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Novel vertebrate nucleoporins Nup133 and Nup160 play a role in mRNA export

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RNA undergoing nuclear export first encounters the basket of the nuclear pore. Two basket proteins, Nup98 and Nup153, are essential for mRNA export, but their molecular partners within the pore are largely unknown. Because the mechanism of RNA export will be in question as long as significant vertebrate pore proteins remain undiscovered, we set out to find their partners. Fragments of Nup98 and Nup153 were used for pulldown experiments from *Xenopus* egg extracts, which contain abundant disassembled nuclear pores. Strikingly, Nup98 and Nup153 each bound the same four large proteins. Purification and sequence analysis revealed that two are the known vertebrate nucleoporins, Nup96 and Nup107, whereas two mapped to ORFs of unknown function. The genes encoding the novel proteins were cloned, and antibodies were produced. Immunofluorescence reveals them to be new nucleoporins, designated Nup160 and Nup133, which are accessible on the basket side of the pore. Nucleoporins Nup160, Nup133, Nup107, and Nup96 exist as a complex in *Xenopus* egg extracts and in assembled pores, now termed the Nup160 complex. Sec13 is prominent in Nup98 and Nup153 pulldowns, and we find it to be a member of the Nup160 complex. We have mapped the sites that are required for binding the Nup160 subcomplex, and have found that in Nup98, the binding site is used to tether Nup98 to the nucleus; in Nup153, the binding site targets Nup153 to the nuclear pore. With transfection and in vivo transport assays, we find that specific Nup160 and Nup133 fragments block poly[A]$^+$ RNA export, but not protein import or export. These results demonstrate that two novel vertebrate nucleoporins, Nup160 and Nup133, not only interact with Nup98 and Nup153, but themselves play a role in mRNA export.

Introduction

The nuclear pore mediates export from the nucleus. For proteins, a soluble nuclear export receptor binds Ran-GTP and a protein cargo bearing a nuclear export sequence (NES)* to form a trimeric export complex (Mattaj and Englmeier, 1998; Gorlich and Kutay, 1999; Damelin and Silver, 2000; Ryan and Wente, 2000; Conti and Izaurralde, 2001; Vasu and Forbes, 2001). The complex translocates through the pore, pausing on the cytoplasmic filaments of the pore. There, Ran-GTP hydrolysis disassembles the complex and completes export. Individual receptors have been tailored for specific cargo, such that Crm1/exportin1 carries proteins bearing leucine-rich NESs, whereas exportin-t carries newly transcribed tRNAs. Multiple different proteins have been implicated in mRNA export (for review see Conti and Izaurralde, 2001).

The vertebrate pore at 120 million daltons is estimated to contain ~30–60 different proteins. Each is present in ≥8–32 copies, giving perhaps 1,000 proteins per pore. Only a subset of vertebrate pore proteins is known (Vasu and Forbes, 2001). Structurally, the pore consists of three stacked rings of ~1,200 Å. The middle ring contains eight thick spokes surrounding a central transporter (Stoffler et al., 1999; Allen et al., 2000; Ryan and Wente, 2000; Vasu and Forbes, 2001). At one face of the pore, cytoplasmic filaments extend to interact with incoming or outgoing receptor complexes. On the opposite or nuclear face of the pore, eight long filaments connect to a 500-Å ring to form the nuclear basket of the pore. A large mRNA/protein cargo, the Balbiani transcript, has been seen to thread through the basket during export (Kiseleva et al., 1996). It is hypothesized that other export cargos follow a similar pathway.

To date, only two basket nucleoporins have been shown to play a critical role in vertebrate RNA export, Nup98 and

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*Abbreviations used in this paper: aa, amino acid(s); AL, annulate lamellae; GFP, green fluorescent protein; IB, immunoblotting; IF, immunofluorescence; LMB, leptomycin B; NES, nuclear export sequence.

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Nup153 (Bastos et al., 1996; Powers et al., 1997; Ullman et al., 1999). Antibodies to Nup98 block mRNA, snRNA, 5S RNA, and preribosome particles export, whereas these antibodies have no effect on tRNA export or NLS-mediated import (Powers et al., 1997). Vertebrate Nup98 resembles three yeast nucleoporins involved in RNA export, Nup100p, Nup116p, and Nup145p, having features in common with each and identity with none (Powers et al., 1995; Radu et al., 1995; Stutz et al., 1996; Iovine and Wente, 1997; Bailer et al., 1998; Pritchard et al., 1999; Zolotukhin and Felber, 1999; Bachi et al., 2000; Fontoura et al., 2000; Strasser et al., 2000). Although all have GLFG repeats capable of binding different transport receptors in vitro, only Nup116p and Nup98 contain a sequence that binds the small transport factor Gle2 (Murphy et al., 1996; Bharathi et al., 1997; Bailer et al., 1998; Zolotukhin and Felber, 1999). Nup98 also resembles yeast Nup145 in that both are synthesized as precursors capable of self-cleavage into two nucleoporins: Nup98 and Nup96 in vertebrates, and Nup145N and Nup145C in yeast (Emtage et al., 1997; Teixeira et al., 1997, 1999; Fontoura et al., 1999; Rosenblum and Blobel, 1999). The yeast GLFG proteins are found on both sides of the yeast pore (Rout et al., 2000), whereas Nup98 is concentrated on the nuclear face of the pore and in the nuclear interior (Powers et al., 1995; Radu et al., 1995; Zolotukhin and Felber, 1999). Clearly, evolution has responded to challenges yet to be elucidated.

Nup153 is the only vertebrate nucleoporin reported to map to the distal ring of the basket (Panté et al., 1994). Functionally, Nup153 is critical for export (Bastos et al., 1996; Ullman et al., 1999). Overexpression of certain domains of Nup153 causes poly[A]$^+$ RNA accumulation in the nucleus (Bastos et al., 1996). Moreover, antibodies to Nup153 block mRNA, snRNA, and 5S RNA export, as well as NES protein export and Rev-dependent HIV RNA export (Ullman et al., 1999). The antibodies do not block tRNA export, recycling of importin β to the cytoplasm, or NLS-mediated import. Nup153 also functions in the terminal steps of nuclear import, binding importin α–β–NLS complexes and transportin (Shah et al., 1998; Shah and Forbes, 1998; Nakielny et al., 1999). Interestingly, the presence of Nup153 on the nuclear pore depends on an intact nuclear lamina (Smythe et al., 2000), whereas photobleaching shows that vertebrate Nup153 can exchange on and off the pore with fast kinetics (Daigle et al., 2001; Lyman and Gerace, 2001), raising further interest in the role of Nup153 on the pore.

In the rat, Nup153 is comprised of a unique NH_2 terminus, four central zinc fingers, and 32 FXFG and FG repeats at the COOH terminus (see Fig. 2 a) (Sukegawa and Blobel, 1993; Bastos et al., 1996; Shah et al., 1998). Yeast has no sequence homologue of Nup153, and no yeast nucleoporins contain Zn fingers. It is possible that yeast Nup1 may fulfill certain of the functions of Nup153, as it is localized to the distal basket and can bind importin β (Davis and Fink, 1990; Rout et al., 2000). However, beyond possessing FG repeats, yeast Nup1 bears no sequence resemblance to Nup153.

Yeast and vertebrate nuclear pores are separated by a billion years of evolution (Gouy and Li, 1989). The vertebrate pore is reported to be five times the volume and twice the longitudinal axis of the yeast pore, containing a number of different structural elements (Yang et al., 1998; Rout et al., 2000). Interestingly, whereas the soluble receptors and factors used in nuclear transport have been relatively well conserved, the nuclear pore proteins themselves have diverged dramatically (Mattaj and Englmeier, 1998; Stoffler et al., 1999; Ryan and Wente, 2000; Conti and Izaurralde, 2001; Vasu and Forbes, 2001). Four different protein scenarios have been observed: (A) A small subset of nucleoporins are fairly similar in sequence in vertebrates and yeast (vNup155/ScNup157/ScNup170 and vNup93/ScNic96; 21 and 24% identity, respectively); (B) Other vertebrate pore proteins such as Nup98 are related to multiple different yeast nucleoporins; (C) Others have no yeast homologues and vice versa (gp210, POM121, POM152); and (D) Yet others, such as Nup153 and Nup214, have no sequence homologues in yeast but are suspected to have analogues. One last difference is that the majority of yeast nucleoporins are symmetrically localized to both sides of the pore (Rout et al., 2000), whereas many vertebrate nucleoporins are found on a specific face of the pore (Vasu and Forbes, 2001). Given the evolutionary divergence in size, architecture, composition, and protein sequence between yeast and vertebrate pores, identifying the proteins of and providing a structure for the 120 million dalton vertebrate pore remains a daunting task.

The importance of Nup98 and Nup153 is clear from the findings that they function in RNA export, protein import, and most recently, viral infection (Petersen et al., 2000; von Kobbe et al., 2000; Gustin and Sarnow, 2001). In the five years since their discovery, little evidence has been found to connect them to one another or to other nucleoporins. A recent exception is Nup50, required for protein export (Guan et al., 2000). Here we report four large proteins that interact with Nup98. The same four proteins also bind Nup153. We demonstrate that all four are nucleoporins, two known and two hitherto unknown, which we now term vertebrate Nup160 and Nup133. All are present in a large subcomplex of the nuclear pore. The complex appears to play a role not only in tethering Nup98 and Nup153 to the nucleus and the pore, but also in vertebrate mRNA export.

Results

Novel molecular partners for the RNA export nucleoporin, Nup98

To more clearly define those components of the nuclear pore required for RNA export, the protein partners of Nup98 were sought. The amino half of Nup98, containing GLFG repeats and a Gle2-binding site, is thought to interact primarily with transport factors. Thus, we focused on the carboxyl half of Nup98 as a likely site of interaction with putative nucleoporins. Recombinant Nup98 fragments complexed to Sepharose beads were used for pull-downs from extracts of *Xenopus* eggs containing the disassembled proteins of $\sim 2.5 \times 10^8$ nuclear pores (Cordes et al., 1995). The Nup98 COOH terminus was found to bind a distinct set of silver staining proteins from *Xenopus* egg extracts, (Fig. 1 b, lanes 3–5). Those <60 kd were determined to be either nonspecifically bound, as they were also pulled down by protein A-Sepharose (Fig. 1 b, lane 2),

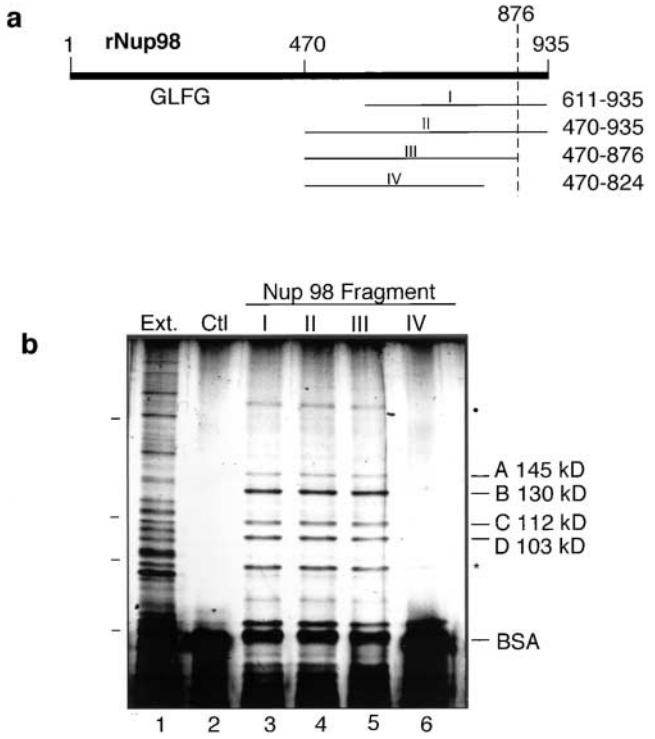

Figure 1. **Proteins A–D bind to the COOH terminus of Nup98.** (a) Subcloned fragments I–IV of rat Nup98. The dotted line shows the cleavage site for Nup98 endoproteolytic activity (Fontoura et al., 1999; Rosenblum and Blobel, 1999). Gle2 binds to Nup98 aa 150–224, whereas GLFG repeats occupy much of the remainder of aa 1–470. (b) Nup98 fragments I–IV coupled to beads were mixed with *Xenopus* egg cytosol in pulldown reactions. The * indicates a ~97-kd Ran-sensitive protein. The filled circle indicates a band variably seen. Proteins A–D bound to fragments I–III (lanes 3–5), but not to fragment IV (lane 6) or *S. aureus* protein A beads (Ctl, lane 2). *Xenopus* egg extract (Ext; 0.02 μl) is shown for comparison (lane 1). Size markers are 205, 116, 97, and 66 kd, respectively (left hatchmarks).

or were obscured by BSA. An ~97-kd protein was also observed to bind (*, Fig. 1 b, lanes 3–5), but because this protein was greatly decreased after Ran-GTP addition (*, Fig. 2 c, compare lanes 3 and 4), it resembled a Ran-sensitive transport receptor and was not analyzed further. Bands present in lesser amounts or variably present from experiment to experiment (dot, Fig. 1 b, lanes 3–5) also were not analyzed further.

Our attention focused on four large proteins, designated A–D, consistently pulled down by the COOH terminus of Nup98 (Fig. 1 b, lanes 3–5). Proteins A–D bound to Nup98 fragments I, II, and III. We found that fragments I and II become self-cleaved in vitro, as occurs with endogenous Nup98, to produce a COOH terminus at amino acid (aa) 876 (unpublished data; Fontoura et al., 1999; Rosenblum and Blobel, 1999). We concluded that the Nup98 binding site(s) for the A–D proteins must be upstream of the $HF_{876}SKY$ cleavage site (Fig. 1 a, dotted line). When Nup98 aa 470–824 was used, proteins A–D no longer bound (Fig. 1 b, fragment IV). Thus, Nup98 aa 825–876 are critical for binding the A–D proteins in that removal of these terminal 52 aa abolishes binding. The fact that all four are lost with the same small deletion first suggested that they might bind to Nup98 as a complex.

The A–D proteins migrate on gels at ~145, 130, 112, and 103 kd, respectively (Fig. 1 b). Their binding to Nup98 fragments is Ran insensitive (Fig. 2 c, lanes 3–4), as well as stable to 500 mM NaCl (unpublished data), indicative of strong protein–protein interactions. Because the Nup98 fragments used above all contain a potential, if abbreviated, RNA-binding motif (Radu et al., 1995), and because Nup98 binds to certain homoribopolymers in vitro (Ullman et al., 1999), we tested for an effect of RNase on the Nup98/A–D interaction; we found none (unpublished data). This suggests that no RNA moiety is required for the formation or the maintenance of the Nup98/A–D interactions.

The basket nucleoporin Nup153 interacts with proteins A–D

Nup153 is localized to the most distal ring of the nuclear pore basket (Panté et al., 1994). The NH_2 terminus of human Nup153 (aa 1–339) is sufficient to target to nuclear pores in transfected cells (Enarson, et al., 1998). To search for nucleoporin partners of Nup153, fragments of the NH_2 terminus (aa 1–339) (Fig. 2 a) were coupled to beads and used in pulldowns from *Xenopus* egg cytosol. Proteins <60 kd were either nonspecifically bound or were obscured by BSA (Fig. 2 b). Strikingly, four large proteins with a mobility identical to proteins A–D bound to aa 1–339 of human Nup153 (Fig. 2 b, lane 4); these were designated A′–D′. A very abundant protein of ~97 kd also bound (*, Fig. 2 b, lanes 2–4), but was not studied further as it was Ran sensitive (*, Fig. 2 c, compare lane 5 with lane 6) and bound even in absence of A′–D′ binding (*, Fig. 2 b, lanes 2 and 3). The A′–D′ proteins did not bind to Nup153 aa 1–245 (Fig. 2 b, lane 3), to a *Xenopus* Nup153 fragment equivalent to aa 431–723 of human Nup153 (Nup153-N′, Fig. 2 b, lane 2), or to Sepharose beads coupled to control proteins (Fig. 2 b, lane 1, and unpublished data). To test whether A′–D′ were identical to A–D, the A–D proteins from a Nup98 column were biotinylated, and added to beads containing either green fluorescent protein (GFP), Nup153 aa 1–339, or Nup153-N′. The A–D proteins bound only to the Nup153 1–339 beads (unpublished data), indicating the A′–D′ and A–D proteins are identical. For Nup153, aa 246–339 are most critical for interaction with the A–D proteins.

Protein purification reveals nucleoporins Nup96 and Nup107

To identify the A–D proteins, small scale pulldown reactions were probed for known vertebrate nucleoporins and transport factors by immunoblotting (IB). The pulldowns did not contain substantial amounts of Nup62, Nup93, Nup98, Nup155, Nup205, Nup214, Nup358, the pore-associated protein Tpr, importin α, importin β, Gle2, Crm1, or the nuclear protein RCC1, although traces of importin α and β, and Nup93 and Nup205 were observed (Table SI, available at http://www.jcb.org/cgi/content/full/jcb.200108007/DC1). Overall, the finding that these other proteins did not bind to the Nup98 and Nup153 fragments in question underlined the specificity of association with proteins A–D.

The A–D proteins were purified and examined by proteolytic cleavage and peptide sequence analysis. The A′–D′ proteins gave the same peptide sequences as the A–D proteins

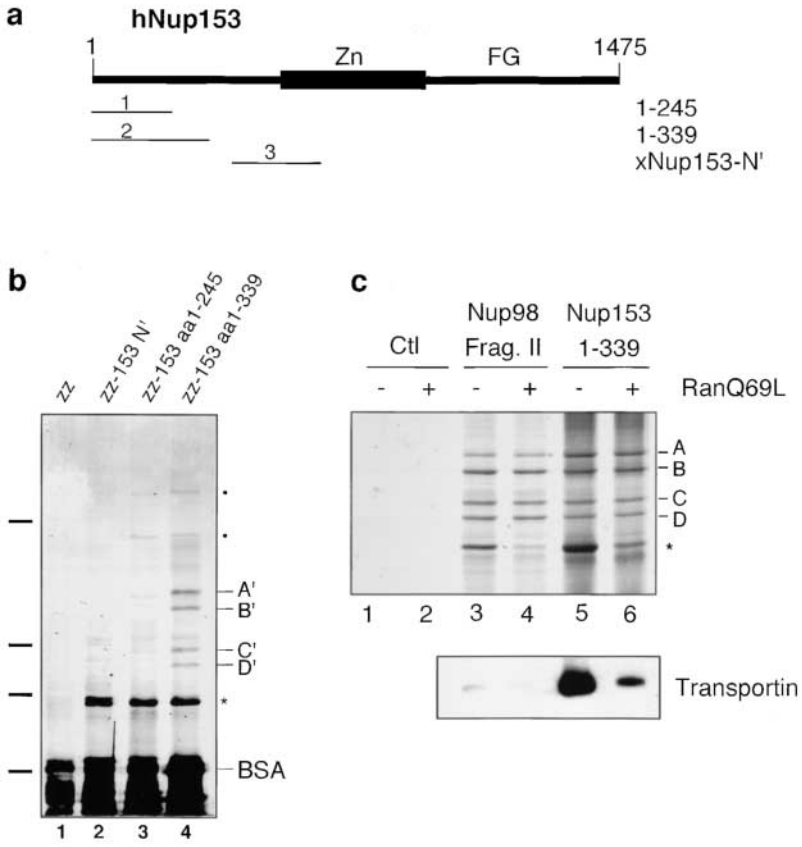

Figure 2. **Nucleoporin Nup153 binds the same four proteins.** (a) A map of Nup153 and the fragments used. (b) Nup153 fragments coupled to beads were used in pulldowns. Nup153 aa 1–339 bound four proteins similar in size to A–D, termed A′–D′ (lane 4). A zz tag control fragment and two Nup153 fragments, xNup153-N′ and human Nup153 aa 1–245, did not (lanes 1–3). Size markers are 205, 116, 97, and 66 kd, respectively. (c) Nup98 fragment II and Nup153 aa 1–339 bind proteins identical in size (upper panel). Each also bound a protein of ~97 kd (*; lanes 3 and 5) that was largely removed by the addition of RanQ69L (lanes 4 and 6). A–D and A′–D′ binding were not sensitive to RanQ69L (lanes 3–6). Control *S. aureus* protein A-Sepharose beads did not bind A–D (lanes 1 and 2). RanQ69L was functional (lower panel), as it dissociated transportin from a Nup153 fragment (Shah and Forbes, 1998). The lower panel is an immunoblot with anti-transportin antibody; compare lane 5 (no Ran) with lane 6 (+Ran).

(unpublished data). *Xenopus* band C produced peptides with high homology to human Nup96 (Fig. 3 a), whereas band D revealed peptides with almost complete identity to rat Nup107 (Fig. 3 b) (Radu et al., 1994; Fontoura et al., 1999; Rosenblum and Blobel, 1999). Human Nup96 migrates at 115 kd, a molecular mass essentially identical to that of *Xenopus* protein C at ~112 kd. Rat Nup107 migrates at ~107 kd, almost identically to *Xenopus* protein D at ~103 kd. We concluded from the size identity, sequence homology, and biochemical behavior of the proteins (see below) that C and D are the *Xenopus* homologues of Nup96 and Nup107, respectively.

A new vertebrate nucleoporin, Nup133

Peptide analysis of *Xenopus* protein B yielded compelling matches to a predicted human protein of 1156 aa with unknown function (AK001676), as well as to mouse ESTs. When a sensitive Psi-BLAST search was done with the human sequence, it revealed relatedness to proteins of identical length in *Drosophila* (1,154 aa; AAF56042; 24% identity, 44% similarity) and *Schizosaccharomyces pombe* (1162 aa; CAB55845.1). Strikingly, the *S. pombe* protein had 22% identity with a *Saccharomyces cerevisiae* nucleoporin, Nup133 (1157 aa) (Doye et al., 1994; Li et al., 1995; Pemberton et al., 1995). All are predicted to migrate at ~130 kd, identical in size to *Xenopus* protein B. The human sequence shows very distant, but discernable sequence relatedness to ScNup133 (18% identity for aa 536–903).

An antibody was raised to aa 777–1105 of the human protein. This recognized a single band of ~130 kd in HeLa cells and in rat liver nuclei (Fig. 4 a). When used to probe Nup98 aa 470–876 pulldowns from *Xenopus* extracts, the antibody recognized a single ~130-kd protein (Fig. 4 b, lane 6) identical in size to *Xenopus* protein B as visualized by silver stain (lane 3). The band was not seen in Nup98 aa 470–824 pulldowns (Fig. 4 b, lanes 2 and 5). Immunofluorescence (IF) of HeLa cells gave a punctate nuclear rim stain (Figs. 4 c and 5 b). We conclude that the 130-kd human

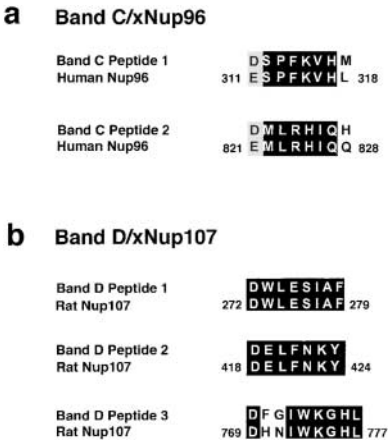

Figure 3. **Proteins C and D are the known vertebrate nucleoporins Nup96 and Nup107.** (a) Two of the peptides obtained from Band C are shown. (b) Three of the peptides obtained from Band B and D are shown and match with near identity to rat nucleoporin Nup107. Identity is boxed and homology is indicated in gray, as defined by Kyte-Doolittle algorithms.

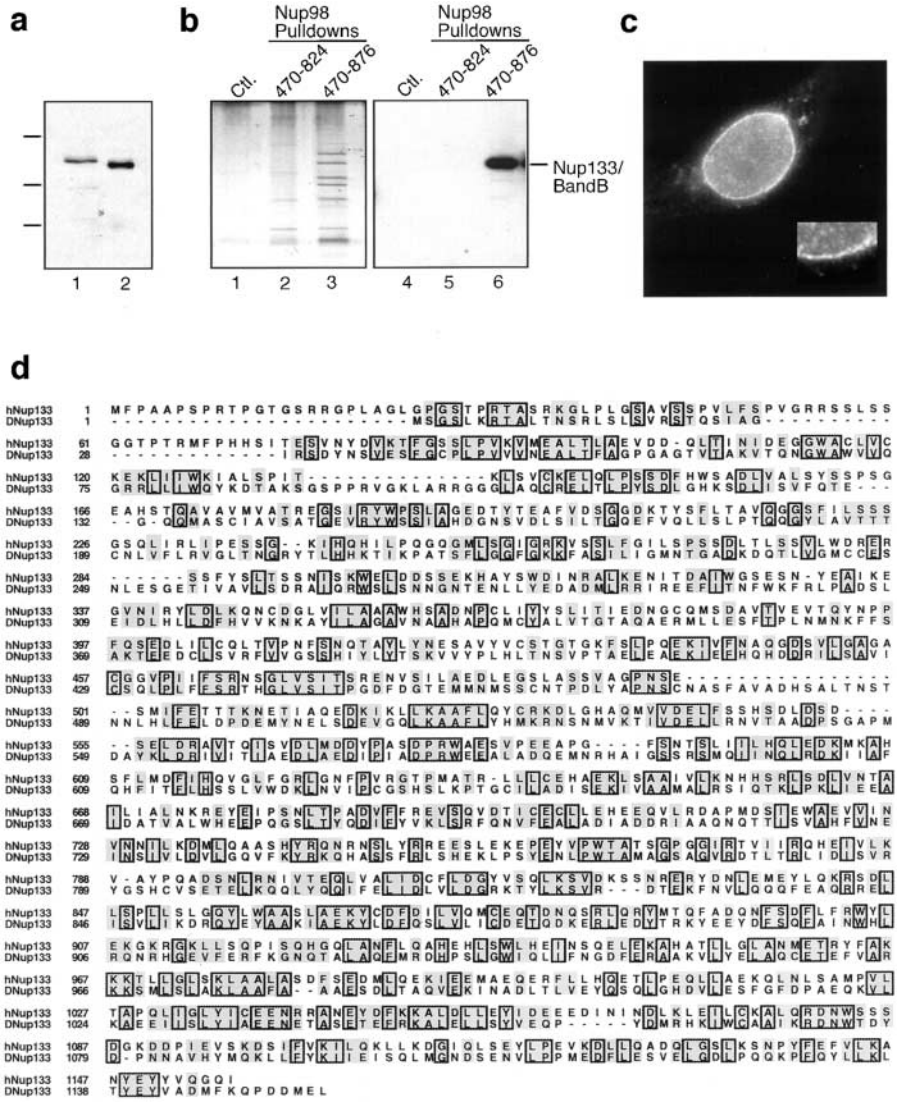

Figure 4. **A novel vertebrate nucleoporin, Nup133.** (a) Antibody to aa 777–1105 of the human protein AK001676, homologous to *Xenopus* protein B, recognized a single ~130-kd protein in HeLa cell extracts (lane 1) and in rat liver nuclei (lane 2). Markers are 205, 116, and 97 kd. (b) Pulldowns with Nup98 fragments were split and electrophoresed on two gels. One was silver stained (lanes 1–3) and one immunoblotted with anti-Nup133 antibody (lanes 4–6). An antibody-reactive band of ~130 kd was observed only in the Nup98 aa 470–876 pull-down (lane 6) and ran coincident with silver-stained protein B (lane 3). Ctl represents a pulldown with *S. aureus* protein A beads. (c) IF on HeLa cells using affinity-purified anti-Nup133 antibody; the inset shows a portion of the same nucleus magnified to reveal the punctate nuclear rim stain. (d) The sequence of the novel human nucleoporin Nup133 (AK001676) is compared with a highly related *Drosophila* homologue (AAF56042). Identity is boxed and homology is indicated in gray, as defined by Kyte-Doolittle algorithms.

and the *Xenopus* B protein are new vertebrate nucleoporins, now termed vertebrate Nup133. A comparison to the *Drosophila* Nup133 homologue is shown in Fig. 4 d. Additionally, we have raised an antibody to a *Xenopus* sequence homologue of human Nup133. This recognizes *Xenopus* protein B (see Fig. 7 c, lane 2), closing the circle and demonstrating that *Xenopus* protein B is xNup133.

Antibodies to vertebrate Nup133 bind to the nucleoplasmic face of the pore

To localize Nup133, IF was performed using digitonin permeabilization of HeLa cells, where only the cytoplasmic side of the pore is accessible to antibody (Fig. 5, exterior), and Triton permeabilization, which renders both sides of the pore accessible (Fig. 5, interior + exterior). Anti-Nup133 stained the nuclear pore only when the nuclear envelope was permeabilized by Triton X-100 (Fig. 5 b) or by exceptionally long digitonin permeabilization (unpublished data). Antibodies to Nup153 (Fig. 5, i–j), Nup98 (Fig. 5, m–n), and lamin B (Fig. 5, c–h, k–p) also stained their antigen only when the nuclear envelope was permeabilized. Monoclonal mAb414, which can recognize Nup214 and Nup358 on the cytoplasmic filaments of pores, gave a punctate nuclear rim in both digitonin- and Triton-permeabilized cells (Fig. 5, e–f). We conclude that Nup133 is primarily accessible on the basket side of the pore.

Identification of a large novel vertebrate nucleoporin, Nup160

Multiple peptides were obtained from *Xenopus* band A (Fig. 6 a). All showed high homology to a putative 160-kd mouse protein (1402 aa; AAD17922) (Fig. 6, a and d), a human protein of unknown function (1314 aa; KIAA0197; 91% identity), a 160-kd *Drosophila* protein (1411 aa; AAF53075.1; 28% identity; 47% homology), and a more distantly related 176-kd *Caenorhabditis elegans* protein (AAB37803.1). A search of *Xenopus* cDNAs revealed a *Xenopus* EST. This encoded a highly homologous *Xenopus* 160-kd protein. Antibody raised to the *Xenopus* 160-kd protein cross reacted with a single protein in *Xenopus* egg cytosol identical in size to Band A (Fig. 7 c, lane 1). IF with both this antibody (unpublished data) and an antibody raised to the human 160-kd protein gave a punctate nuclear rim stain (Fig. 6 c). Thus, we have designated *Xenopus* band A and its

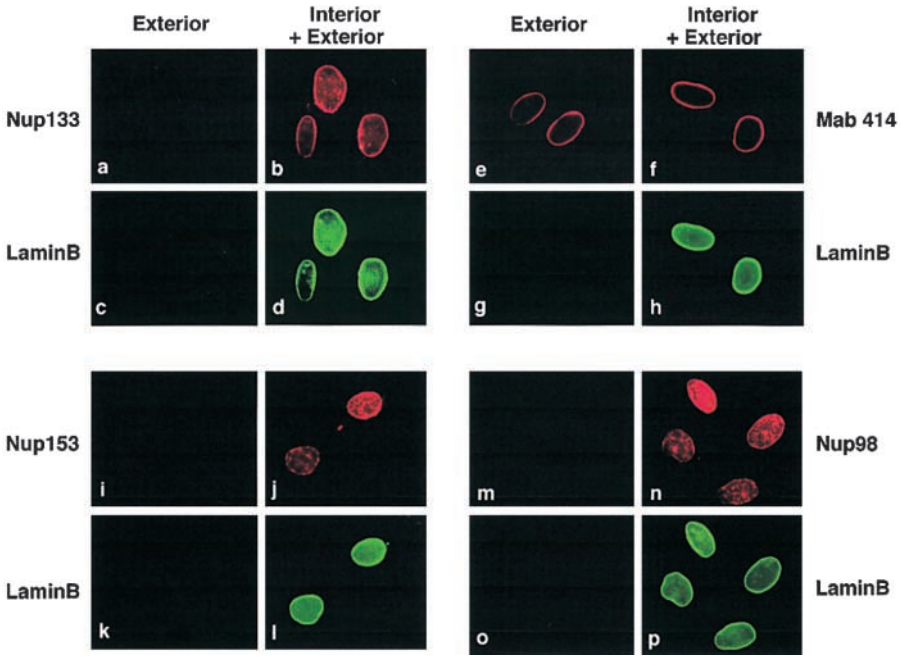

Figure 5. **Antibody to Nup133 localizes to the nuclear side of the pore.** HeLa cells were permeabilized with digitonin to allow antibodies access only to the exterior face of the nuclear envelope (Exterior). The monoclonal antibody mAb414 detects the presence of Nup214 and Nup358 on the cytoplasmic face of the nuclear pores (e). Nup133, Nup153, or Nup98 were not detected on this side of the pores (a, i, and m, respectively). As a control for nuclear envelope integrity, the cells were co-stained with the anti–lamin B antibody, LS-1 (c, g, k, and o). To probe the nuclear interior, Triton X-100 (Interior + Exterior) or longer digitonin (unpublished data) permeabilization was used. Anti–lamin B stain confirms nuclear envelope penetration of the antibody (d, h, l, and p) after TX-100. Nup153 and Nup98 are detected on the intranuclear face of pores, as expected (j and n). Nup133 is detected on the interior (b), but not the exterior (a) of the nuclear envelope.

relatives in mice, humans, and *Drosophila* as nucleoporin Nup160. A comparison of mouse and *Drosophila* Nup160 is shown in Fig. 6 d. Like Nup133, Nup160 is primarily accessible to antibody only on the nuclear side of the pore (Fig. 6 c, right panel).

We searched for homology to any known yeast nucleoporins. Yeast homologues of Nup96 and Nup107 are normally in complex with four other nucleoporins, Nup120p (120 kd), Nup85p (85 kd), sec13p (33 kd), and seh1p (39 kd) (Siniossoglou et al., 1996, 2000), all seemingly too small to be a homologue of metazoan Nup160. Mouse Nup160 showed no sequence homology to yeast Nup85p, sec13p, or seh1p. A highly sensitive Psi-BLAST search using *S. cerevisiae* Nup120 (1037 aa) (Aitchison et al., 1995; Heath et al., 1995; Altschul et al., 1997) brought up no *Drosophila* or vertebrate homologues, but did show NH_2-terminal homology with aa 100–445 of an *S. pombe* sequence of 1136 aa (PIR T40355) (Fig. 6 b). When a standard BLAST was done using this putative *S. pombe* Nup120p, it identified *S. cerevisiae* Nup120 and the same *Drosophila* and *C. elegans* sequences we had observed with mouse Nup160. When a more sensitive Psi-BLAST search was done starting with the putative SpNup120p (rather than ScNup120p), only five sequences were observed as related: the mouse 160-kd protein, the related human protein, the *Drosophila* and *C. elegans* sequences, and ScNup120. The metazoan Nup160 proteins and ScNup120p contain no FG or other repeat sequences.

Direct comparison of the metazoan Nup160 proteins and the shorter yeast Nup120 proteins shows that they differ greatly in sequence. The highest homology between the putative *S. pombe* Nup120p and the Nup160 proteins is at the *S. pombe* COOH terminus (gray box, Fig. 6 b). *S. cerevisiae* Nup120p (aa 756–1033) has quite good homology to *Drosophila* Nup160 in this region (aa 861–1130) (black rimmed boxes; Fig. 6 b), but not to human or mouse Nup160. The NH_2 termini of *S. cerevisiae* and putative *S. pombe* Nup120 show the most resemblance to one another, but show little similarity to the same region in the Nup160 proteins. The central domain of ScNup120p (aa 426–755) has diverged the most, both from the Nup160 proteins and from the *S. pombe* protein. Most strikingly, the metazoan Nup160 proteins contain an additional 33 kd at their COOH termini (Fig. 6 b). Clearly, of the four A–D nucleoporins, Nup160 has undergone the most changes through evolution.

A Nup160 complex of nucleoporins in vertebrates

To determine whether the A–D proteins interact with Nup98 and Nup153 individually or as a complex, egg extract was subjected to gel filtration, and 54 fractions were collected. The odd fractions were subjected to pulldown with Nup153 aa 1–339 beads and the bound proteins were analyzed by SDS-PAGE and silver staining. All four nucleoporins A–D were pulled down from the same fractions (33–41, Fig. 7 a) and peaked in fraction 37 (dots). An immunoblot using anti-hNup133 antibody on the even fractions (i.e., total cytosol with no pulldown) gave essentially the same pattern of migration for Nup133 (Fig. 7 b).

Immunoprecipitation was performed from unfractionated egg extract under native conditions (Fig. 7 c). Anti-Nup160 antibody coimmunoprecipitated Nup133 (Fig. 7 c, lane 5), whereas anti-Nup133 antibody coimmunoprecipitated Nup160 (Fig. 7 c, lane 6). A control anti-Nup62 antibody did not immunoprecipitate either Nup160 or Nup133 (lane 7). Thus, Nup160 and Nup133 exist in the same complex. From this and the cofractionation of proteins A–D in pulldowns (Figs. 1, 2, 4, and 7 a), we designate *Xenopus* Nup160, Nup133, Nup107, and Nup96 proteins as members of a Nup160 complex. The *Xenopus* Nup160 complex migrates at ~700–800 kd.

As stated above, the yeast homologues of Nup96 and Nup107 are extracted from yeast pores in complex with yeast Nup120p, Nup85p, sec13p, and seh1p (Pryer et al., 1993; Siniossoglou et al., 1996, 2000). By silver stain, we never ob-

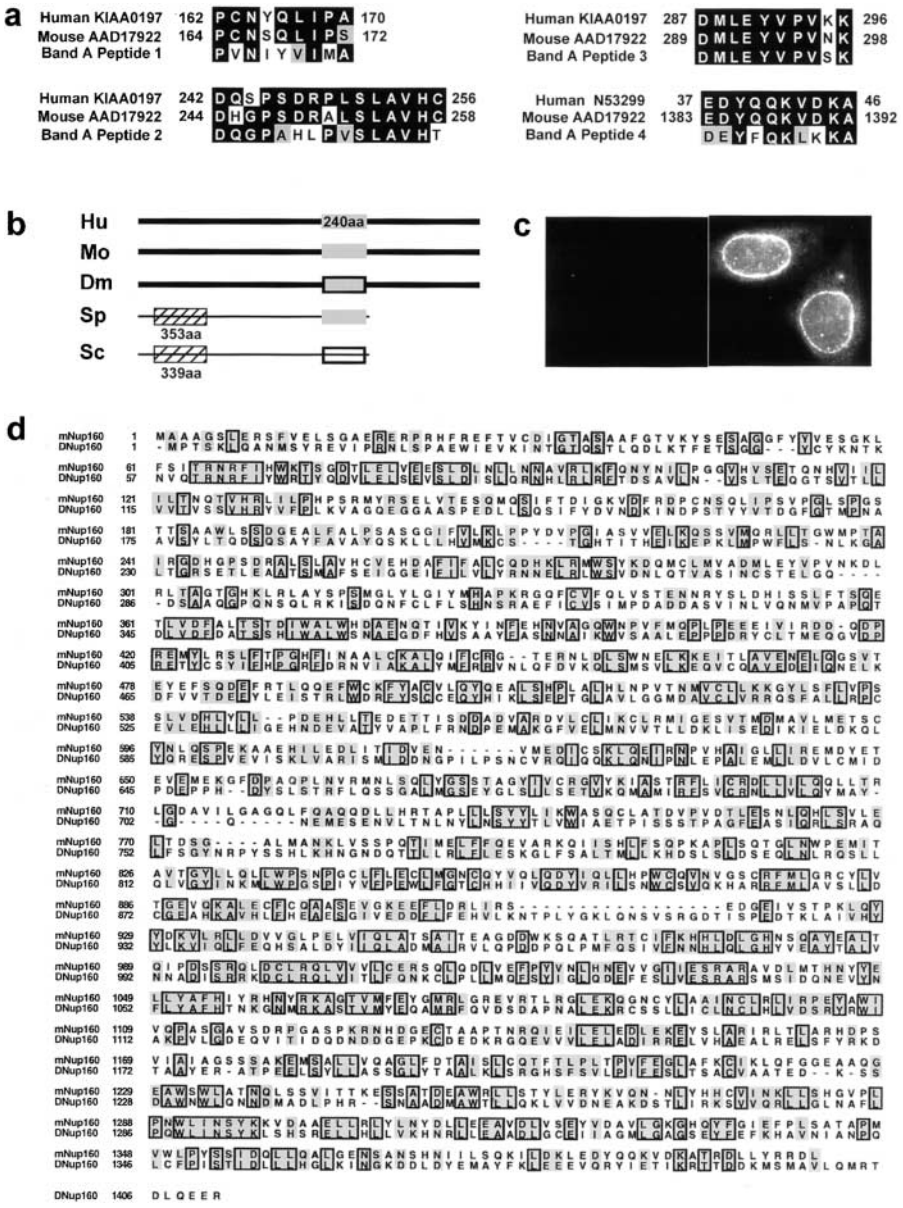

Figure 6. **A new vertebrate nucleoporin, Nup160.** (a) Peptides from *Xenopus* band A match sequences of a 160- kd unknown mouse protein (AAD17922), a human clone (KIAA0197), and an overlapping human EST (N53299). (b) A schematic comparison of the human (Hu), mouse (Mo), and *Drosophila* (Dm) Nup160 proteins and distantly related *S. cerevisiae* ScNUP120 and putative *S. pombe* Nup120 proteins. Thick lines indicate relatedness, hatched boxes show relatedness between the two yeast proteins, gray boxes indicate a region of moderate homology between *S. pombe* and metazoans, and the black outlined box indicates a region of homology between ScNup120 and *Drosophila* Nup160. The hatched box and thin lines of the yeast proteins show little relatedness to the vertebrate proteins. (c) Antibody raised to the putative human Nup160 protein gives a punctate nuclear rim stain on TX-100 permeabilized HeLa cells (right panel), but not on digitonin-permeabilized cells (left panel), indicating localization of Nup160 on the basket face of the pore. (d) A comparison of mouse Nup160 and the highly related *Drosophila* homologue, aligned using Clustal W. Identities are boxed, homologies are in gray.

serve a vertebrate 85-kd protein in our pulldowns. However, we have recently identified a sequence with homology to ScNup85, implying the Nup160 complex may also contain vertebrate Nup85 protein.

Putative proteins of the size of sec13 and seh1 would have been obscured by small nonspecifically bound proteins in our silver-stained pulldowns (Figs. 1 and 2). Although the existence of a vertebrate seh1 is controversial, human sec13 has been cloned (Shaywitz et al., 1995). Using anti–human sec13 antibody (unpublished data), we probed the gel filtration fractions of total *Xenopus* egg cytosol and found that human sec13 migrates in a region identical to that of Nup133 (Fig. 7 b) and to A–D (Fig. 7 a). Sec13 protein is present in Nup98 aa 470–876 pulldowns (Fig. 7 d, lane 3), but not in Nup98 aa 470–824 pulldowns (Fig. 7 d, lane 4). Similarly, sec13 is present in Nup153 aa 1–339 pulldowns (Fig. 7 d, lane 5), but not in Nup153 aa 1–245 pulldowns (Fig. 7 d, lane 6). Most convincingly, sec13 is coimmunoprecipitated by both anti-Nup160 and Nup133 antibodies (Fig. 7 c, lanes 5 and 6). Thus, sec13 is a member of the vertebrate Nup160 complex, which now minimally contains vertebrate Nup160, Nup133, Nup107, Nup96, sec13, and likely a vertebrate Nup85 (unpublished data).

Neither Nup98 nor Nup153 were found in anti-Nup160 or anti-Nup133 immunoprecipitates (Fig. 7 c, lanes 9 and 10; see also Table I in the online supplement), as determined by IB. The absence of Nup98 and Nup153 from the Nup160 complex is entirely consistent with previous findings that, when nuclear pores disassemble at mitosis, Nup98 is found primarily in a pore subcomplex containing the transport factor Gle2, both in egg extract (unpublished data) and in human mitotic extracts (Matsuoka et al., 1999). Similarly, *Xenopus* Nup153 is found primarily in complex with the transport receptors importin α, β, and transportin in egg extract (Shah et al., 1998; Shah and Forbes, 1998).

Assembled pores contain the Nup160 complex

We examined assembled pores for the Nup160 complex. Annulate lamellae (AL), cytoplasmic stacks of membranes

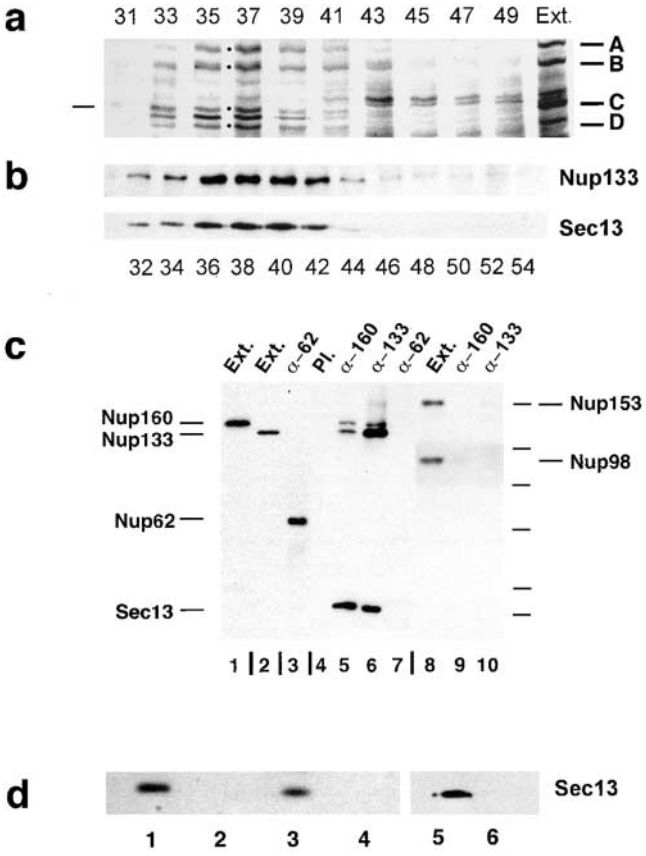

Figure 7. **The Nup160 nuclear pore subcomplex.** (a) After gel filtration of *Xenopus* egg cytosol, odd fractions were subjected to Nup153 aa 1–339 pulldown, SDS-PAGE, and silver staining. A–D fractionated together and peaked at fraction 37 (dots). The left hatchmark indicates a 116-kd mw marker protein. When size controls were monitored for migration on the gel filtration column (unpublished data), Nup214 was seen to fractionate in a complex peaking at ~1,000 kd (peak fraction = 34) and Nup98 fractionated in a complex peaking at ~450 kd (peak fraction = 44), as expected (Macaulay et al., 1995). The dark band between C and D was not seen in pulldowns of unfractionated extract (Figs. 1 and 2) and is likely a breakdown product. (b) The even fractions (of total egg extract) were probed with anti-Nup133 and anti-sec13 antibodies. Nup133 peaks coincident with the peak of the silver-stained bands A–D indicated by dots in panel (a). The majority of soluble sec13 in *Xenopus* extracts also peaks in this region. (c) Immunoprecipitation from *Xenopus* cytosol using anti-xNup160 (lanes 5 and 9), anti-xNup133 (lanes 6 and 10), anti-xNup62 (lanes 3 and 7), and a mix of the preimmune sera for xNup160 and xNup133 (lane 4). The top portion of the blot corresponding to lanes 4–7 was probed with both anti-xNup160 and anti-xNup133 antibodies and the bottom with anti-sec13 antibody. The blot of lanes 8–10 was probed with both anti-rNup98 and anti-rNup153. *Xenopus* cytosol (0.2 μl) is shown in lanes 1, 2, and 8. Lane 1 was probed with anti-xNup160, lane 2 with anti-xNup133, and lane 3 with anti-xNup62. The molecular markers (left) were 200, 120, 90, 68, 53, 36, and 32 kd. Nup153 runs aberrantly high at ~180 kd (Sukegawa and Blobel, 1993) (d) To ask whether sec13p is in Nup98 pulldowns, Nup98 aa 470–876 pulldowns were done from solubilized AL (lane 1), solubilized mock AL made in the presence of the pore assembly inhibitor BAPTA (lane 2), and egg extract (lane 3). A pulldown from egg extract using Nup98 aa 470–824 beads was done in lane 4. Pulldowns from *Xenopus* extract were also done with Nup153 aa 1–339 (lane 5) and Nup153 aa 1–245 (lane 6). All pulldowns were probed with anti–human sec13p antibody.

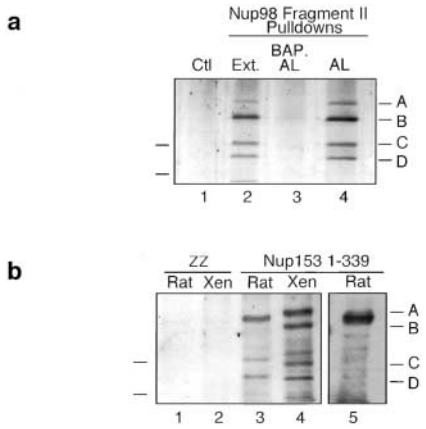

Figure 8. **The Nup160 complex is in assembled pores.** (a) AL assembly reactions were done in the presence or absence of BAPTA. AL were isolated and partially solubilized with 0.5 M NaCl which does not destabilize the A–D complex. Solubilized AL was clarified by centrifugation and used for pulldowns with Nup98 fragment II beads. Proteins A–D were pulled down from egg extract (lane 2, Ext) and from AL (lane 4) by Nup98 beads, but not by *S. aureus* protein A beads (lane 1, Ctl) or from AL reactions in which pore assembly had been inhibited by BAPTA (lane 3). Mw markers are 116 and 97 kd, respectively. (b) Rat liver nuclei were partially disassembled with 2% Triton. The clarified supernatant was incubated with Nup153 aa 1–339 beads. Three rat proteins in the relevant size range were observed by silver staining to be pulled down (lane 3), two similar to *Xenopus* C and D (lane 4), as well as a thick band intermediate between *Xenopus* A and B. None bound to zz-tag control beads (lanes 1 and 2). A portion of the Nup153 aa 1–339 pulldown from solubilized rat liver nuclei was probed with anti–human Nup133 antibody and gave a single immunoreactive band (lane 5) coincident with the thick band observed by silver stain (lane 3), indicating that this is rat Nup133.

that contain abundant pores identical to nuclear pores, can easily be assembled in *Xenopus* extracts in vitro (Dabauvalle et al., 1991; Cordes et al., 1995; Meier et al., 1995; Miller et al., 2000; Miller and Forbes, 2000). AL were formed in egg extract, purified, and treated with 0.5 M NaCl to partially solubilize the pores (Miller et al., 2000). When this mixture was added to Nup98 beads (fragment II), all four A–D proteins were pulled down (Fig. 8 a, lane 4), as was sec13 (Fig. 7 d, lane 1). Neither A–D (Fig. 8 a, lane 3) nor sec13 (Fig. 7 d, lane 2) were pulled down from AL reactions done in the presence of the pore assembly inhibitor BAPTA (Macaulay and Forbes, 1996; Goldberg et al., 1997). The A–D proteins were pulled down from normal AL pores by Nup153 aa 1–339 beads (unpublished data). We conclude that proteins A–D and sec13 are present together in the assembled pores of AL. Also indicative of this, we found that the A–D proteins, when purified and biotinylated, could be incorporated into AL in vitro (unpublished results).

Rat liver nuclei were also gently solubilized with Triton X-100, and the extracted proteins were added to Nup153 aa 1–339 beads. Two rat proteins very similar in size to *Xenopus* Nup96 (C) and Nup107 (D) were observed by silver stain to pulldown (Fig. 8 b, compare lane 3 with lane 4). A dark band intermediate in size between *Xenopus* Nup160 (A) and Nup133 (B) was also observed in the rat pulldowns (Fig. 8 b, lane 3, upper band). This band reacted strongly

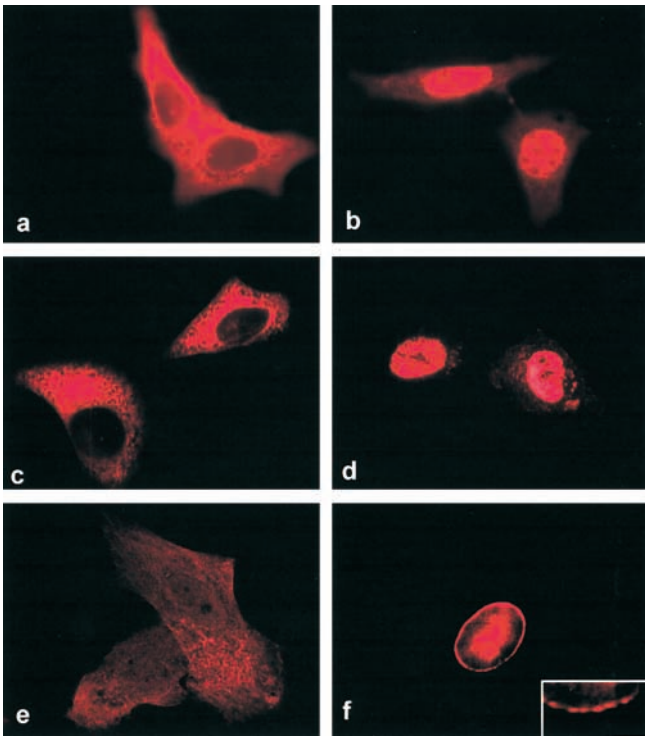

Figure 9. **Nup98 and Nup153 tether in the nucleus and to the pore, respectively, using their Nup160 complex binding domains.** After transfection, myc-tagged Nup98 aa 470–824 is cytoplasmic (a and c), whereas Nup98 aa 470–876 is nuclear (b), like endogenous Nup98 (Powers et al., 1995). Nup98 aa 470–824 has access to the nucleus since it localizes there upon LMB addition (LMB; 100 nM, l hr) (d). Fragment 1C3 from the COOH terminus of *Xenopus* Nup153 (Shah et al., 1998) localized throughout the cell with no nuclear rim (e). Human Nup153 aa 1–339, which binds the Nup160 complex, localized to nuclear pores (f), as in Enarson et al. (1998).

with our anti–human Nup133 antibody (Fig. 8 b, lane 5). Thus, rat liver nuclei contain three proteins similar in size and/or immunogenicity to those of the *Xenopus* Nup160 complex that are pulled down by Nup153 beads, consistent with the existence of a Nup160 complex in assembled rat nuclear pores.

Nup153 and Nup98 are tethered to nuclear sites by their Nup160 complex binding domains

To determine the importance of its Nup160 complex binding site to Nup153, myc-tagged Nup153 constructs were transfected into cells. A Nup153 fragment lacking the Nup160 complex binding site, when transfected, was cytoplasmic (Fig. 9 e). However, Nup153 aa 1–339, which binds the Nup160 complex, localized to the nuclear rim in a punctate pattern typical of a nuclear pore stain (Fig. 9 f), consistent with a previous localization study (Enarson et al., 1998). In that study, Nup153 aa 1–245 localized to the nuclear rim, but not to the pore. Thus, the Nup153 aa that are required for A–D binding, aa 246–339, are also essential for Nup153's targeting to the nuclear pore (Fig. 9 f).

When a Nup98 fragment that cannot bind the Nup160 complex was transfected into cells, it showed diffuse cytoplasmic localization (Nup98 aa 470–824; Fig. 9, a and c). However, Nup98 aa 470–876, which contains the additional 52 aa that allow the Nup160 complex to bind, localized to the nucleus (Fig. 9 b). This pattern mirrors that of endogenous Nup98, i.e., a rim and intranuclear stain (Powers et al., 1995; Radu et al., 1995; Zolotukhin and Felber, 1999). We tested whether the shorter 470–824 fragment might simply lack an NLS by testing localization of the shorter fragment plus and minus leptomycin B (LMB), a drug that inhibits NES-mediated export (Fornerod et al., 1997a). When LMB was present, the shorter Nup98 aa 470–824 fragment localized to the nucleus (Fig. 9 d), indicating that this shorter fragment can normally shuttle between the nucleus and cytoplasm. We conclude that Nup98 aa 470–876 localizes to the nucleus, not because it contains an NLS that aa 470–824 lacks, but because it is actively tethered to the nucleus by the ≥52 COOH-terminal aa critical for binding the Nup160 complex.

Smaller Nup98 constructs were made. Nup98 aa 611–703 did not bind the Nup160 complex (unpublished data), but aa 611–876 both bound the complex and localized to the nucleus upon transfection (unpublished data). We conclude that aa 611–876 are sufficient for binding the Nup160 complex and for tethering the Nup98 fragment within the nucleus. In particular, aa 824–876 are essential for both complex binding and nuclear tethering.

Fragments of Nup160 and Nup133 inhibit nuclear export of poly[A]$^+$ RNA

The novel vertebrate nucleoporins Nup160 and Nup133 were discovered through their interaction with Nup98 and Nup153, both critical for vertebrate mRNA export. To ask whether Nup160 and Nup133 play a role in RNA export, myc-tagged fragments of the genes were transfected into HeLa cells and poly[A]$^+$ RNA was monitored by hybridization to Texas red oligo[dT]$_{50}$. Cells were stained with FITC anti-myc antibody to reveal successful transfection. Untransfected cells (Fig. 10 for those not stained by anti-myc antibody), as well as cells transfected with the negative control malate dehydrogenase gene (Fig. 10, e and h), showed diffuse cytoplasmic poly[A]$^+$ RNA staining with intranuclear spots. This pattern is typical of poly[A]$^+$ RNA localization in normal cells (Heath et al., 1995; Bastos et al., 1996; Watkins et al., 1998; Pritchard et al., 1999). A Nup98 fragment containing the Gle2 binding site (aa 150–224), which is known to cause nuclear poly[A]$^+$ RNA accumulation (Pritchard et al., 1999), served as a positive control for inhibition of mRNA export (Fig. 10, f and i). A fragment containing residues 1–1149 of human Nup133 had little effect on poly[A]$^+$ RNA (Fig. 10, g and j). However, human Nup133 residues 587–936, when transfected into HeLa cells, caused strong nuclear poly[A]$^+$ accumulation (transfected cells, Fig. 10 d). Transfection of residues 317–697 of human Nup160 also caused nuclear poly[A]$^+$ RNA accumulation (Fig. 10 b, transfected cells). None of the constructs above affected the hormone-inducible nuclear import or the export, upon hormone withdrawal, of a shuttling reporter protein RGG (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200108007/DC1). In RGG, HIV Rev is fused to GFP and to the hormone binding domain of the glucocorticoid

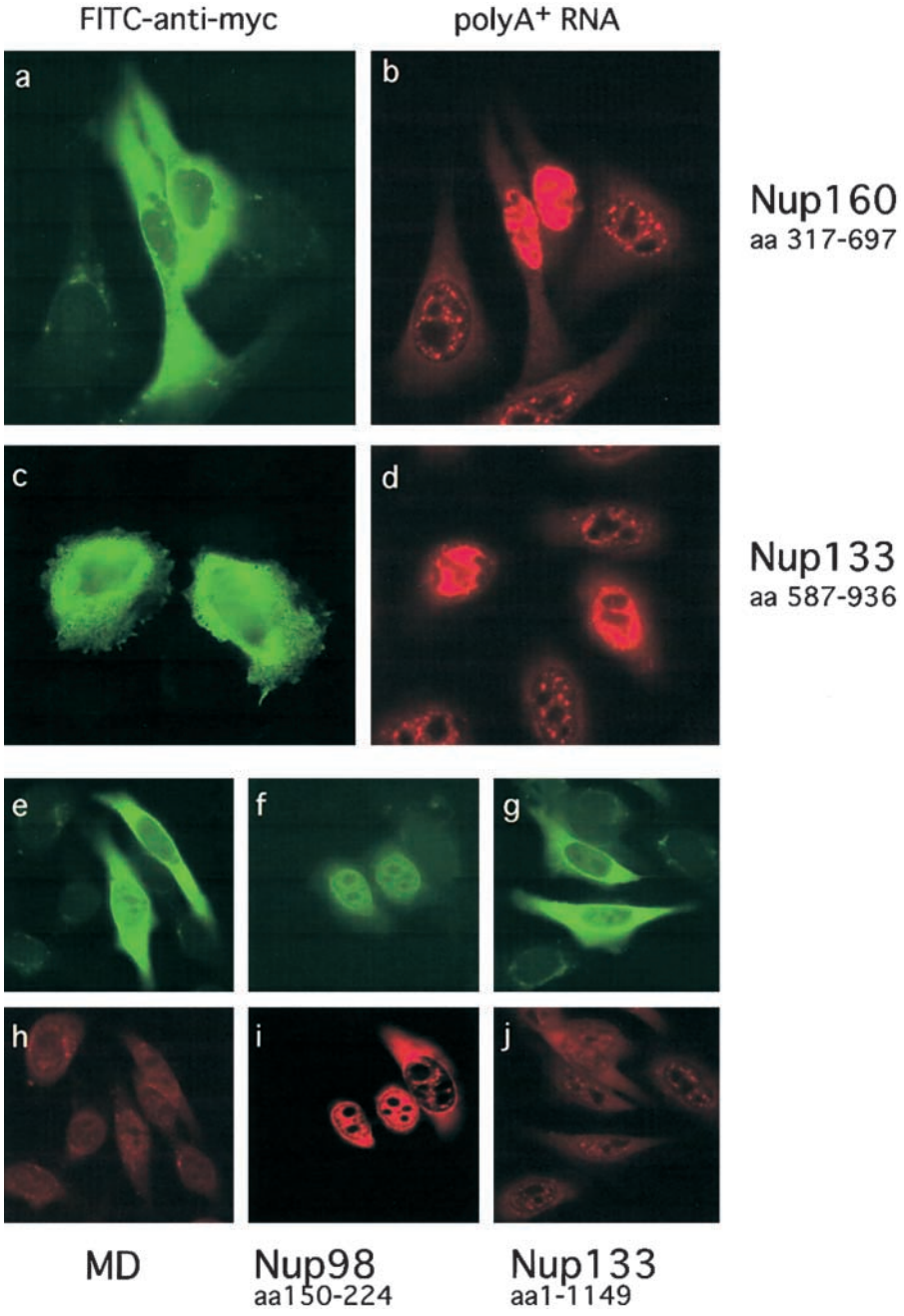

Figure 10. **Dominant negative fragments of Nup133 and Nup160 cause nuclear accumulation of poly[A]$^+$ RNA.** HeLa cells were transfected with pCS2MT vectors containing: human Nup160 aa 317–697 (a and b) and 133 aa 587–936 (c and d), malate dehydrogenase (negative control; e and h), rat Nup98 aa 150–224 (positive inhibitory control; f and i), or human Nup133 aa 1–1149 (g and j) in myc-tagged form. Successful transfection was assayed by IF with FITC–anti-myc antibody (a, c, and e–g), whereas the localization of poly[A]$^+$ RNA was assessed by hybridization of the coverslips with Texas red oligo[dT]$_{50}$ (b, d, and h–j). Both human Nup160 aa 317–697 (b) and Nup133 aa 587–936 (d) caused a striking accumulation of poly[A]$^+$ RNA in the nucleus, identical to that caused by Nup98 aa 150–224 (i).

receptor (Love et al., 1998; Gustin and Sarnow, 2001). Export of the RGG reporter protein could be inhibited by the FG domain of Nup214, which is known to inhibit NES protein export (Zolotukhin and Felber, 1999) (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200108007/DC1). We conclude that fragments of the novel nucleoporins Nup133 and Nup160 elicit a dominant negative effect on poly[A]$^+$ RNA export, while leaving RGG protein import and export pathways intact.

Discussion

Only two proteins of the nuclear pore basket, Nup98 and Nup153, have been identified previously as playing a role in RNA export. We have now identified four large nucleoporins that interact with Nup98 and Nup153. Two are Nup96 and Nup107, whereas two are nucleoporins previously unknown in vertebrates, now designated vertebrate Nup160 and Nup133. Antibodies localize the novel proteins to the basket side of the nuclear pore. Nup133 is distantly related to *S. cerevisiae* Nup133, whereas Nup160 is very remotely related to yeast Nup120. Pulldowns, gel filtration, and coimmunoprecipitation all show that *Xenopus* Nup160, Nup133, Nup107, Nup96, and the small secretory protein sec13 form a complex, the Nup160 complex. The complex is present in *Xenopus* egg cytosol, as well as in assembled pores. We have mapped the sites that are required for binding the Nup160 subcomplex: in Nup98, the binding site tethers Nup98 to the nucleus; in Nup153, the binding site targets Nup153 to the nuclear pore. When fragments of the

novel nucleoporins Nup160 and Nup133 are overexpressed, they cause strong intranuclear accumulation of poly[A]$^+$ RNA, indicating that the Nup160 complex plays a functional role in mRNA export.

The existence of the Nup160 complex was suggested from the short stretch of aa in Nup98 and Nup153 essential for pulldown of the A–D proteins. Nup107, Nup96, and sec13 had been observed previously among ~30 proteins extracted from rat nuclear envelopes (Fontoura et al., 1999). However, in that study it was not possible to determine an association, as all 30 were present in only four protein-containing fractions, and fifteen proteins were present with Nup107, Nup96, and sec13p. Here, extensive gel filtration followed by a Nup153 pulldown of each fraction showed that Nup160, Nup133, Nup107, Nup96, and sec13 are all pulled down from the same fractions with an apparent complex mw of ~700–800 kd. Definitive proof of a complex came from the coimmunoprecipitation of Nup160, Nup133, and sec13. We conclude that a Nup160 complex exists and contains at a minimum *Xenopus* Nup160, Nup133, Nup107, and Nup96, and sec13p. A vertebrate Nup85 may also be present (unpublished data)

The related yeast Nup84 complex contains six members: Nup107 and Nup96 homologues, as well as Nup120, sec13, Nup85, and seh1 proteins (Siniossoglou et al., 1996, 2000). Of these, we find Nup107, Nup96, Nup160 (a relative of Nup120), and sec13. We cannot yet analyze whether a potential vertebrate seh1 homologue is present. Instead, we find an unexpected nucleoporin, the newly discovered vertebrate Nup133. Vertebrate Nup133 is distantly related to *S. cerevisiae* Nup133, which when mutant causes defects in mRNA export (Doye et al., 1994; Li et al., 1995; Pemberton et al., 1995). ScNup133p is not present in the yeast Nup84 complex. Either the pore basket differs in yeast and vertebrates or, alternatively, the mitotic fracture of vertebrate pores that produces the Nup160 complex occurs along different "fault lines" than those that create the yeast Nup84 complex.

In considering how the different subcomplexes of the vertebrate pore basket connect one to another, we do not think that Nup98 and Nup153 bind to the same Nup160 complex protein. Excess soluble Nup153 fragment (aa 1–339) has no effect on Nup98 pulldowns of A–D and visa versa (unpublished data). Based on the affinity of recombinant Nup96 and Nup98 (Rosenblum and Blobel, 1999), Nup96 may be the protein of the Nup160 complex that interacts with Nup98. Careful examination of Nup153 pulldowns indicates that Nup160 is more enriched than in Nup98 pulldowns (Fig. 2 c, band A, lanes 3–6). This suggests that Nup160 may be the complex protein that interacts with Nup153.

We found no coimmunoprecipitation of the Nup160, Nup98, and Nup153 subcomplexes with one another (Fig. 7). This is consistent with the finding that nuclear pore assembly requires the presence of membranes (Dabauvalle et al., 1991; Meier et al., 1995; Macaulay and Forbes, 1996). Pore subcomplexes do not assemble into multisubcomplex structures unless membranes are present and subcomplex sizes are identical in both interphase and mitotic extracts (Macaulay et al., 1995; Matsuoka et al., 1999). Possibly the affinity of one pore subcomplex for another is too low when they are present as monomers dilute in interphase cytosol. However, when subcomplexes are faced with the multiple adjacent copies of a partner subcomplex in a forming pore, we predict that the affinity or avidity increases, allowing binding between subcomplexes. In the yeast pore, 8–52 copies of any given subcomplex are present (Rout et al., 2000). In our experiments, numerous closely apposed Nup98 or Nup153 molecules on beads may mimic the multiple copies of these proteins found in the forming pore, promoting the binding of the Nup160 complex.

Functionally, the Nup160 complex plays at least three important roles. First, the ability of Nup98 to tether in the nucleus coincides with its ability to bind the Nup160 complex. Second, the ability of Nup153 to target to the pore requires its Nup160 complex–binding site. Most importantly, the Nup160 complex plays a role in RNA export: fragments of Nup160 and Nup133 act as dominant negative inhibitors of mRNA export. In yeast, the central portion of Nup133 is required for mRNA export (Doye et al., 1994; Li et al., 1995). Here we find that a fragment from a similar region of vertebrate Nup133 blocks mRNA export in mammalian cells.

Nup133 and Nup160 join a very small handful of vertebrate nucleoporins involved in RNA export: Nup214, Nup98, and Nup153 (Conti and Izaurralde, 2001; Vasu and Forbes, 2001). Nup214 on the cytoplasmic filaments of the pore acts as a late docking site for the mRNA export factor TAP (Katahira et al., 1999; Conti and Izaurralde, 2001). Nup98 and Nup153 also bind TAP in vitro and may do so in vivo (Bachi et al., 2000; Strasser et al., 2000; Tan et al., 2000). They are also presumed to bind other export receptors and proteins, such as Gle2. Nup133 and Nup160 may function either directly by interacting with specific factors or receptors involved in mRNA export, indirectly by tethering Nup153 and Nup98 to the pore, or both.

It is worth reflecting on a recent mouse mutant engineered to produce Nup98 that lacks the Gle2 binding site (exon 3 of 31) but contains all other exons (Fontoura et al., 2001; Wu et al., 2001). Yet unanswered in this mutant is whether the bulk of the Nup98 protein is produced. Because nucleoporin Nup96 is found to be present, the Nup 98 aa 715–920 that autocatalytically cleave their common precursor must be also be present. Thus, at the very least, aa 715–876 of the Nup160 binding site on Nup98 continue to be present in the mutant mouse.

To form the nuclear pore basket, eight 1,000-Å filaments connect to a 500-Å distal ring (Stoffler et al., 1999). Nup98 and Nup153 at ~60 and 80 Å, if globular, are clearly much smaller. ImmunoEM places Nup153 on the ring of the basket and Nup98 somewhere over the basket. In one model, multiple copies of Nup98 could comprise the basket filaments and use connectors to join to the 500-Å Nup153 ring. A molecular connector linking Nup98 and Nup153 has previously been lacking. The Nup160 complex, which binds both Nup98 and Nup153, could fill the role of that connector. The related Nup84 complex of yeast has a three-legged or triskelion structure, perhaps suggesting a role for the yeast complex at structural vertices (Siniossoglou et al., 2000). In the future, the above proteins, together with the new Nup50 and the finding that Nup153 cycles on and off

the pore (Daigle et al., 2001), must be fit into the enigmatic puzzle that forms the nuclear pore basket.

In a last consideration, the vertebrate Nup160 complex may play a role in intranuclear architecture. This stems from the finding that the yeast homologue of vertebrate Nup96, SrNup145C, is instrumental in organizing multiple structures and functions within the yeast nucleus. Yeast Nup-145C attaches intranuclear filaments to the yeast pore and these in turn anchor telomeres, dsDNA repair enzymes, and silenced genes to the yeast nuclear periphery (Galy et al., 2000). In strains lacking Nup145C, yeast telomeres are released from the nuclear periphery, double strand break repair is defective, and silenced genes become unsilenced. Other intranuclear roles for vertebrate Nup96 have also been suggested (Fontoura et al., 2001).

In summary, the novel nucleoporins Nup160 and Nup133 appear intimately involved in mRNA export and, as members of a large complex, interact with the basket nucleoporins Nup98 and Nup153. This is among the first evidence linking subcomplexes of the vertebrate nuclear pore and, as such, allows models of the vertebrate pore to be proposed and tested. Future work may determine the Nup160 complex to be a central anchoring point, both for the pore and for pore-associated proteins.

Materials and methods

cDNA cloning and protein expression of Nup98 and Nup153

A partial rat Nup98 construct, 98-1 (aa 43–824) (Radu et al., 1995), was converted to full-length Nup98 as follows: the complete rat Nup98 COOH terminus was obtained by reverse transcription of total rat RNA, using an oligo specific to the 3′ end of the ORF in the Nup98 GenBank/EMBL/DDBJ accession no. L39991, and spliced onto Nup98-1 to give Nup98 aa 1–935. This was sequenced to verify authenticity. Oligonucleotides were used to produce subfragments of Nup98, which were cloned as EcoR1–Xho1 fragments into vector pET28B (In Vitrogen) for bacterial expression or as Nco1–Xho1 fragments in pCS2MT for mammalian expression. A cDNA of human Nup153 aa 1–800 was reverse transcribed from HeLa total RNA, amplified by PCR, and cloned into pET28. Oligonucleotides were used to amplify Nup153 aa 1–339 from this cDNA clone, whereas Nup153 aa 1–245 were cloned as an EcoR1 fragment from the original clone. These Nup153 subfragments were separately cloned into a pET28 vector containing the *Staphylococcus aureus* protein A zz domain fused to a 6-His tag (zz-pET28) to produce zz-tagged fragments. Cloning of *Xenopus* Nup153 N′ (Fig. 2 a) was described previously (Shah et al., 1998). Recombinant protein was expressed in bacteria and isolated on Ni-NTA-agarose (QIAGEN).

Nup98 and Nup153 pulldowns

Nup98 protein fragments were coupled to CnBr–Sepharose CL4B beads (Amersham Pharmacia Biotech). Beads (20 μl) containing Nup98 fragment (20 μg) were blocked with BSA (1 h; 20 mg/ml). *Xenopus* extract, diluted 1:50 in PBS, 1 mg/ml BSA, 1 μg/ml aprotinin, and 1 μg/ml leupeptin, was added and incubated at 4°C with tumbling. The beads were washed five times with PBS. We found cleavage at HF_{876}SKY to cause the Nup98 fragments I and II used here to be truncated at aa 876 (unpublished data). For Nup153 pulldowns, each fragment (5 μg; zz-Nup153 1–339, zz-Nup153 N′, or zz-Nup153 1–245) was prebound to IgG Sepharose (Amersham Pharmacia Biotech) for 1 h at room temperature in Nup153 PDB (150 mM NaCl, 1 mM MgOAc, 0.2% Tween-20, 50 mM Tris, pH 8.0, 1 mg/ml BSA, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). *Xenopus* extract (diluted 1:50 in PDB) was incubated with the beads at 4°C with tumbling. Pulldowns were washed (three times with PDB; one time with PBS), and proteins were eluted with 100 mM glycine, pH 2.5. SDS-PAGE and IB were performed as in Shah et al. (1998).

Protein purification, peptide sequence analysis, and column chromatography

Proteins A–D were purified from *Xenopus* egg extract (diluted as above to 40 ml) by binding to Nup98 fragment II beads (6 ml), washing five times with PBS, and eluting with 100 mM glycine, pH 2.5 (12 ml). A–D were concentrated with a Millipore Centrifugal Filter (30-kd cutoff), and resolved on a 7% SDS-PAGE gel. After transfer to polyvinylidene difluoride and staining with amido black, A–D were excised individually and digested with endoproteinase Asp-N. Peptides were sequenced by automated Edman degradation on a PE Biosystems Procise 494 (Fischer et al., 1991). Proteins A′–D′ were purified from a large scale zz-Nup153 aa 1–339 pulldown from *Xenopus* egg extract (2 ml); they proved to contain identical peptides to proteins A–D (unpublished data). Peptides from proteins A, C, and D are provided in Figs. 3 and 6. Protein B peptides and their mouse and human matches include: *Xenopus* Peptide/mouse EST BE532781/human AK001754 peptides: DLAVNQISV/DKAVTQISV/$_{558}$DRAVTQISV$_{566}$; *Xenopus* peptide 2/mouse EST AA536824/human AK001754 peptides: VSLSSVSKSSRQAV/SQLKSLEKSSDQER/$_{818}$SQLKSVDKSSNRER$_{831}$; and *Xenopus* peptide 3/mouse EST AA536824/human AK001754 peptides: DMNYAQKRS/EVEYLQKRS/$_{836}$EMEYLQKRS$_{844}$.

For gel filtration, *Xenopus* egg extract (200 μl) was fractionated on a Sephacryl S400-HR column (30 × 1 cm, 0.11 ml/min; Amersham Pharmacia Biotech) equilibrated in column buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM $MgCl_2$, 0.1% Tween-20). 54 fractions (400 μl) were collected. Even fractions (30 μl) were fractionated on gels and immunoblotted (Fig. 7 a); odd fractions were subjected to Nup153 aa 1–339 pulldown and the bound proteins were eluted, electrophoresed, and silver stained (Fig. 7 a; BioRad Laboratories).

Transfection and IF

HeLa cells on coverslips were transfected with Nup98 or Nup153 subclones in pCS2MT using TFX-20 transfection reagent (Promega) or $CaPO_4$. LMB was added as noted in Fig. 9. After ~16 h, transfected cells were fixed with 4% formaldehyde (5 min), permeabilized with PBS/0.2% Triton X-100, and blocked 10 min with PBS/0.2% Triton X-100/5% fetal calf serum. Transfected cells were stained with anti–c-myc tag antibody (9E10; Calbiochem) (l hr, 1:500 dilution) and detected with rhodamine-labeled goat anti–mouse IgG (l hr, 1:500; Southern Biotechnology Associates). Coverslips were mounted on Vectashield (2 μl; Vector Laboratories) and visualized with a Zeiss Axioskop 2 microscope. Digitonin and Triton X-100 permeabilization of HeLa cells was performed as in Bastos et al. (1996).

Constructs, expression, and antibody production to Nup133 and Nup160

A cDNA clone encoding aa 777–1105 of putative human Nup133 (Fig. 4 d) was prepared by reverse transcription of HeLa total RNA, amplification by PCR, and cloning into pET28c. Recombinant protein was expressed in *Escherichia coli* BL21/DE3, purified on Ni-NTA resin (QIAGEN), and used to immunize a rabbit. Antibodies were affinity purified against the same hNup133 protein fragment coupled to CnBr-Sepharose (Amersham Pharmacia Biotech). The complete human Nup133 sequence was RT-PCR cloned using 5 and 3′ oligos derived from GenBank/EMBL/DDBJ accession no. AK01676 and total HeLa cell RNA. Antibody was raised to *Xenopus* Nup133 protein expressed from *Xenopus* EST AW635680. Subclones of human Nup133 and Nup160 were prepared by using specific oligonucleotides and PCR or by restriction digestion, followed by subcloning into pCS2MT. A globular protein structure for Nup133 and Nup160 was predicted by PredictProtein through Columbia University's Bioinformatics Center (New York, NY).

A presumptive full-length *Xenopus* cDNA clone homologous to the mouse 160-kd protein (GenBank/EMBL/DDBJ accession nos. AAD17922) was prepared by reverse transcription of *Xenopus* total RNA using oligos derived from the extreme 5 and 3′ aa sequences of the GenBank *Xenopus* clones (GenBank/EMBL/DDBJ accession no. BF048903 and BF049549) (Results), amplification by PCR, and cloning into pET28. After expression, antibodies were raised and affinity purified against the same *Xenopus* protein. Human Nup160 aa 317–697 was subcloned from GenBank/EMBL/DDBJ accession no. KIAA0197 into pCS2MT for transfection and into pET28 for production of antisera in rabbits. We note that the KIAA0197 clone lacks 88 aa in regions around the COOH terminus of mouse Nup160, but we found human EST clones that contain these missing aa (Fig. 6 a).

Immunoprecipitation was done using *Xenopus* egg cytosol prepared as in Shah et al. (1998). Affinity-purified IgG against xNup160, xNup133, and xNup214, or preimmune antisera (1 μg) was coupled to 10 μl protein A-Sepharose beads (Amersham Pharmacia Biotech). The antibody beads were blocked (1 h; 4°C; 20 mg/ml BSA). Blocked beads (10 μl) were incubated in egg extract (20 μl) and 500 μl PBS (2 h; 4°C), washed five times in PBS, and eluted with 0.1 M glycine, pH 2.5 (50 μl). 5 μl were loaded per lane for Western blots; 15 μl was loaded for silver-stained gels. SDS-PAGE (7 or 8%) protein gels were used throughout. We note that not all verte-

brate Nup133 may be contained in the Nup160 complex, as a greater amount of Nup133 was observed in Fig. 7 c, lane 6 than in lane 5.

Poly[A]$^+$ RNA accumulation, protein import, and protein export assays

Poly[A]$^+$ RNA accumulation was monitored after transfection of HeLa cells with nucleoporin subclones, using fixation and permeabilization conditions from Dr. Susan Wente (Washington University, St. Louis, MO), modified as below. Cells were grown on coverslips for ~16 h, then transfected for 16 h with control or nucleoporin gene fragments in pCS2MT using QIAGEN Effectene. These were fixed (3% formaldehyde in PBS, 20′, on ice), permeabilized (0.5% Triton X-100 in PBS), incubated 5 min with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7), and then prehybridized with 50% formamide, 2 × SSC, 1 mg/ml BSA, 1 mg/ml yeast tRNA (Bethesda Research Laboratories), 1 mM vanadyl ribonucleoside complexes (GIBCO BRL), and 10% dextran sulfate (1 h, 37°C). The cells were hybridized with Texas red–oligo[dT]$_{50}$ (MWG Biotech) at 50 pg/μl in the same buffer (16 h, 37°C), washed three times in 2 × SSC (37°C, 5 min each), then refixed as above. Transfected expressed proteins were detected with FITC–anti-myc antibody (1:500; Calbiochem). Effects on nuclear protein import and export were tested by cotransfection (16 h) of a pXRGG plasmid obtained from Dr. Bryce Paschal (University of Virginia, Charlottesville, VA) with either control or nucleoporin-encoding plasmids. After transfection cells were treated with 1 mM dexamethasone (Calbiochem; 60 min), the cells were visualized by fluorescence microscopy for nuclear import of the RGG fusion protein. Parallel cells were treated with dexamethasone for 60 min to allow RGG import, washed, then incubated with media lacking hormone (2 hr, 37°C) to allow export (Gustin and Sarnow, 2001).

Antibodies

The antibodies used were affinity-purified anti–human Nup133 aa 777–1105 (1:100 for IB; 1:1,000 for IF); anti-*Xenopus* Nup133 (1:4,000, IB; 10 μg/immunoprecipitation); anti–human Nup160 (1:100, IF); anti-*Xenopus* Nup160 (1:1,000, IB and 5 μg/immunoprecipitation); anti–rat Nup98 aa 43–470 (1:100, IF); anti–rat Nup155 aa 295–578 (1:1,000, IB); anti-Nup153 and anti-Tpr (Shah et al., 1998); anti-Nup62 and anti-Nup214 (Macaulay et al., 1995); anti-Nup205 and anti-Nup93 (Miller et al., 2000); anti–mouse Gle2 (1:100 IB), a gift of M. Powers (Emory University, Atlanta, GA); mAb414 (1:500, IF; 1:2,000, IB; Babco); anti-importin α and β, anti-transportin and anti-Ran (1:2,000, IB; Transduction Laboratories); and anti–lamin B and RCC1 from Dr. John Newport (University of California at San Diego, San Diego, CA). Anti-Crm1 (1:5,000, IB) and RanQ69L were gifts of D. Gorlich (Heidelberg University, Heidelberg, Germany). The anti-sec13p antibody was the gift of Dr. Bill Balch and Jacques Weismann (The Scripps Research Institute, La Jolla, CA) before publication. Rabbit antibodies were detected using rhodamine or fluorescein goat anti–rabbit IgG (1:200) for IF, and goat anti–rabbit HRP (1:2,000) for blots (Jackson ImmunoResearch Laboratory).

Accession numbers

Nup160 homologues were non–full-length human KIAA0197 (GenBank/EMBL/DDBJ accession no. BAA12110), a human EST (N53299), a full-length 160-kd mouse protein (GenBank/EMBL/DDBJ accession no. AAD17922), a 176-kd *C. elegans* protein (GenBank/EMBL/DDBJ accession no. AAB37803.1), and a 160-kd *Drosophila* protein (GenBank/EMBL/DDBJ accession no. AAF53075). A *Xenopus* EST with homology to mouse Nup160 is encoded in GenBank/EMBL/DDBJ accession nos. BF048903 and BF049549. Nup133 homologues were: the full-length human gene AK001676, related ESTs from *Xenopus* (AW635680), and mouse (GenBank/EMBL/DDBJ accession no. AA536824), *Drosophila* AAF56042, and *S. pombe* GenBank clone CAB55845. (A partial human Nup133 clone, GenBank/EMBL/DDBJ accession no. AK001754, was used in early analysis.) Sequences were aligned and compared using Clustal W and SeqVu with Kyte-Doolittle algorithms for homology. No homology was observed between vertebrate Nup160 and Nup133, unlike that reported for the yeast proteins Nup120p and anti-Nup133p (Aitchison et al., 1995).

Online supplemental material

To determine which *Xenopus* proteins bind to fragments of Nup98 or Nup153, egg extract was added to beads conjugated to individual Nup98 or Nup153 fragments. Bound proteins were assessed by immunoblotting (Table S1). Only Bands A–D, i.e., Nup160, Nup133, Nup96, and Nup107, as well as sec 13, bound in significant amounts to the beads; they did so only to Nup98 aa 470–876 and to Nup153 aa 1–339 beads. Cotransfection (Fig. S1) was performed to assess the effect of Nup133 and Nup160 fragments on the shuttling reporter protein RGG. No effect was observed on the nuclear import or export of RGG.

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Note added in proof. Recent studies by Belgareh published after acceptance of this work also identify the novel vertebrate nucleoporins Nup133 and Nup160, the latter of which they designate hNup120 (Belgareh, N., G. Rabut, S.W. Bai, M. van Overbeek, J. Beaudouin, N. Daigle, O.V. Zatsepina, F. Pasteau, V. Labas, M. Fromont-Racine, J. Ellenberg, and V. Doye. 2001. *J. Cell Biol.* 154:1147–1160). We have designated it vertebrate Nup160, as it shows very slight homology to yeast Nup120 and is larger, containing an additional 32 kd at its COOH terminus, as determined by the NCBI Blast 2 Sequences algorithm. We find Nup133 and Nup160 inaccessible to antibody in digitonin-permeabilized cells, indicating that they are either localized to the nuclear pore basket or, if symmetrically localized, their epitopes are masked on the cytoplasmic side of the pore.

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