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.

Key words: Nup133; Nup160; mRNA export; Nup98; Nup153

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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; Baier 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; Baier 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 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.

In the rat, Nup153 is comprised of a unique NH2 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 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 ~2.5 × 106 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),
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 NH₂ 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 NH₂ 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 nucleoprotein RCC1, although traces of importin α and β, and Nup93 and Nup205 were observed (Table S1, 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 (asterisks, 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 antitransportin antibody; compare lane 5 (no Ran) with lane 6 (+Ran).

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 D and match with near identity to rat nucleoporin Nup107. Identity is boxed and homology is indicated in gray, as defined by Kyte-Doolittle algorithms.

(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
and the Xenopus B protein are new vertebrate nucleoporins, now termed vertebrate Nup133. A comparison to the Droso-
phila Nup133 homologue is shown in Fig. 4 d. Addition-
ally, 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 demon-
strating 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 per-
meabilization 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 exception-
ally long digitonin permeabilization (unpublished data). An-
tibodies to Nup153 (Fig. 5, i–j), Nup98 (Fig. 5, m–m), and
lamin B (Fig. 5, e–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 hu-
man protein of unknown function (1314 aa; KIAA0197;
91% identity), a 160-kd Droso-
phila 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
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 NH2-terminal homology at the nuclear side of the pore (Fig. 6 d). Like Nup133, Nup160 is primarily accessible to antibody only on the nuclear side of the pore (Fig. 6 c, right panel).

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-
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
probed with anti–human sec13p antibody. All pulldowns were done from solubilized AL (lane 1), solubilized AL 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 Nup98 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 Nup98 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
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]+ 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.
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 targets 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 \textit{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 \textit{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 pull-downs (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 Nup153 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 Nup133 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, SnNup145C, is instrumental in organizing multiple structures and functions within the yeast nucleus. Yeast Nup145C 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 pore, 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 modeling 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 cDNA, 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/DDJB accession no. L39991, and spliced onto Nup98-1 to give Nup98 aa 1–915. This was sequenced to verify authenticity. Oligonucleotides were used to produce subfragments of Nup98, which were cloned as EcoR1–Xho1 fragments from vector pET28B (In Vitrogen) for bacterial expression or as oligo specific to the 3’ end of the ORF in the Nup98 GenBank/EMBL/DDJB accession no. KIAA0197 into pCS2MT for transfection and into pET28 for expression in Escherichia coli (New York, NY). Recombinant protein was expressed in bacteria and isolated on Ni-NTA-agarose (Qiagen). 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 Nup133 protein fragment coupled to CNBr-Sepharose (Amersham Pharmacia Biotech) (H9262). The complete human Nup133 sequence was RT-PCR cloned using 5’ and 3’ oligos derived from GenBank/EMBL/DDJB accession no. AA01676 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 glycosylated 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/DDJB 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/DDJB accession no. BF048903 and BF049549). Resultants, amplification by PCR, and cloning into pET28. After reverse transcription, antibodies were raised and affinity purified against the same Xenopus protein. Human Nup160 aa 317–697 was subcloned from GenBank/EMBL/DDJB accession no. KIAA0197 into pCS2MT for transfection and into pET28 for protein expression. For immunofluorescence, note that we detected Nup133 and Nup160 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 (100 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 (50.0 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/ml 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 h, 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 aa 1,400–1,800; and beaded beads. 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 Belague after acceptance of this work also identify the novel vertebrate nucleoporins Nup133 and Nup160, the latter of which they designate hNup120 (Belague, N., G. Rabut, S.W. Bai, M. van Overbeek, J. Beaudouin, N. Daigle, O.V. Zatsepina, F. Paepe, 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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