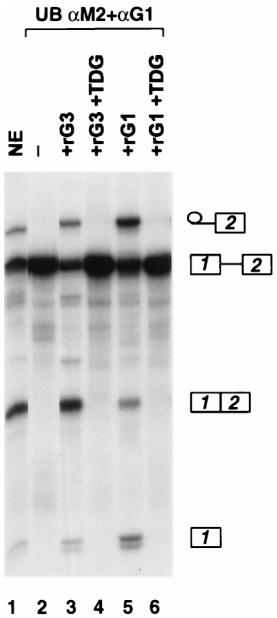

FIG. 4. Comparison of the splicing activities of NE, NE immunodepleted by antibodies against galectins-1 and -3, and depleted NE reconstituted with rG1 or rG3. Lanes: 1, control splicing reaction of nondepleted NE; 2 to 6, unbound (UB) fraction when NE is subjected to immunodepletion with anti-M2 (αM2) and anti-G1 (αG1); 2, no additions; 3 and 4, addition of 12 μM rG3 in the absence (lane 3) or presence (lane 4) of 150 mM TDG; 5 and 6, addition of 26 μM rG1 in the absence (lane 5) or presence (lane 6) of 150 mM TDG. The positions of migration of the pre-mRNA substrate, the splicing intermediates (exon 1 and lariat-exon 2), and the mature RNA product are highlighted on the right.

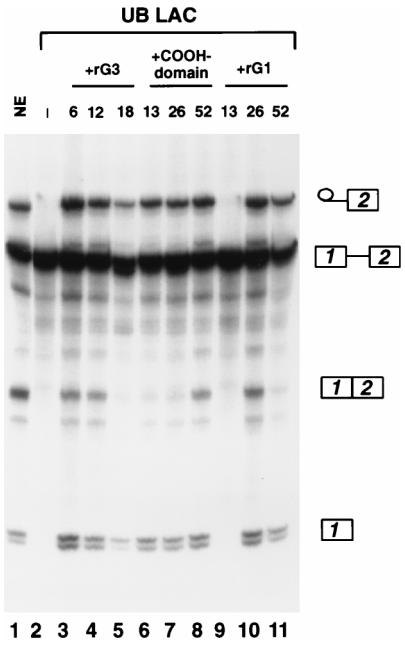

FIG. 5. Comparison of the splicing activities of NE, NE depleted by Lac affinity adsorption, and depleted NE reconstituted with rG1, rG3, or the carboxyl-terminal (COOH) domain of rG3. Lanes: 1, control splicing reaction of nondepleted NE; 2 to 11, the unbound (UB) fraction when NE is subjected to Lac affinity adsorption; 2, no additions; 3 to 5: addition of rG3; 6 to 8, addition of the COOH domain of rG3; 9 to 11, addition of rG1. The concentration (micromolar) of the recombinant proteins added is indicated at the top. The positions of migration of pre-mRNA substrate, splicing intermediates (exon 1 and lariat-exon 2), and mature RNA product are highlighted on the right.

Comparison of the concentration dependence of galectin-1 and galectin-3 in reconstituting splicing activity. In this series of experiments, we compared the concentrations of galectin-1 and galectin-3 required to reconstitute a splicing-deficient NE depleted of the galectins by Lac affinity adsorption. We also compared the intact polypeptide of galectin-3 (M_r, ~30,000) with its carboxyl-terminal CRD (M_r, ~15,000) (1) in terms of splicing reconstitution. Using galectin-3 concentrations ranging from 2 to 18 μM, we found that the minimal threshold concentration required for restoration of splicing activity was ~3 μM. The optimal concentrations for reconstitution were 6 to 12 μM (Fig. 5, lanes 3 and 4). Product formation was inhibited at 18 μM, consistent with our previous observations that high concentrations of galectin-3 inhibit splicing reconstitution (12).

On the basis of our observations that a higher concentration of rG1 than rG3 was required to reconstitute the splicing-deficient NE, derived from double-antibody depletion experiments (Fig. 4), the concentration range tested for galectin-1 was 13 to 52 μM. In contrast to the results obtained with galectin-3, 13 μM was insufficient to reconstitute splicing activity (Fig. 5, lane 9). Splicing activity was observed with 26 μM galectin-1 (Fig. 5, lane 10), which was comparable to or even better than that observed with the optimal concentration of galectin-3. Finally, 52 μM showed inhibition of splicing, as was observed with galectin-3 at the high concentration end.

Although the carboxyl-terminal CRD of galectin-3 can reconstitute the splicing activity of a deficient NE, it required a much higher concentration of protein than was required of the intact galectin-3 polypeptide. Thus, we observed reconstitution of splicing only at the highest concentration tested (52 μM) (Fig. 5, lane 8). These results suggest that the galectin-3 polypeptide, with a Gly-, Pro-rich domain fused onto the CRD (corresponding to galectin-1 or the carboxyl-terminal domain of galectin-3), was most efficient in the reconstitution of splicing activity.

Double-immunofluorescence analyses: galectins versus splicing factor SC35. Since we identified galectins-1 and -3 as components of NE prepared from HeLa cells and as required factors in pre-mRNA splicing, we further investigated their intranuclear distribution relative to that of another known splicing component, SC35 (16). HeLa cells were permeabilized with Triton X-100 (without fixation) and then were fixed and stained simultaneously with anti-M2 and anti-SC35. The binding of anti-M2 was revealed with FITC-conjugated goat anti-rat immunoglobulin, yielding the green fluorescence pattern in Fig. 6A. The binding of anti-SC35 was revealed with cy3-conjugated sheep anti-mouse immunoglobulin, yielding the red fluorescence pattern in the same figure. In the composite of the double-immunofluorescence patterns, the yellow represents regions of coincidence of the green and red stains. Careful analysis of such doubly stained photomicrographs indicates (i) the red anti-SC35 staining appears speckled; (ii) there is coincidence between the red anti-SC35 spots and the green anti-M2 spots, leading to a speckled yellowish composite; and (iii) the anti-M2 staining for galectin-3 appears to be more diffuse, giving rise to green areas in the composite containing no red staining. The latter point is important, for it suggests that the sheep anti-mouse immunoglobulin reagent does not cross-react with the rat monoclonal anti-M2 antibody. This conclusion is supported by control experiments checking for cross-reactivity between the sheep anti-mouse and goat anti-rat immunoglobulins and the rat and mouse immunoglobulins, respectively.

A similar double-immunofluorescence analysis was carried out with anti-G1 and anti-SC35. The binding of anti-G1 was revealed with FITC-conjugated goat anti-rabbit immunoglobulin (green fluorescence); the binding of anti-SC35 was revealed with cy3-conjugated sheep anti-mouse immunoglobulin (red fluorescence) (Fig. 6B). The speckled SC35 pattern contains regions of coincidence with the anti-G1 staining, giving rise to yellowish spots on the composite. However, there are also spots where SC35 staining is not matched by the presence of galectin-1. These appear as reddish orange spots on the composite. Conversely, there are also green areas that appear to be devoid of any red SC35 staining.

DISCUSSION

The key findings documented in the present study include the following. (i) NEs capable of carrying out pre-mRNA splicing contain both galectins-1 and -3. (ii) Depletion of both galectins from NE, either by Lac affinity adsorption or by double-antibody adsorption, results in the concomitant loss of splicing activity. (iii) Depletion of either galectin-1 or galectin-3 by specific antibody adsorption fails to remove all of the splicing activity, and the residual activity is still sensitive to saccharide-specific inhibition. (iv) Either galectin-1 or galectin-3 alone is sufficient to reconstitute, at least partially, the splicing activity of NE depleted of both galectins; (v) although a CRD (either as galectin-1 or the carboxyl-terminal domain of galectin-3) is sufficient to restore the splicing activity to galectin-depleted NE, the concentration required for reconstitution is far greater than that of the full-length galectin-3 polypeptide.

These results suggest that the splicing activity of nuclear galectins is functionally redundant and implicate a modulatory role for the amino-terminal domain of galectin-3 in splicing

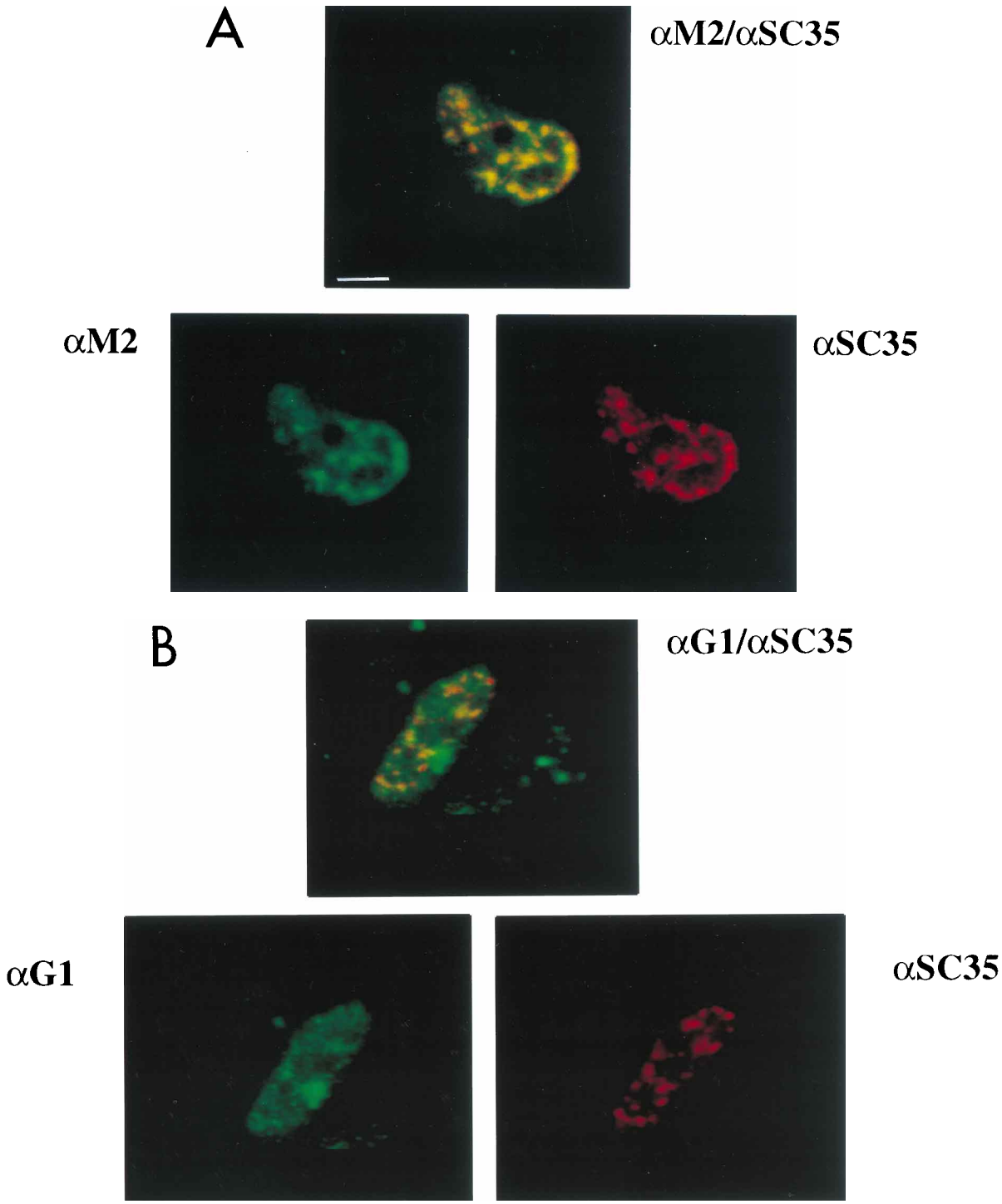

FIG. 6. (A) Double-immunofluorescence analysis of galectin-3 and SC35. HeLa cells were permeabilized with 0.05% Triton X-100 (without fixation) and then were fixed with paraformaldehyde (4%) and stained simultaneously with anti-M2 (αM2) and anti-SC35 (αSC35). The binding of anti-M2 was revealed by FITC-conjugated goat anti-rat immunoglobulin, yielding the panel with the green fluorescence; the binding of anti-SC35 was revealed by cy3-conjugated sheep anti-mouse immunoglobulin, yielding the panel with the red fluorescence. A composite of the two panels is also shown. Bar, 5 μm. (B) Double-immunofluorescence analysis of galectin-1 and SC35. Anti-G1 binding was revealed by FITC-conjugated goat anti-rabbit immunoglobulin (green panel). Anti-SC35 binding was revealed by cy3-conjugated sheep anti-mouse immunoglobulin (red panel). A composite of the two panels is also shown. The magnification is the same as that in panel A.

activity. The notion of functional redundancy between galectins-1 and -3 is consistent with the results of experiments with transgenic mice in which a null mutation in the gene encoding galectin-1 has been introduced by homologous recombination in embryonic stem cells (9, 31). Homozygous animals carrying the mutant allele lacked galectin-1; however, there was no apparent damage in terms of development. The mice were viable and fertile. Thus, the function or functions assigned to

galectin-1 seem to have been complemented by a redundant relative, possibly galectin-3.

Although polypeptides corresponding to the CRD are sufficient to reconstitute splicing activity, the minimum concentrations required are four to eight times higher than that of the intact galectin-3 polypeptide, which contains a Gly-, Pro-rich domain fused onto the CRD. Several possibilities need to be considered to account for this apparent difference in efficiency. First, the predominant structural feature of the amino-terminal domain, the Pro-Gly-Ala-Tyr-Pro-Gly-Xxx-Xxx repeats (3, 21), may play a role in the interaction between galectin-3 and components of the splicing machinery. Although immunoblotting of subnuclear fractions and immunofluorescence and immunoelectron microscopy suggest that galectin-3 is associated with RNP complexes (19, 23, 38), and although there is a recent report that galectin-3 interacts directly with RNA and single-stranded DNA (37), the specific spliceosomal component that interacts with galectin-3 has not yet been identified. Therefore, we can only speculate that if this domain indeed plays a role, then galectin-1 and the carboxyl-terminal CRD of galectin-3 must overcome their lack of such a structural feature on the basis of mass action.

Second, it is known that endogenous cellular galectin-3 exists as two isoelectric variants: a pI 8.7 species corresponding to the unmodified polypeptide, which is found exclusively in the nucleus, and a pI 8.2 species representing a singly phosphorylated derivative, which is localized to both the nucleus and the cytoplasm (11). Chemical studies with the canine homolog have identified a major site of phosphorylation at Ser-6 (~90%) and a minor site at Ser-12 (~10%) (20). If phosphorylation plays a role in enhancing galectin-3's interaction with protein and/or RNA components of spliceosomes, then galectin-1 or the carboxyl-terminal CRD of galectin-3 would again have to compensate by mass action.

The third consideration is that besides the possible interaction between the galectins and other spliceosome components, these structural features (phosphorylation and/or Pro-, Gly-rich repeats) may play a role in self-association of the galectin polypeptide. Self-association of the rat and mouse homologs of galectin-3 has been inferred from the concentration-dependent hemagglutination activity (18, 30) and positive cooperativity in the binding of the lectins to multivalent glycoproteins (18, 27). Both the cooperative binding experiments (18, 27) and cross-linking studies carried out with hamster galectin-3 (28) implicate the amino-terminal domain as being responsible for the oligomerization of the lectin. Thus, polypeptides lacking the structural features of the amino-terminal domain (galectin-1 or the carboxyl-terminal CRD of galectin-3) would require other mechanisms, including self-association via sequences contained in the CRD, to achieve the same degree of multivalency.

Several studies indicate that galectin-1 from various species forms homodimers (2, 4, 25, 32), and recent crystallographic structures of bovine galectin-1 are consistent with this conclusion (5, 26). In a detailed analysis, Cho and Cummings (8) showed that both rG1 produced in an *E. coli* expression system, as well as galectin-1 endogenous to the cytosol of Chinese hamster ovary cells, existed in a monomer-dimer equilibrium. The equilibrium dissociation constant and equilibration time were estimated to be about 7 μM and 10 h, respectively. Although the concentration dependence was studied in less detail, it has also been shown that the carboxyl-terminal CRD of galectin-3 can undergo monomer-dimer association (36). Inasmuch as our present data showed that 26 μM rG1 was required to reconstitute the splicing activity of a galectin-depleted NE, it appears that the protein was functional in the dimeric state. We should hasten to note, however, that the concentration of galectin-1 in NE is estimated to be in the nanomolar range, and much of the requirement for a high concentration of the reconstituting protein may have to do with the inefficient steps of dialysis and reassembly of the spliceosome, as discussed previously (12).

The identification of galectins-1 and -3 as splicing factors is also supported by the observation that at least a fraction of each of the nuclear galectins colocalizes in speckles with the non-snRNP splicing factor SC35 (33, 34). Xing et al. (39) have suggested that the SC35-containing domains of nuclei correspond to regions of intron and exon sequences for specific RNA transcripts and are near the sites of transcription. On the other hand, however, more recent studies (41) have concluded that bulk transcription and splicing occur throughout the nucleoplasm during periods of active transcription and that factors involved in these two processes coalesce to the speckles for recycling or reactivation during periods of less active transcription. In any case, the speckled structures have been found to correspond, at the ultrastructural level, to interchromatin granule clusters and perichromatin fibrils (14). Perichromatin fibrils are readily labeled with short pulses of [^{3}H]uridine (15) and anti-RNA polymerase II antibodies (35), suggesting that they represent nascent transcripts at the sites of mRNA synthesis and early events of pre-mRNA processing. Immunogold labeling at the ultrastructural level has found galectin-3 in perichromatin fibrils (19). It would therefore be of obvious interest to determine if a similar localization can be achieved for galectin-1.

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