

## RNA Association Defines a Functionally Conserved Domain in the Nuclear Pore Protein Nup153\*

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**Traffic between the nucleus and cytoplasm takes place through a macromolecular structure termed the nuclear pore complex. To understand how the vital process of nucleocytoplasmic transport occurs, the contribution of individual pore proteins must be elucidated. One such protein, the nucleoporin Nup153, is localized to the nuclear basket of the pore complex and has been shown to be a central component of the nuclear transport machinery. Perturbation of Nup153 function was demonstrated previously to block the export of several classes of RNA cargo. Moreover, these studies also showed that Nup153 can stably associate with RNA *in vitro*. In this study, we have mapped a domain within Nup153, encompassing amino acids 250–400 in human Nup153, that is responsible for RNA association. After cloning this region of *Xenopus* Nup153, we performed a cross-species analysis. Despite variation in sequence conservation between *Drosophila*, *Xenopus*, and human, this domain of Nup153 displayed robust RNA binding activity in each case, indicating that this property is a hallmark feature of Nup153 and pointing toward a subset of amino acid residues that are key to conferring this ability. We have further determined that a recombinant fragment of Nup153 can bind directly to RNA and that this fragment can interact with endogenous RNA targets. Our findings identify a functionally conserved domain in Nup153 and suggest a role for RNA binding in Nup153 function at the nuclear pore.**

or RNA cargoes, bridging contact between cargo and the pore and facilitating translocation through the NPC (4). Receptor-cargo complexes are thought to contact specific pore proteins en route through the pore, yet little is known about how individual pore proteins function in the transport process. In addition to cargo signals and receptors, specific accessory factors are also crucial to the control of transport. The small GTPase Ran, for example, regulates the interactions between cargo, receptors, and the NPC (5–7).

The vertebrate NPC is a macromolecular structure of 120 MDa. A central plug, or transporter, is embedded within the pore, extending from the inner nuclear membrane to the outer nuclear membrane. Ring-like structures surround this central region on both the cytoplasmic and nucleoplasmic faces. Attached to the cytoplasmic ring are filaments that extend into the cytoplasm. Fibers that emanate from the nucleoplasmic face of the pore are anchored at a distal ring, forming the nuclear basket. Currently, 25 of an estimated 50 different nuclear pore proteins (nucleoporins) have been identified, each predicted to be present in multiple copies due to the 8-fold symmetry of the pore (8). Many nuclear pore proteins have already been localized to structural domains within the NPC by immunoelectron microscopy. Nup88, Nup214, and Nup358 have been localized to the filaments extending from the cytoplasmic ring (9–13). Nup62, Nup58, and Nup54 are present in the central channel region (14, 15). Nup50, Nup93, Nup96, Nup98, Nup153, and Nup205 have been found on the nucleoplasmic face of the pore (16–21). Further progress in delineating pore architecture and in analyzing the functional contribution made by individual nuclear pore proteins to the transport machinery is essential to a comprehensive understanding of the nucleocytoplasmic transport process.

One nucleoporin that has been studied in some detail is Nup153. This protein consists of three distinct domains: a unique N terminus within which a pore targeting region has been identified (22); a central C<sub>2</sub>-C<sub>2</sub> zinc finger-like domain that has been designated as a RanGDP binding domain (23); and a C-terminal domain housing the nucleoporin-characteristic FG-repeat motifs that are thought to function as receptor docking domains (23–25).

A multifunctional role for Nup153 in transport has been well established. Involvement of Nup153 in nuclear import was demonstrated when two distinct Nup153 fragments were shown to act as dominant-negative inhibitors of either NLS or M9-mediated import (26). A role for Nup153 function in import is also supported by the observation that Importin  $\beta$  bridges an association between Nup153 and NLS cargo-receptor complexes (24). Underscoring the modular nature of Nup153, distinct binding sites for Transportin, Exportin1, and Importin  $\alpha$  have also been identified on Nup153 (23, 26, 27). Evidence for Nup153 function in export was found when initial studies demonstrated that overexpression of a C-terminal fragment of

Communication between the nuclear and cytoplasmic compartments of the eukaryotic cell is critical to normal cell function. By providing a channel through which selective molecular traffic can take place, the nuclear pore complex (NPC)<sup>1</sup> serves as the gateway for communication between the nucleus and cytoplasm (1–3). A wide array of cargo passes through the pore, each cargo type harboring localization signals that dictate directionality and route of passage. These localization signals are recognized by soluble transport receptors that bind to protein

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<sup>1</sup> The abbreviations used are: NPC, nuclear pore complex; NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RNP, ribonucleoprotein; GFP, green fluorescent protein.

Nup153 causes nuclear accumulation of poly(A)<sup>+</sup> RNA (28). It was further shown that antibodies to Nup153, when injected into *Xenopus* oocytes, block the export of mRNA, 5 S rRNA, and U1 small nuclear RNA, demonstrating a central role for Nup153 in the export of several classes of RNAs (29).

Interestingly, a stable association between Nup153 and RNA *in vitro* was found using homopolymeric RNA (29). Homoribopolymers have been extensively used to classify, characterize, and purify RNA-binding proteins. The effectiveness of using homopolymeric RNA for biochemical characterization became evident when the ribosomal protein S1 and the termination factor  $\rho$  were isolated on poly(C) cellulose (30). Homoribopolymers have since been used to reveal a role for RNA association in the function of a variety of proteins, including the product of the fragile X gene (FMR1) and the *Xenopus* zinc finger protein XFG5-1 (31, 32). In addition, many of the heterogeneous nuclear RNPs have been categorized and differentiated on the basis of homopolymeric RNA-based purification (33). Homoribopolymers allow a way to probe for the ability of a protein to associate with RNA without prior knowledge of the recognition motif. Association with a subset of homoribopolymers usually reflects a sequence or structural recognition preference. In this case, Nup153 was found to associate with poly(G) and to a lesser extent poly(U), although whether this was a direct or indirect interaction remained an open question (29).

To begin to bridge the observation of the *in vitro* RNA binding activity of Nup153 to a role for such an association *in vivo*, we have mapped the site of RNA association and determined that Nup153 can interact directly with RNA. A comparison of the domain for RNA association between Nup153 from *Drosophila*, *Xenopus*, and human demonstrated that, despite varying degrees of conservation in the amino acid sequence, the property of RNA association is well conserved at this site. Together, these results suggest that the ability to associate directly with RNA is important to the function of Nup153.

#### EXPERIMENTAL PROCEDURES

**Homoribopolymer Binding Assay**—Egg extracts and tissue culture cell lysates were prepared as described below. Agarose beads coupled to the homoribopolymer poly(G) (Sigma) were equilibrated in binding buffer (25 mM HEPES, pH 7.8, 100 mM KCl, 0.5% Triton X-100, 10 mg/ml ovalbumin) and then incubated with extract or lysate with or without <sup>35</sup>S-radiolabeled protein for 60 min at 4 °C. The RNA resins were centrifuged at 6750 × g for 30 s at 4 °C to pellet and separate the beads from unbound proteins. The pellets were washed twice in wash buffer (25 mM HEPES, pH 7.8, 200 mM KCl, 0.5% Triton X-100, 10 mg/ml ovalbumin) containing 3 mg/ml heparin sulfate as follows: one was a rapid wash, and the second wash was for 10 min at 4 °C. The beads were again pelleted as above and were washed one final time in wash buffer without heparin sulfate or ovalbumin. When recombinant protein was used in the assay, the binding buffer contained 300 mM KCl and 3 mg/ml heparin sulfate in addition to other components listed above. In addition, the wash buffer was modified to 300 mM KCl instead of 200 mM KCl. Proteins that remained associated with the beads were eluted in SDS sample buffer and analyzed by SDS-PAGE. To monitor <sup>35</sup>S-labeled proteins, gels were fixed, amplified (Amplify; Amersham Pharmacia Biotech), and dried, and the results were visualized by autoradiography and quantified by PhosphorImager (Molecular Dynamics). Western blotting was performed to monitor recombinant protein or protein from extract or lysate as follows. Proteins were transferred to polyvinylidene fluoride membrane (PerkinElmer Life Sciences). The membrane was blocked in 5% non-fat dried milk, 0.1% Tween in phosphate-buffered saline for 45 min at room temperature. Incubation with primary antibody was performed for 45 min at room temperature or overnight at 4 °C in blocking buffer. Detection was carried out using an horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories Inc.) and chemiluminescent substrate (Renaissance ECL; PerkinElmer Life Sciences).

**Analysis of Interaction between Endogenous RNA and Recombinant RNA Binding Domain**—Anti-T7 Tag antibody coupled to agarose beads (Novagen) was equilibrated in binding buffer (except 10 mg/ml bovine

serum albumin was used instead of ovalbumin) for 1 h at room temperature. Purified T7-tagged recombinant protein was then incubated with the pre-equilibrated anti-T7-agarose beads for 1 h at 4 °C. The beads were pelleted by centrifugation and washed twice rapidly in binding buffer without bovine serum albumin. The beads were then resuspended in binding buffer containing 3 mg/ml heparin sulfate and 80 units of recombinant RNasin (Promega) and incubated for 1 h at 4 °C with 24 μg of total cellular RNA extracted with Trizol (Invitrogen) from *Xenopus* A6 tissue culture cells. This immunoprecipitation reaction was briefly centrifuged, and the pellets were rapidly washed three times in wash buffer (see above).

After the final wash, each pellet was split in half; one sample underwent RNA isolation, and the other sample underwent protein analysis. The first pellet was resuspended in NTS buffer (300 mM NaCl, 100 mM Tris-HCl, pH 8.0, 2% SDS), and acid phenol/chloroform extracted for 15 min at 37 °C. Precipitated RNA was resuspended in RNA loading buffer and analyzed on a 6% denaturing, acrylamide gel (ISC Bioscience). RNA was stained by SYBR Gold nucleic acid stain (Molecular Probes) for 15 min at room temperature and visualized using the Bio-Rad Gel Documentation system/Quantity One software (Bio-Rad). The second pellet was resuspended in SDS sample buffer and analyzed by SDS-PAGE, followed by Western blotting with anti-T7 antibody. Detection was carried out using horseradish peroxidase-conjugated protein A (Bio-Rad) as described above.

**Antibodies**—Primary antibodies for Western blotting included monoclonal antibody 414 (Covance) to detect Nup358, Nup214, Nup153, and Nup62 and anti-T7 epitope tag (Novagen) to detect recombinant proteins. Anti-Nup153 antibodies analogous to antibody 2 (29) were independently raised and affinity-purified for use as a *Xenopus* Nup153-specific probe (antibody 2 plasmid construct (Nup153 amino acids 334–828, by old numbering) was a kind gift of Drs. S. Shah and D. Forbes); anti-Nup98 (kind gift of Dr. M. Powers), anti-Nup93 (kind gift of Dr. M. Powers), and anti-RIP/Rab antibodies (Santa Cruz Biotechnology, Inc.) were also used. For transport factor detection, the following antibodies were used: anti-CAS (Transduction Laboratories), anti-Transportin (Transduction Laboratories), and anti-Gle2/RAE1 (kind gift of Dr. M. Powers). Anti-Exportin1/Crm1 and anti-REF antibodies were raised against the following peptide antigens: anti-Exp1/Crm1, (C)RQAQEEKH-KLQMSVPGI, and anti-REF/Aly, (C)RGRGRGAGRNPQQQLS, and affinity-purified prior to use as primary antibodies in Western blot analysis.

**Construction of N-terminal Truncations and PCR Templates**—Full-length human Nup153 harbored within the pET28b plasmid was created by subcloning a *XhoI/BglII* fragment from pCMV-hNup153 (kind gift of Dr. B. Burke) into pBluescript *XhoI/BamHI* sites. Nup153 was then subcloned of pBluescript using the *XhoI/NotI* sites and placed into pET28b cut at its *SalI/NotI* sites. pET28-hNup153 was used as a template for *in vitro* transcription and translation using the TnT Quick-coupled Reticulocyte Lysate system (Promega). Translation reactions were done in the presence of [<sup>35</sup>S]methionine (ICN); production of full-length protein product was verified by SDS-PAGE and autoradiography as described above.

hNup153 N-terminal truncations were generated by PCR amplification using a 5' primer to which an *XhoI* site was engineered and a 3' end primer flanking a *KpnI* site. PCR products were digested with *XhoI* and *KpnI*. A *KpnI/NotI* hNup153 fragment cut out of pET28-hNup153 was purified and ligated to the *XhoI/KpnI* cut PCR product and pET28b plasmid cut at its *SalI/NotI* sites. N-terminal truncations in pET28 served as templates for coupled TnT as described above. All 5'-truncation constructs were sequenced at the University of Utah DNA Sequencing Core Facility.

PCR templates for TnT reactions were generated using a 5' primer into which a T7 promoter and a Kozak sequence were engineered. PCR products were then purified (PCR Purification Kit, Qiagen) and used as templates in coupled TnT reactions.

PCR was used to isolate a fragment encompassing the RNA association domain from a *Drosophila* embryonic cDNA library (kind gift of Dr. K. Clark) and was cloned into the pCR2.1 vector (Invitrogen) by TA cloning and confirmed by DNA sequence analysis (DNA sequencing core facility, University of Utah). This *Drosophila* Nup153 fragment was generated in TnT reactions as above.

**Cloning the Full-length Open Reading Frame of *Xenopus laevis* Nup153**—The missing 5' end of the open reading frame for *Xenopus* Nup153 was cloned by PCR from a random-primed embryonic library (kindly provided by Drs. D. Wettstein and C. Kintner). A primer complementary to a sequence 5' of the polylinker in the library vector pJG4 (5'-GACTGGCTGAAATCGAATGG-3') was used in conjunction with a primer complementary to a proximal region in the currently available *Xenopus* sequence (5'-GTGCTTGTCTGGCTGTATTCTGCGACTG-3').

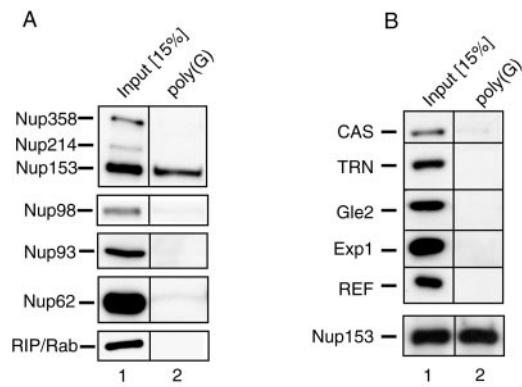
By using 75 ng of the plasmid library, 1  $\mu$ M each primer, and *Taq* polymerase (Promega), a PCR product was obtained and cloned into pCR2.1 (Invitrogen) using the TOPO-TA cloning kit (Invitrogen). Plasmids with inserts corresponding to Nup153 were isolated and sequenced at the University of Utah Sequencing Facility. A fragment containing ~500 base pairs of new sequence was obtained, and a new primer was designed based on the proximal end of this sequence (5'-GGAGAATCACCAAGCTGACG-3'). The new primer was used in conjunction with the library primer as before, and another fragment containing ~700 nucleotides of new Nup153 sequence was obtained. Conceptual translation of this fragment identified a methionine that aligned with the start methionine from human and rat Nup153; however, no upstream stop codon was present in this clone. To confirm that this was the true start methionine, we designed another primer based on the most proximal new sequence (5'-GCGAGGTAGAGTCCTT-GATC-3'), and the procedure was repeated by PCR amplifying with the library vector primer and the new primer. Two clones were sequenced from this round, and both had stop codons within 50 amino acids upstream of the candidate start methionine. To obtain a clone containing the new 5' end of the open reading frame was synthesized with an additional *SacI* site for cloning purposes (5'-GCCCGAGCTCGATG-GCGGAGCCGGAGG-3'). This primer was used in conjunction with a primer complementary to a region ending at nucleotide 906 in the original *Xenopus* Nup153 clone (24). The oligonucleotides were used to PCR an ~2-kilobase pair fragment from 250 ng of linearized library using *PfuI* polymerase (Promega). After cloning PCR products into pCR2.1, sequence was obtained along the length of the new sequence in both directions. This sequence, depicted in Fig. 5, was used in these experiments and deposited in GenBank™ accession number AF434196. Two nucleotides (and consequently two amino acids) differed between our original *Taq*-generated fragments and our *PfuI*-generated fragment (Q38R and S198T). This could be due to polymerase errors or allelic differences.

**Preparation of *Xenopus* Egg Extract and Cell Lysates**—High speed extract prepared from *Xenopus* eggs was done as described previously (34). Cell lysates were made from *Xenopus* A6 and human HT29 cell lines. Cell pellets were resuspended in lysis buffer (100 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 15 mM MgCl<sub>2</sub>, 1% Triton X-100) and sonicated 3 times for 30 s at 4 °C, followed by a 10-min incubation on ice. The cells were then centrifuged for 5 min at 30,000  $\times g$  at 4 °C, and the supernatant was collected for subsequent use in the homoribopolymer binding assay.

**Recombinant Protein Expression and Purification**—*Xenopus* Nup153 fragments (amino acids 249–375 and amino acids 436–717 (amino acids 53–334, by old numbering)) were purified from *Escherichia coli* as follows (the latter expression construct was a kind gift of Drs. S. Shah and D. Forbes). Expression plasmids were transformed into BL21(LysS) cells. Transformants were grown in LB overnight at 37 °C and then diluted (1:10) and grown for an additional 45 min at 37 °C. Expression was induced using 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside for 3 h at 37 °C. Following centrifugation at 5000  $\times g$  for 5 min at 4 °C, the pellets were washed in 1 $\times$  phosphate-buffered saline, resuspended in lysis buffer (0.5 M NaCl, 20 mM Tris, pH 8, 5 mM imidazole, 1 mg/ml lysozyme), and sonicated 4 times for 30 s at 4 °C. The lysates were centrifuged at 10,000  $\times g$  for 20 min at 4 °C, and the supernatant was collected and then incubated with nickel-nitrilotriacetic acid beads (Qiagen) for 2 h at 4 °C. Following 2 washes in wash buffer (0.5 M NaCl, 20 mM Tris, pH 8, 20 mM imidazole), elution buffer (0.5 M NaCl, 20 mM Tris, pH 8, 10 mM EDTA, 250 mM imidazole) was used to elute the protein from the nickel-nitrilotriacetic acid resin. Eluted protein was then concentrated in an Ultrafree-15 Centrifugal Filter Unit (Biomax 5K, Millipore).

## RESULTS

**Association with RNA Distinguishes Nup153 from Other Nucleoporins Tested**—It was shown previously that Nup153 has a central role in the export of several classes of RNA and that a stable association between Nup153 and RNA can be detected (29). In particular, Nup153 was shown to associate preferentially with poly(G). To determine how prevalent this property of RNA association is among proteins involved in nucleocytoplasmic trafficking, we surveyed several proteins for their activity in the homoribopolymer binding assay. *Xenopus* egg extract, which provides a vast supply of soluble NPC components, was incubated with poly(G) immobilized on agarose

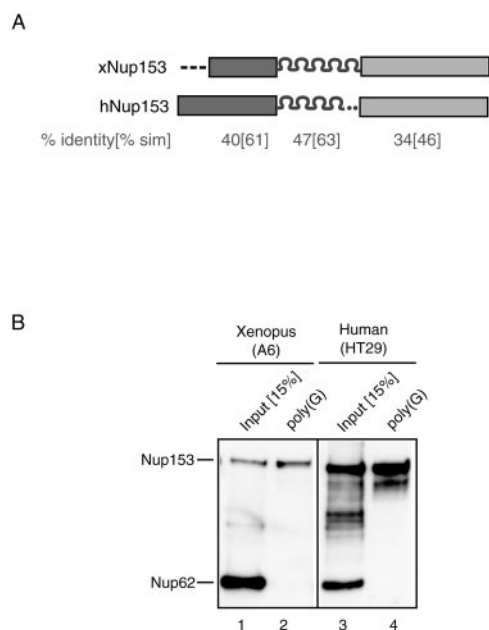


**FIG. 1. RNA association distinguishes Nup153 from other proteins tested.** A, poly(G) immobilized on agarose beads was incubated with *Xenopus* egg extract. After the beads were extensively washed, proteins that remained associated were eluted with SDS sample buffer, separated by SDS-PAGE, and analyzed by Western blot (see “Experimental Procedures”). Nup358, Nup214, Nup153, Nup98, Nup93, Nup62, and the nucleoporin-like protein RIP/Rab were detected in the extract (lane 1). Of these proteins, only Nup153 was detectably associated with the poly(G) beads (lane 2). B, interactions with transport factors were also tested in the binding assay (described in A) using specific antibodies (see “Experimental Procedures”). The transport receptors CAS, Transportin (TRN), and Exportin1/Crm1 (Exp1) were detected in the extract (lane 1) but not in association with the RNA resin (lane 2). In addition, the export factors Gle2/RAE1 and REF were also not detectably retained on the RNA beads (lane 2).

beads. Following washes in heparin sulfate-containing buffer to reduce nonspecific binding to the beads, the poly(G)-associated proteins were eluted, separated by SDS-PAGE, and analyzed by Western blot first using antibodies specific for several nuclear pore proteins. This panel included some nucleoporins that had been monitored previously (Nup358, Nup214, Nup153, Nup98, and Nup62) and was extended here to include Nup93 and the nucleoporin-like protein RIP/Rab. Of the seven proteins examined, only Nup153 was found to associate with poly(G) (Fig. 1A, lane 2). Nup358, Nup214, Nup98, Nup93, Nup62, and RIP/Rab showed no significant level of association with the RNA. This suggests that among vertebrate nucleoporins Nup153 is unique, or among a specialized subset of nucleoporins, in its ability to associate with poly(G) RNA.

**Transport Factor Interactions with Poly(G)**—To investigate the range of transport-related proteins that might have a similar profile of RNA association, either independently or in a complex with Nup153, Western blots of the homoribopolymer binding assay were performed with antibodies directed against members of the Importin  $\beta$ /Karyopherin  $\beta$  family of transport receptors or specific RNA-associated export factors. No significant interaction with RNA was detected in this assay for CAS, Transportin, Gle2/RAE1, Exportin1/Crm1, or REF/Aly (Fig. 1B, lane 2).

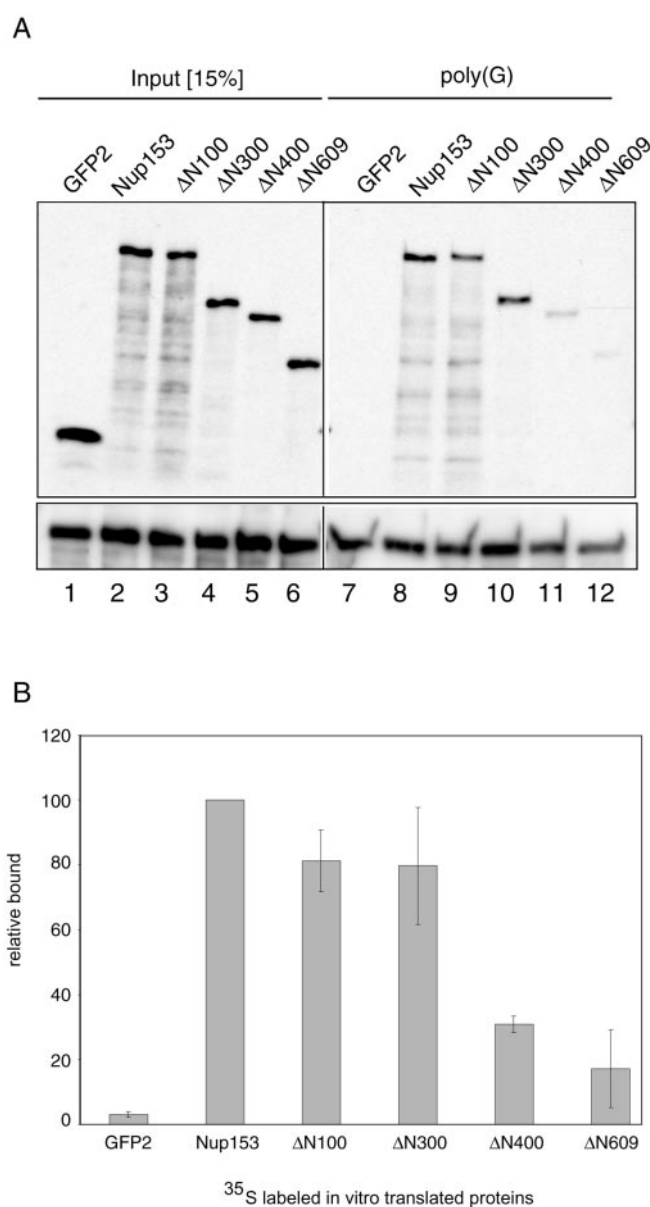
**Association with RNA Is Conserved in Both *Xenopus* and Human Nup153**—To probe further the importance of Nup153 association with RNA, we next asked whether this feature is conserved in human Nup153. Alignment of hNup153 with the available (incomplete) sequence of *Xenopus* Nup153 showed a relatively low level of identity in the N-terminal domain (40%), in the Zinc finger region (47%), and in the C-terminal region (34%) (Fig. 2A). To determine whether the property of RNA association is well conserved despite the divergence in sequence, Nup153 from the *Xenopus* A6 cell line and from the human HT29 cell line was screened in the homoribopolymer binding assay. Lysates made from these cell lines were incubated with the poly(G) resin and assayed as described above. Nup153 in both cell lysates was seen to associate with poly(G) in similar proportions (Fig. 2B, compare lanes 2 and 4 with 1



**FIG. 2. RNA association is a conserved property in *Xenopus* and human Nup153.** *A*, schematic alignment of full-length hNup153 with the incomplete xNup153 protein sequence, missing its N terminus (dashed line), illustrates a low level of sequence identity in the unique N terminus, the central zinc finger motif, and the FG C-terminal domain. Percent identity and percent similarity between regions of *Xenopus* and human Nup153 are indicated. *B*, Nup153 in the somatic cell lines, *Xenopus* A6 and human HT29, was assayed for RNA association in the homoribopolymer binding assay. Cell lysates were independently incubated with poly(G) immobilized onto agarose beads, separated on a 6.3% SDS-PAGE gel, and processed as described in Fig. 1*A*. In each case, Nup153 was found to associate with the RNA resin, whereas Nup62 remained unbound (lanes 2 and 4). The primary antibody, monoclonal antibody 414, reacts with other protein species in HT29 extract that migrate between Nup153 and Nup62. These are likely to be breakdown products of Nup153, some of which are able to associate with poly(G).

and 3, respectively). Furthermore, RNA association of both *Xenopus* and human Nup153 from tissue culture cells (somatic origin) recapitulates the binding efficiency of Nup153 in *Xenopus* egg extract (compare Fig. 1*A* with Fig. 2*B*).

**Deletion Analysis of Human Nup153 Indicates an N-terminal Region Important for RNA Association**—We next set out to map the region within Nup153 responsible for mediating RNA association by generating a series of 5' deletions in the human *NUP153* gene. These N-terminal truncation mutants were *in vitro* translated and incubated with poly(G) resin and *Xenopus* egg extract and monitored for their ability to associate with the RNA as described above. Proteins associated with the resin were eluted, separated by SDS-PAGE, and analyzed by autoradiography. In parallel, xNup153 endogenous in the egg extract was monitored by Western blot on samples of the same elutions. This provided an internal control for the specificity of any reduction in binding. Nup153 mutants lacking the first 400 or 609 amino acids demonstrated a significant loss of association to poly(G) compared with full-length Nup153 or mutants lacking up to the first 300 amino acids (Fig. 3*A*, compare lanes 11 and 12 with lanes 8–10). This finding indicated that a region within amino acids 300–400 in the N terminus is important for mediating RNA association. A GFP dimer construct (GFP2) with no known RNA-binding property was used as a negative control and showed no significant association with the poly(G) resin (Fig. 3*A*, lane 7). Consistent levels of RNA association by endogenous xNup153 were detected in each sample (Fig. 3*A*, bottom right panel). Results of three independent experiments were quantified by PhosphorImager analysis (Molecular Dy-



**FIG. 3. Regions important for RNA association are located in the N terminus of hNup153.** *A*, truncations of hNup153 were *in vitro* translated and incubated with poly(G)-agarose beads and *Xenopus* egg extract (see “Experimental Procedures”). Samples were split and run on two separate 6.3% SDS-PAGE gels. Labeled *in vitro* translated proteins associated with the poly(G) resin were monitored by autoradiography (upper panels). *In vitro* translated proteins are seen in lanes 1–6. hNup153 mutants missing the first 400 and 609 amino acids lose poly(G) association (lanes 11 and 12) compared with full-length Nup153 (lane 8) and mutants ΔN100 and ΔN300 (lanes 9 and 10). A green fluorescent protein (GFP2) dimer construct served as a negative control (lane 7). Endogenous xNup153 was monitored in parallel by Western blot with anti-xNup153-specific antibodies and was shown to be unaffected in the presence of the translated mutants (bottom panel, lanes 7–12). *B*, quantitation of three experiments performed as in *A* was done using a PhosphorImager (Molecular Dynamics).

namics), normalized for input levels, and graphed relative to full-length Nup153 (Fig. 3*B*). Deletion of N-terminal regions up to amino acid 400 reduced RNA association by 69%.

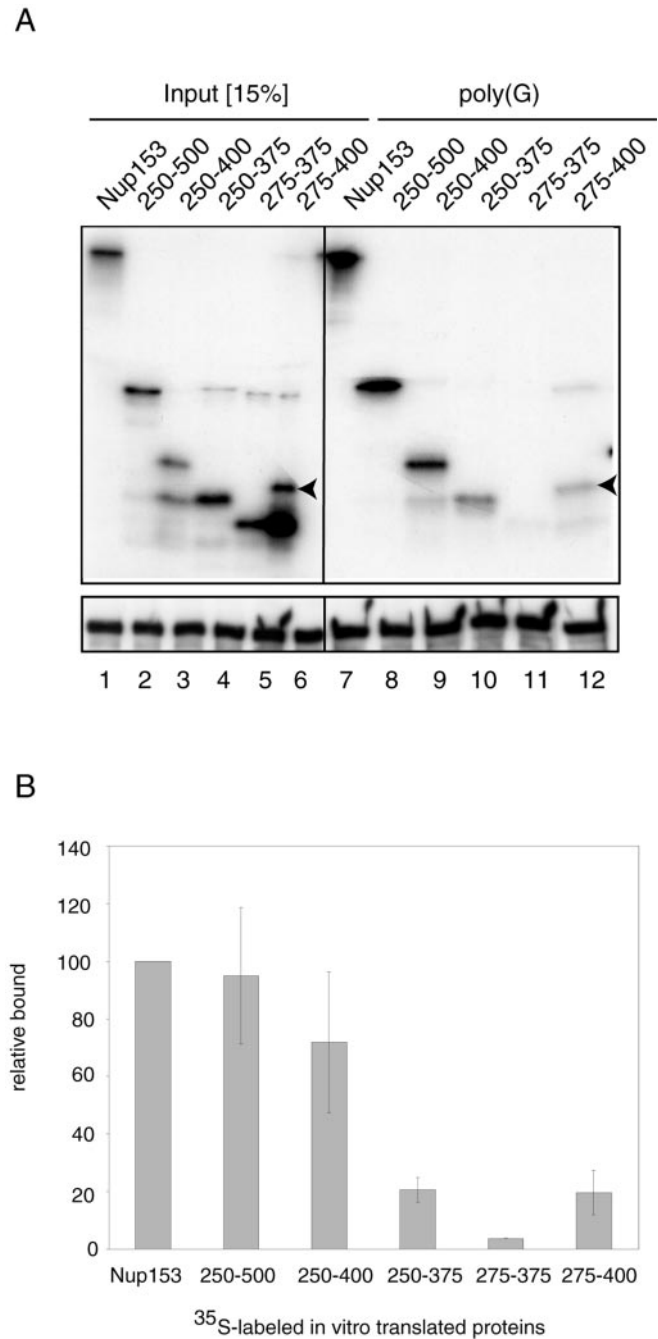
**Mapping Studies Delineate a 150-Amino Acid Domain Sufficient for RNA Association**—To identify the minimal region sufficient to confer RNA association, subsequent homoribopolymer binding assays were done using a panel of small fragments to narrow down the interaction domain within the hNup153 N terminus. Because the ΔN400 truncated protein

showed reduced affinity for RNA, regions upstream of and overlapping with amino acid 400 were tested in the binding assay. The templates for these fragments were generated by PCR in which a T7 promoter and Kozak sequence were engineered at the 5' end. *In vitro* translated fragments incubated with *Xenopus* egg extract and poly(G) beads were processed and analyzed as described above. In some cases smaller products are seen by gel analysis. These are likely due to internal initiation sites, premature translation termination, or proteolytic breakdown. For purposes of quantitation, the incomplete products were disregarded. Fragments encompassing amino acids 250–400 were found to be sufficient for RNA association (Fig. 4A, lane 9; 99% of full-length binding activity), whereas fragments composed of amino acid 250–375 or 275–400 lost significant efficiency in RNA association (Fig. 4A, lanes 10 and 12; 25 and 29%). A fragment composed of amino acids 275–375 did not exhibit RNA association (Fig. 4A, lane 11; 4%), indicating that this central core requires flanking sequences. Either N-terminal or C-terminal short flanking sequences (found in 250–375 or 275–400 fragments) provide sufficient additional sequence for low level association of this core fragment, but clearly a fragment encompassing amino acids 250–400 most closely mirrors the RNA association activity of full-length Nup153. This pinpoints a 150-amino acid domain responsible for the property of RNA association (Fig. 4, A and B).

**Cloning of the *Xenopus* Nup153 N Terminus**—The RNA association domain maps to an N-terminal region in hNup153 that was not present in the existing xNup153 clone, which lacks the 5' region of the cDNA. To address further questions regarding the conservation of the ability of this domain to associate with RNA, it was therefore necessary to obtain the rest of the N-terminal sequence of xNup153. The 5' region missing from the original cDNA clone of xNup153 was cloned from a *Xenopus* library by PCR as detailed under “Experimental Procedures.” Conceptual translation (Fig. 5A) and alignment between the newly identified *Xenopus* sequence and the sequence for human Nup153 shows a relatively high degree of identity at 63%.

**RNA Association by Nup153 Is Evolutionarily Conserved**—With the full-length sequence for xNup153 in hand, a minimal fragment corresponding to the region sufficient for RNA association in hNup153 was generated. This *Xenopus* fragment was found to retain the ability to associate with the poly(G) beads (Fig. 5C, lane 7), demonstrating functional conservation of this RNA association domain (amino acids 250–400 in human and 260–410 in *Xenopus*).

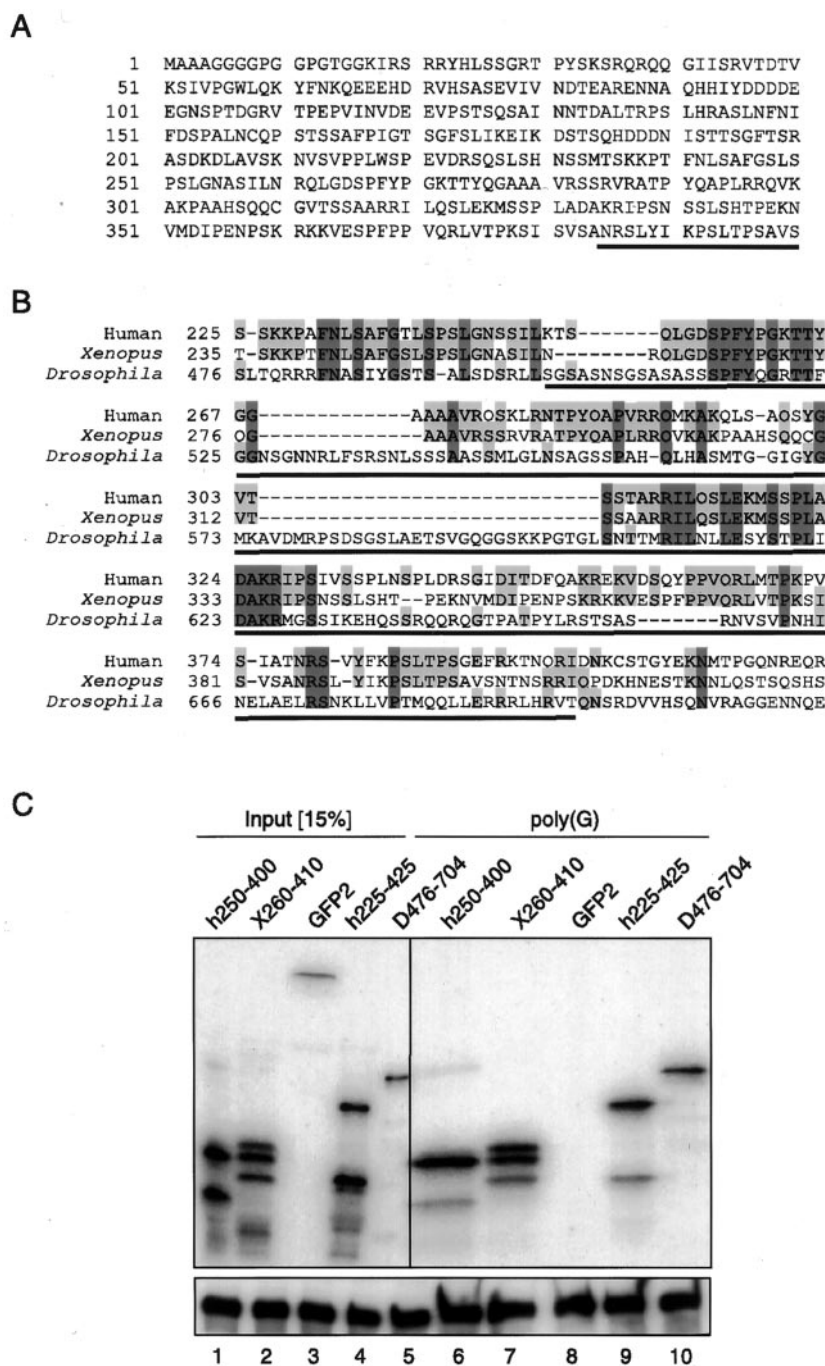
To investigate further the evolutionary conservation of Nup153 RNA association, a fragment of Nup153, designed to encompass the putative RNA association domain, was isolated from a *Drosophila* cDNA library. A similar fragment was generated from hNup153 for comparison. Note that due to the insertion of sequence in dNup153 relative to regions of alignment (Fig. 5B), this is a larger fragment in *Drosophila* in the region encompassing the RNA association domain (Fig. 5C, lanes 4 and 5). The *Drosophila* Nup153 fragment was found to associate with poly(G) (Fig. 5C, lane 10), underscoring the conserved nature of RNA binding in Nup153 across species. Sequence comparison of Nup153 shows a marked difference in overall identity between *Xenopus*, human, and *Drosophila* in the region encompassing the RNA association domain (Fig. 5B). However, isolated spans of amino acid residues show high levels of sequence conservation and will provide insight into future structure-function studies.



**FIG. 4. hNup153 amino acids 250–400 are sufficient for RNA association.** A, fragments of hNup153 were translated *in vitro*, incubated with poly(G)-agarose beads and *Xenopus* egg extract, separated on either 15 (upper panel) or 6.3% (lower panel) SDS-PAGE, and processed as described in Fig. 3. A fragment of hNup153 encompassing amino acids 250–400 was found to retain RNA association (lane 9), whereas a fragment encompassing amino acids 275–375 could not (lane 11). Low levels of association were found with fragments composed of amino acids 250–375 (lane 10) and 275–400 (lane 12). The arrowhead indicates where the full-length 275–400 product migrates on the gel. As described in Fig. 3A, endogenous xNup153 was monitored by Western blot (lower panel). B, quantitation of three experiments performed as in A was done using a PhosphorImager (Molecular Dynamics).

**The RNA Association Domain within Nup153 Has Intrinsic Affinity for RNA**—*Xenopus* egg extract was initially included in the homoribopolymer binding assay to account for any possible adapter or receptor protein that might act as a bridging factor for Nup153 binding to RNA. To determine whether a bridging factor is required to mediate binding, a fragment of xNup153 was purified from bacteria and used as a tool to ask whether a

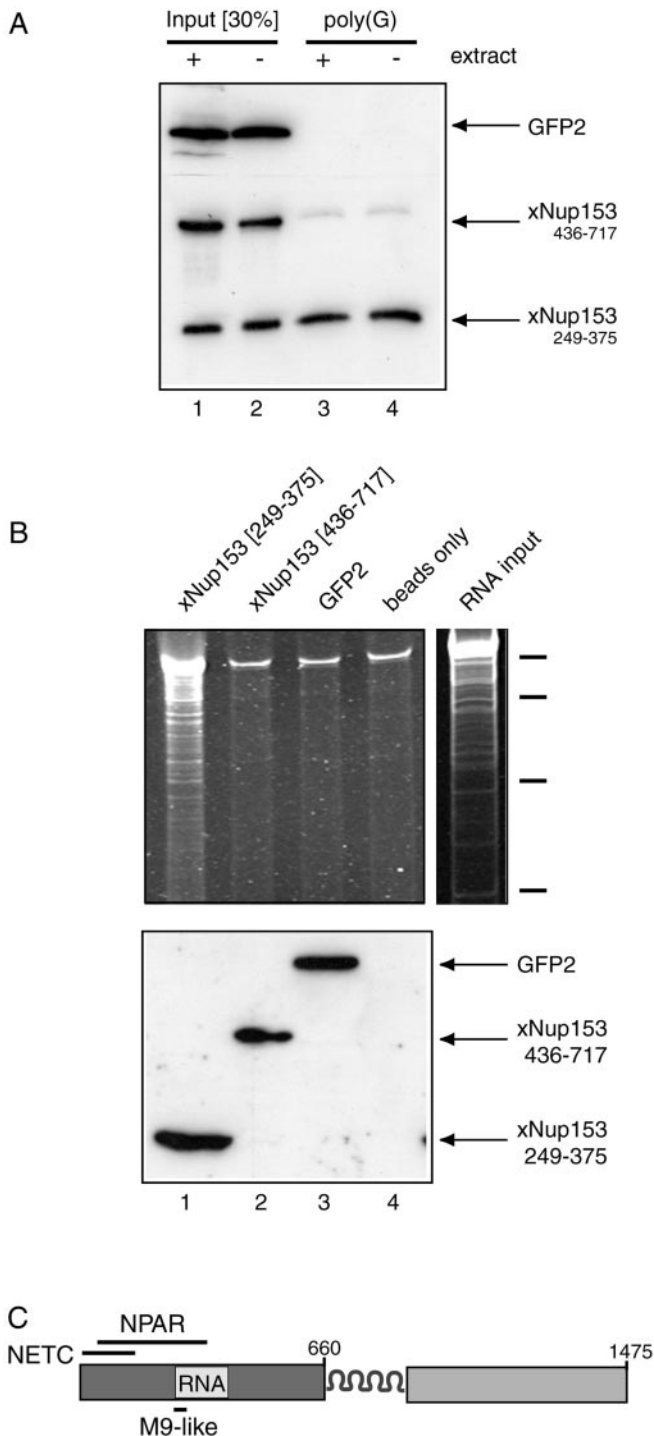
**FIG. 5. Cross-species analysis of RNA association.** A, cloning of the N terminus of *Xenopus* Nup153 was completed using PCR techniques (see "Experimental Procedures"). The new N-terminal sequence of xNup153 is depicted. Sequence from the original clone is *underlined*. B, cross-species sequence comparison of a region encompassing the minimal Nup153 RNA association domain. Amino acids conserved in all three species are *darkly shaded*; amino acids conserved between two sequences are more *lightly shaded*. In addition, there are many instances of highly conserved residues. The *underlined region* indicates the minimal RNA association domain. C, an xNup153 fragment encompassing the amino acids equivalent to the RNA association domain in hNup153 was generated by *in vitro* translation and analyzed as described in Fig. 4. This xNup153 fragment shows efficient association with the poly(G) resin (*lane 7*), comparable with its human counterpart (*lane 6*). A fragment of Nup153 encoding the putative RNA association domain from *Drosophila* Nup153 was *in vitro* translated and processed as described in Fig. 3. The *Drosophila* fragment was found to associate with RNA (*lane 10*) comparably to an approximately equivalent hNup153 fragment encompassing amino acids 225–425 (*lane 9*). GFP2 showed no significant association with the RNA resin (*lane 8*).



direct binding interaction between Nup153 and RNA could be detected. We found that this fragment (amino acids 249–375) bound to poly(G) resin both in the presence and in the absence of *Xenopus* egg extract (Fig. 6A, lanes 3 and 4) demonstrating an intrinsic ability of Nup153 to bind RNA. As a control, two recombinant proteins were tested in the assay. A distinct Nup153 fragment (amino acids 436–717), lacking the domain that mediates RNA association, showed no significant association with poly(G) (Fig. 6A, lanes 3 and 4). Another recombinant protein, GFP2, with no known RNA binding domain, was used as an additional control and showed no significant binding to poly(G) (Fig. 6A, lane 3 and 4).

**The RNA Association Domain within Nup153 Can Interact with Endogenous RNA**—We next wanted to determine whether the newly defined RNA binding region could interact with physiological RNA targets. The recombinant fragments of

*Xenopus* Nup153 (amino acids 249–375 and 436–717) and the GFP dimer, GFP2, were independently immobilized onto agarose beads and incubated with RNA isolated from *Xenopus* A6 cells. Samples were then assessed for the presence of RNA. Endogenous RNAs were found to associate with the xNup153-(249–375) fragment (Fig. 6B, lane 1, upper panel). In comparison, the two control fragments, xNup153-(436–717) and GFP2 (Fig. 6B, lanes 2 and 3, upper panel), showed no significant interaction with endogenous RNA above the level of background accounted for by the agarose beads themselves (Fig. 6B, lane 4, upper panel). Of note, RNase digestion confirmed that all major species detected were RNA (data not shown). To characterize specificity further, DNA was also tested for binding to the recombinant proteins. Under equivalent conditions, an array of DNA fragments generated from a plasmid digest showed no association with the recombinant Nup153 fragment



**FIG. 6. Nup153 can bind directly to RNA.** *A*, fragment encompassing amino acids mapped to the RNA association domain of xNup153 (amino acids 249–375) was expressed and purified from bacteria (see “Experimental Procedures”). This fragment was incubated with the poly(G) resin both in the presence and in the absence of *Xenopus* egg extract, along with another recombinant fragment of Nup153 (amino acids 436–717; this corresponds to amino acids 53–334 from the original clone) and a recombinant GFP construct (*GFP2*). After incubation and washing, proteins were eluted with SDS sample buffer, separated on a 12% SDS-PAGE gel, and monitored by Western blot using anti-T7 antibodies. The N-terminal fragment of amino acids 249–375 was found to efficiently bind the RNA resin under both conditions tested (*lanes 3 and 4*), compared with the fragment of amino acids 436–717 (*lanes 3 and 4*) and a GFP construct (*lanes 3 and 4*). Recombinant proteins loaded onto the RNA resin are shown in the presence of extract (*lane 1*) and in the absence of extract (*lane 2*). *B*, immobilized recombinant proteins were incubated with endogenous RNA isolated from *Xenopus* A6 cells. After incubation and washes, half of each sample was

(amino acids 249–375) demonstrating that the RNA binding domain of Nup153 does not confer a general affinity for nucleic acid (data not shown).

#### DISCUSSION

Addressing the role of individual nuclear pore proteins is essential to reaching a mechanistic understanding of how transport machinery at the NPC works. In this study, we have focused on Nup153 and have characterized a novel domain within this pore protein that can associate directly with RNA. The use of homopolymeric RNA immobilized onto agarose beads offers an *in vitro* assay in which a stable association between proteins and RNA can be detected in an unbiased, sequence-independent fashion (33). Employment of this *in vitro* RNA binding assay as a tool with *Xenopus* egg extract at the outset offers two important advantages. First, an abundance of nuclear pore complex components in soluble form allows comparison of various nucleoporins for their ability to associate with RNA. Second, an interaction with RNA is likely to be detected regardless of the molecular complexity involved. In other words, *Xenopus* egg extract is a rich source of proteins that may serve as bridging factors to RNA. Nup153 shows relatively strong association with poly(G) in contrast to other vertebrate nucleoporins in the panel tested, which originally included Nup358, Nup214, Nup153, Nup98, and Nup62 (29) and was both confirmed and expanded here to also include Nup93 and the nucleoporin-like protein RIP/Rab (Fig. 1A).

Several transport receptors in the Importin  $\beta$  superfamily are known to have docking sites on Nup153 and thus were candidates for participating in an indirect interaction between Nup153 and RNA. However, when Importin  $\beta$  (data not shown), Transportin, and Exportin1/Crm1 (Fig. 1B) were tested in the homoribopolymer binding assay, none were found to associate with RNA in this assay. Similarly CAS, another member of this family, was not detected in the pull-down assay (Fig. 1B). In addition, potential adapter proteins, Gle2/RAE1 (35) and REF/Aly (36, 37), did not detectably associate with poly(G) under the conditions tested (Fig. 1B). Coilin, an RNA-binding protein that was shown previously to bind poly(G) and characterized to be a partner of U7 small nuclear RNA (38) was detected in the pull-down assay as expected (data not shown). However, coilin was not found to co-immunoprecipitate with Nup153 (data not shown), suggesting that coilin does not serve to bridge the association between RNA and Nup153. This analysis of poly(G)-associated proteins did not rule out the possibility that other proteins are involved in mediating Nup153 interaction with RNA but provided an indication that this interaction may not require a bridging adapter protein. To answer definitively the critical question of whether the interaction between Nup153 and RNA is direct or indirect, a fragment (amino acids 249–375) of xNup153 that mapped to the

subjected to RNA isolation and analysis on a 6% denaturing, acrylamide gel (*upper panel*). A sample of input RNA (1.2  $\mu$ g) is shown in *lane 5* (exposed 4 times shorter than *lanes 1–4*). xNup153 fragment (amino acids 249–375) was found to bind endogenous RNA from the A6 cells (*lane 1*), compared with the xNup153 fragment of amino acids 436–717 (*lane 2*), the GFP2 construct (*lane 3*), and the antibody-coupled agarose beads alone (*lane 4*) that showed no significant RNA binding activity. Migration of RNA markers is indicated at *right*. Dashes correspond to markers at 1770, 1520, 1200, and 780 nucleotides. To ensure equal recovery, parallel samples underwent protein analysis on a 12% SDS-PAGE gel, followed by Western blot with anti-T7 antibodies (*lower panel*). *C*, domains mapped in the N-terminal region of human Nup153. The nuclear envelope targeting cassette (*NETC*, amino acids 3–144), the nuclear pore complex assembly region (*NPAR*, amino acids 39–339), an M9-like localization signal (*M9-like*, amino acids 247–290), and the newly identified RNA association domain (*RNA*, amino acids 250–400) are indicated.

RNA association domain (see below) was engineered, expressed, and purified from bacteria. This recombinant protein was found to bind to poly(G) both in the presence and the absence of egg extract, demonstrating an intrinsic affinity for RNA (Fig. 6A). Moreover, we have also found that this recombinant fragment of Nup153 can interact directly with RNA isolated from cultured *Xenopus* cells (Fig. 6B). Although Nup153 lacks a requirement for bridging factors, the direct interaction with RNA may certainly be facilitated/regulated *in vivo* by adapter proteins that mediate cooperative interactions at other sites on Nup153 and/or different pore components.

To determine the importance of RNA association originally observed with Nup153 from the *Xenopus* egg extract, we began by testing its conservation in human Nup153. By analyzing Nup153 in somatic cell lines of both *Xenopus* and human origin, we found that Nup153 from *Xenopus* A6 cells and human HT29 cells could recapitulate the RNA association seen with Nup153 endogenous in *Xenopus* egg extract (Fig. 2B). Therefore, despite their overall divergence in sequence, both *Xenopus* and human Nup153 maintain RNA association, suggesting that this biochemical property reflects a significant functional feature of Nup153 rather than being a fortuitous consequence of the sequence. This point is further underscored by our observation that a region from the *Drosophila* homologue of Nup153 also exhibits the ability to associate with RNA *in vitro* (Fig. 5C, see below).

To map the domain within Nup153 responsible for mediating RNA association, hNup153 N-terminal deletion mutants were tested for RNA association in the homoribopolymer binding assay. Whereas a truncation mutant lacking the first 300 amino acids retained the ability to associate with RNA, a mutant lacking the first 400 amino acids showed significant reduction in RNA association, implicating residues within the 300–400-amino acid region of Nup153 as important for mediating this specialized property (Fig. 3A, lanes 10 and 11). Even when the entire N-terminal domain of Nup153 was removed, some residual RNA association activity remained however (Fig. 3A, lane 12; 11%). This residual binding activity was often observed and could represent a secondary site for RNA association in Nup153. These C-terminal sequences contain a zinc finger-like motif and an FG repeat domain. Although the zinc finger region seemed a likely site for RNA binding, when this domain was tested it had no activity in our assay (data not shown), implicating a region in the C-terminal FG domain as a possible weaker affinity site for RNA association. Interestingly, previous studies (39) have characterized the ability of the rat Nup153 zinc finger domain to bind to DNA in a zinc-dependent manner. The primary sites for DNA and RNA interaction with Nup153, therefore, appear to be distinct.

To delineate further the region necessary and sufficient for mediating RNA association, N-terminal fragments were tested. A domain encompassing human Nup153 amino acid positions 250–400 was shown to be sufficient for mediating RNA association (Fig. 4A, lane 9). The sequence between 400 and 500 appears to enhance RNA association further, and this contribution may explain why the first 300 amino acid residues are dispensable when C-terminal sequences are present (Fig. 3A, lane 10). Several other functional domains have also been identified within the hNup153 N terminus (Fig. 6C). The nuclear envelope targeting cassette, a domain responsible for guiding Nup153 to the nuclear envelope, has been mapped to amino acids 3–144 (22). An M9-like localization signal, which mediates interaction with Transportin, has been mapped to amino acids 247–290 in human Nup153 (23), overlapping with a part of the newly identified RNA association domain. The previously characterized nuclear pore complex assembly region at amino

acids 39–339 (22) also overlaps to some extent with the RNA association domain (Fig. 6C). However, the nuclear pore complex assembly region and RNA association domain are apparently separable in that we have found RNA association is retained in Nup153 lacking the first 300 residues, whereas this protein would be predicted to have lost its pore targeting ability.

Although pore targeting and RNA association appear to require distinct regions, the proximity of these two domains raises the question of how accessible the RNA association domain is at the NPC. Different observations suggest, however, that once targeted to the pore, Nup153 could be reconfigured to further expose the RNA association domain. For instance, the pore basket itself has been shown to adopt different conformations (40, 41) raising the possibility that protein-protein interactions at the pore have some flexibility. Indeed, Nup153 has been proposed to be a mobile component of the pore (23, 42). To probe further the domain accessibility of Nup153 at the pore, we are currently involved in performing immunoelectron microscopy with domain-specific antibodies.

Although still poorly understood, there is a precedent for RNA association with pore proteins and pore-associated proteins. The yeast GLFG nucleoporins, Nup145p and Nup116p, have been found to associate with poly(G) and poly(U) homoribopolymers (43). Moreover, depletion of Nup145p *in vivo* results in poly(A)<sup>+</sup> RNA accumulation in the nucleus. Although Nup153 and Nup145p share the biochemical property of RNA association, this ability is based on distinctly different sequence motifs. The vertebrate GLFG protein, Nup98, has been shown to function in the export of several classes of RNAs (44) and also contains an RNP1-like motif similar to that in yeast GLFG proteins. Whereas Nup98 does not share the property of association with poly(G) *in vitro* (see Ref. 29 and Fig. 1A), this assay reflects only a subset of RNA interactions. Indeed, Nup98 may interact indirectly with RNA *in vivo*. This indirect association could be mediated by its partner protein Gle2/RAE1 (35), which has been found to associate with mRNA by cross-linking studies (45). The pore-associated protein Dbp5 is another example of a protein at the pore that likely provides an interface with RNA. Dbp5, a DEAD box protein that can interact with and unwind RNA in a nucleotide-dependent manner, is thought to play a role in mRNA export (46–48). RNA cargo packaged into RNP complexes may have exposed regions of RNA available for contact with pore machinery. Alternatively, reconfiguration of RNP structure could be a prerequisite for interactions with pore components. Indeed, interactions between RNA and proteins at the NPC may reflect a role in remodeling of RNA cargo prior to and during export. Nup153 could be involved either as a chaperone or as a passive platform in this process, although future studies are needed to test these models. Alternatively, *in vitro* RNA binding could mimic interaction with a novel structural RNA component of the NPC that normally associates with Nup153. This model would predict that a homogeneous population of RNA is in association with Nup153 in egg or tissue culture cell extracts. We have found no evidence to support this however (data not shown). Certainly, under the conditions of the RNA binding assay here (Fig. 6B), Nup153 is able to associate with a broad range of RNA species. These observations are more consistent with a role for Nup153 in contacting a range of RNA cargo during the process of transport.

Sequence alignment of Nup153 from *Drosophila*, *Xenopus*, and human reveals that considerable sequence divergence has occurred at the RNA association domain; however, isolated clusters maintain high levels of identity (Fig. 5B). Amino acid sequence of the conserved residues does not appear to match

any known RNA-binding motifs, and therefore, we believe this region of Nup153 comprises a novel RNA binding domain. Future studies will be directed at understanding the nature of this association with RNA and at elucidating how this specialized property contributes to the function of Nup153 at the nuclear pore complex.

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