netic Monitoring and Screening in the Workplace, OTA-BA-455 (U.S. Government Printing Office, Washington, DC, 1990).
11. *Genetic Information and Health Insurance: Report of the Task Force on Genetic Information and Insurance* (National Institutes of Health–Department of Energy Working Group on the Ethical, Legal, and Social Implications of Human Genome Research, 10 May 1993).
12. See, for example, P. R. Billings *et al.*, *Am. J. Hum. Genet.* **50**, 476 (1992).
13. As the interviews progressed and it was apparent that few members of ethnic or racial minorities were volunteering, additional telephone outreach was made to support groups to increase awareness of the project and opportunities for volunteering. This was only partially successful as seen in the demographic data.
14. There were no statistically significant differences in the responses on the telephone and written interviews so they are reported here together.
15. Of the 332 respondents, 80% were female, 57% have at least a bachelors degree, and 90% were Caucasian. Other characteristics were: 75% were married and living with their spouses, 76% have children, and 63% work outside the home. Family relationship, whether or not the respondent or family members were affected, age of diagnosis, and current age were also recorded.
16. Precise data on the demographics of genetic support groups are not available. Impressions are from staff of the Alliance of Genetic Support Groups based on their conversations and communications with the member organizations and attendance at national, regional, and local meetings.
17. Religious preferences were Roman Catholic, 26%; Protestant, 41%; Christian-other, 9%; Jewish, 11%; other, 2%; and none, 12%.
18. The interviewers were trained in interview techniques by the principal investigator and participated in pretesting the questionnaire.
19. The questions on possible genetic discrimination were taken from a questionnaire developed by Dr. Dorothy C. Wertz, The Shriver Center, Waltham, MA, entitled, *Ethical Issues in Genetics, Part I*, p. 33, No. 34, and used with permission of Dr. Wertz (letter of 16 December 1993).
20. This definition of genetic discrimination differs from the one used by Billings *et al.* (*12*) as they did not include actions against persons who were symptomatic or visibly affected by their genetic disorders. The design of our questionnaire does not permit analysis according to the definition of Billings *et al.* (1993). Because the questions on discrimination ask about all family members at once, the questions do not distinguish among: (i) the direct consequences of ongoing genetic disease or conditions, (ii) the effect of genetic disease on other family members, and (iii) the consequences of genetic information gained through testing.
21. M. J. Ellis Kahn, in a video by the HuGEM Project, *An Overview of the Human Genome Project and Its Ethical, Legal, and Social Issues* (Georgetown University Medical Center, Washington, DC, 1995).
22. K. H. Rothenberg, *J. Law Med. Ethics* **23**, 313 (1995).
23. The Ad Hoc Committee on Genetic Testing/Insurance Issues, *Am. J. Hum. Genet.* **56**, 328 (1995).
24. "Report of the ACLI-HIAA Task Force on Genetic Testing," The American Council of Life Insurance and The Health Insurance Association of America (1991).
25. Many respondents said they had never applied for life insurance because they assumed they would be turned down.
26. Since 1990, the Americans with Disabilities Act (ADA) has provided protection for persons with disabilities in the workplace. The ADA prevents employers from openly denying employment or firing an individual solely on the basis of a "disability" if there are "reasonable accommodations" that can be made in the work setting to allow the person to perform his or her job. In April 1995, the ADA was interpreted by the U.S. Equal Employment Opportunity Commission to include healthy people who are carriers of genetic disorders. Implementation in general relies on employers and employees knowing and similarly interpreting the law as well as having good faith efforts to comply.
27. We wish to thank the HuGEM Advisory Committee: N. A. Holtzman, F. Neal-Smith, E. O. Nightingale, K. H. Rothenberg, and L. Walters, for assistance in planning and carrying out the study. We also wish to thank L. Palincsar, M. A. Wilson, S. Rennert, and M. Volner, for their assistance with the telephone interviews and M. J. Verdieck and M. O'Reilly for research assistance. Most of all, we wish to thank the consumers who provided the data. Funded by the National Institutes of Health at the National Center for Human Genome Research in the branch of Ethical, Legal and Social Issues, grant number RO1-HG00786-03. This support does not constitute an endorsement of the views expressed in this article.

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Kap104p: A Karyopherin Involved in the Nuclear Transport of Messenger RNA Binding Proteins

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A cytosolic yeast karyopherin, Kap104p, was isolated and shown to function in the nuclear import of a specific class of proteins. The protein bound directly to repeat-containing nucleoporins and to a cytosolic pool of two nuclear messenger RNA (mRNA) binding proteins, Nab2p and Nab4p. Depletion of Kap104p resulted in a rapid shift of Nab2p from the nucleus to the cytoplasm without affecting the localization of other nuclear proteins tested. This finding suggests that the major function of Kap104p lies in returning mRNA binding proteins to the nucleus after mRNA export.

Transport across the nuclear envelope occurs through nuclear pore complexes (NPCs) and is governed by the interaction of soluble transport proteins (karyopherins) with the transport substrate and the NPC (*1–12*). Most of our understanding of the mechanism of translocation comes from studying protein import in semipermeablized cells (*1*) of model karyophilic proteins that carry a nuclear localization signal (NLS) from either the SV40 large T antigen or nucleoplasmin (*2*). These classical NLSs are recognized by karyopherin α in a dimeric cytosolic complex with karyopherin β (*3–8*). The complex docks at the NPC through its interaction with nucleoporins that contain characteristic repeated peptide motifs (*6–11*). The small guanosine triphosphatase, Ran, and p10 are required for the subsequent translocation of the substrate (and karyopherin α) through the NPC (*11*, *12*).

Distinct saturable and noncompeting pathways for the import of different karyophiles have been uncovered through the use of microinjection studies in oocytes (*13–15*). Similarly, saturable noncompeting pathways exist for the export of macromolecules from the nucleus (*14*, *16*, *17*). The signals that mediate many of these processes are different from classical NLSs (*14*, *15*, *17–19*) and thus may use recognition factors other than karyopherin α and karyopherin β for nuclear transport. Here we characterize the first such factor, which we term Kap104p and which is required for the import of at least two yeast nuclear mRNA binding proteins.

The *Saccharomyces cerevisiae* proteins Kap60p and Kap95p are homologs of mammalian karyopherin α and karyopherin β (*20*). Sequence comparisons of Kap95p with the complete yeast genome database uncovered three additional proteins that are structurally similar to Kap95p; two of these, which we term Kap123p and Kap104p, have not been previously characterized (*21*), and the third, Pse1p, was identified as a multicopy enhancer of protein secretion (*22*). The sequence alignment of Kap104p with Kap95p is shown (Fig. 1A). The proteins bear substantial similarity over their entire lengths, and secondary structural predictions suggest that Kap95p and Kap104p share the same overall domain structure of HEAT motifs (*23*).

Deletion of *KAP104* resulted in a severe growth defect and temperature sensitivity (*24*). Immunofluorescence microscopy (*25*) with antibodies specific for Kap104p (in wild-type cells) showed that Kap104p was mainly cytosolic and was apparently absent from the nucleus (Fig. 1B). However, in *nup120Δ* cells, which cluster their NPCs to a region of the nuclear envelope opposite the nucleolus (*26*), Kap104p colocalized with the nucleoporin Nsp1p (*27*) (Fig. 1C). The ability to detect coincident staining of the nucleoporins and Kap104p under these conditions likely was due to an interaction of Kap104p with NPCs.

Subcellular fractionation (*28*) was consistent with the distribution of Kap104p detected by immunofluorescence. Kap104p was present mainly in the cytosolic fraction,

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but cofractionated partly with the nucleus (*29*). Further fractionation of the nucleus caused Kap104p to be released into a soluble fraction, and so it did not copurify with NPCs (*28*, *29*). This result is in contrast to that for Kap95p and Kap60p, which were found in the highly enriched NPC fraction (see below) (*30*). Thus, the interaction of Kap104p with the NPC was weaker than that of the Kap95p-Kap60p complex.

We used an overlay blot assay to investigate which (if any) NPC proteins are bound by Kap104p. Both Kap95p and Kap104p were tagged with a functional in-frame COOH-terminal fusion of protein A (*31*). Cytosolic extracts (*28*) containing the chimeras were incubated with NPC proteins immobilized on nitrocellulose, and bound karyopherins were detected by means of the tag (Fig. 2A) (*10*). For reference, the positions of repeat-containing nucleoporins identified through the use of well-characterized monoclonal antibodies are shown (*28*). Kap104–protein A bound most strongly to proteins in the NPC fraction that comigrated with Nup116p, Nup100p, Nup145p (65-kD fragment), and Nup57p. Although Kap95–protein A (likely complexed with Kap60p) (*8*) bound to these nucleoporins, it also bound strongly to proteins comigrating with Nup1p, Nsp1p, and Nup49p. Kap104p expressed as a glutathione-S-transferase (GST) fusion and purified from *Escherichia coli* bound to the same set of nucleoporins as the cytosolic extract containing tagged Kap104p. Thus, whereas Kap95p has been reported to bind to nucleoporins in a complex with Kap60p, Kap104p bound directly to these proteins and required no additional factors from yeast cytosol. The strong signal, with a relative molecular mass of ~60,000, observed in NPCs with Kap95–protein A was identified by immunoblotting as Kap60p (*29*), confirming that Kap95p binds Kap60p. In contrast, Kap104–protein A did not bind Kap60p. Similarly, purified Kap104p did not bind to immobilized Kap60p, whereas purified Kap95p did (*29*). This result strongly suggests that whereas Kap60p binds Kap95p, it does not bind Kap104p.

Because of the comigration of Kap104p binding proteins with the repeat-containing nucleoporins, we expressed GLFG, XFXFG, and PSFG repeat–containing nucleoporins in *E. coli* and compared the ability of Kap104– and Kap95–protein A to bind to each (Fig. 2B). Kap104p bound to Nup116p,

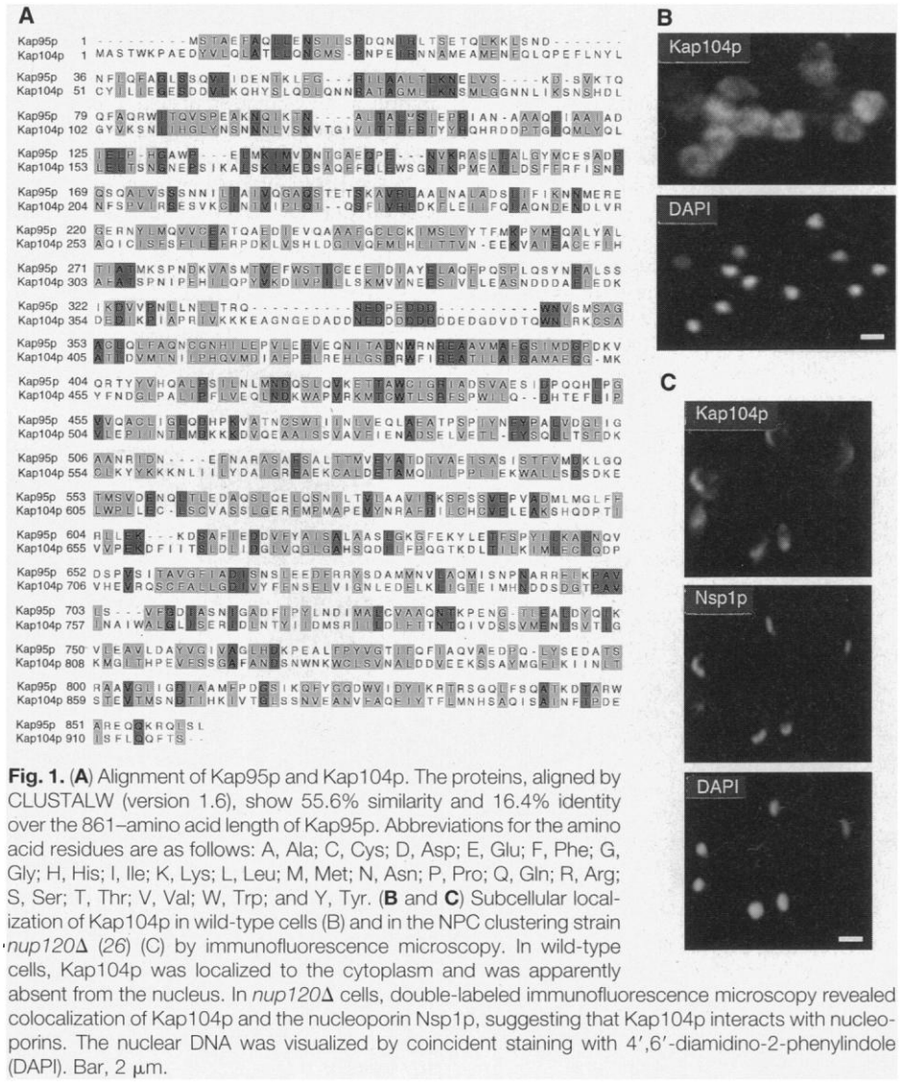

Fig. 1. (**A**) Alignment of Kap95p and Kap104p. The proteins, aligned by CLUSTALW (version 1.6), show 55.6% similarity and 16.4% identity over the 861–amino acid length of Kap95p. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (**B** and **C**) Subcellular localization of Kap104p in wild-type cells (B) and in the NPC clustering strain *nup120Δ* (*26*) (C) by immunofluorescence microscopy. In wild-type cells, Kap104p was localized to the cytoplasm and was apparently absent from the nucleus. In *nup120Δ* cells, double-labeled immunofluorescence microscopy revealed colocalization of Kap104p and the nucleoporin Nsp1p, suggesting that Kap104p interacts with nucleoporins. The nuclear DNA was visualized by coincident staining with 4′,6′-diamidino-2-phenylindole (DAPI). Bar, 2 μm.

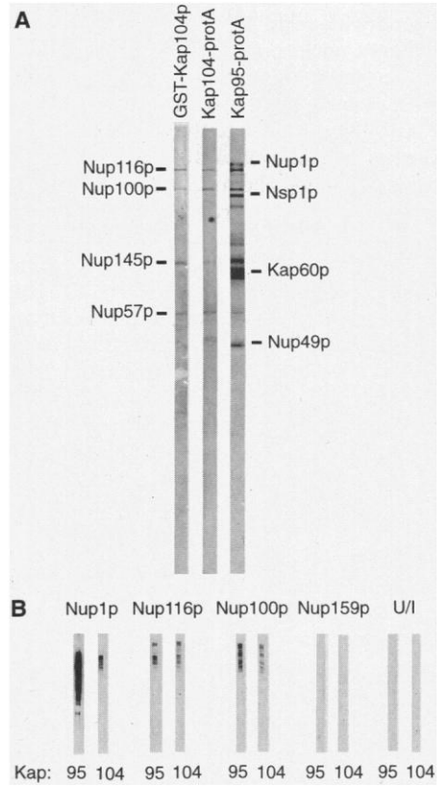

Fig. 2. Kap104p binds a subset of repeat-containing nucleoporins as shown by blot-overlay binding of Kap104p to a subset of nucleoporins. (**A**) Proteins of yeast NPCs (*28*) were separated by SDS-PAGE and transferred to nitrocellulose. Strips were probed with cytosol from *KAP95-A*, *KAP104-A* cells or purified *E. coli*–expressed GST-Kap104p. Bound proteins were detected with rabbit IgG, in the case of the yeast cytosols, or by monoclonal antibodies (mAbs) to GST followed by horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence. Additional strips were immunoblotted with mAbs 414 and 350, and mAb 192 (*29*), which recognize the peptide repeat motifs of various nucleoporins (*28*), to identify the position of the nucleoporins shown. (**B**) Repeat-containing nucleoporins [full-length Nup100p and Nup116p (*9*), amino acids 432 to 816 of Nup1p (*8*), and amino acids 537 to 622 of Nup159p (*39*)] were expressed in *E. coli*, and total lysates from each strain or from uninduced cells (U/I) were assayed for binding to Kap95–protein A and Kap104–protein A as described in (A).

Nup100p, and to the XFXFG repeat–containing region of Nup1p. Kap95p bound most strongly to the XFXFG repeat–containing region of Nup1p. Neither Kap95p nor Kap104p bound to PSFG repeats from Nup159p or to any *E. coli* proteins present in lysates lacking an expressed nucleoporin. It is difficult to assess the relative binding strength of each Kap to the nucleoporins in this assay because each protein may have renatured on the blot to a different extent. It is clear, however, that Kap104p bound to repeat-containing nucleoporins in this assay, and it is likely that Kap95p and Kap104p bound with relatively different affinities to different nucleoporins.

To study the interaction of Kap104p with soluble proteins, we purified the Kap104–protein A fusion from the postnuclear cytosolic extract of *KAP104-A* cells using immunoglobulin G (IgG)–Sepharose (*32*). The same procedure was used to isolate proteins bound to Kap95p (*30*). Two major proteins (p68 and p70) specifically bound to Kap104p–protein A (Fig. 3). The behavior of these proteins in this isolation procedure matched that of Kap60p bound to Kap95–protein A, suggesting that the strength and nature of the interaction of p68 and p70 with Kap104p was similar to that of Kap60p with Kap95p. Quantitation of the recovery of Kap104–protein A by imunoblotting showed that under these conditions, 60 to 70% of the total cellular Kap104p was recovered. Also, the Coomassie-blue staining intensity of p68 and p70 compared with that of Kap104p suggests that they were present in approximately equimolar amounts. Thus, most of Kap104p in the cytosol was bound to p68 and p70, although it remains to be determined whether p68 and p70 bind to Kap104p separately or as a complex. By partial protein sequencing, p68 and p70 were identified as Nab2p and Nab4p (also called Hrp1p) (*33*, *34*). These proteins have been shown to be nuclear mRNA binding proteins, both with similarity to a class of heterogeneous nuclear ribonucleoproteins (hnRNPs) that shuttle between the nucleus and cytoplasm of mammalian cells. Nab4p is the closest structural homolog of hnRNPA1 in yeast (*33*). Although neither Nab2p nor Nab4p contains the M9 sequence shown to be important for shuttling of hnRNPA1 (*19*), both have glycine-rich regions similar to the M9 sequence of hnRNPA1. Furthermore, searches of the yeast database with the M9 sequence revealed no direct homologs in yeast, indicating that this part of the "shuttling" sequence may not be highly conserved between yeast and mammals.

Because Nab2p and Nab4p are nuclear in the steady state (*33*), and the cytosolic fraction used for immunoisolation of Kap104–protein A was prepared under conditions that keep the majority of nuclei intact (*28*), the cytosolic fraction we recovered bound to Kap104p cannot represent the major fraction of these proteins. The similarity of Nab4p and Nab2p to the shuttling hnRNPs suggests that the cytosolic fraction of each protein bound to Kap104p may represent a cytoplasmic pool of these mRNA binding proteins.

Temperature-sensitive mutants of Kap104p were constructed by plasmid shuffling and assayed for their effect on Nab2p (*35*). One of these mutants (*kap104-16*) was of particular interest, because after the temperature shift to 37°C, Kap104p was rapidly turned over, presumably degraded after misfolding of the protein. Immunoblotting of total cell lysates demonstrated that 1 hour after the temperature shift, less than 10% of Kap104p remained (Fig. 4A), and it was barely detectable after 3 hours. Immunofluorescent localization of Nab2p demonstrated that depletion of Kap104p was coincident with redistribution of Nab2p from the nucleus to the cytoplasm (Fig. 4B). Other nuclear marker proteins, including Nsr1p, Nop1p, Npl3p (*36*), and the SV40 large T NLS fused to the green fluorescent protein were not mislocalized under these conditions (*29*). Thus, depletion of Kap104p after the temperature shift resulted in the specific depletion of Nab2p from the nucleus in these cells, which suggests that the accumulation of Nab2p in the cytosol was a direct result of the inability to reimport Nab2p after it was exported from the nucleus. Similar results were obtained when Kap104p was depleted from a strain containing *KAP104* under the glucose-repressible *GAL1* promoter (*29*). Electron microscopy of these cells showed no apparent morphological defects (*29*). In addition, different alleles of *kap104ts* mutants mislocalized Nab2p to a varying extent. Thus, Kap104p appeared to play a role in the import of Nab2p, but not its export. This result is also consistent with the cytosolic location of Kap104p.

We examined the distribution of polyadenylated [poly(A)$^+$] mRNA by in situ hybridization of oligo(dT) in the *kap104-16* strain. Poly(A)$^+$ RNA accumulated within the nucleus after the temperature shift, but in only a subpopulation of cells after 3 hours (*29*), considerably slower than the rapid mislocalization of Nab2p and loss of detectable Kap104p, suggesting that Kap104p depletion did not directly affect mRNA ex-

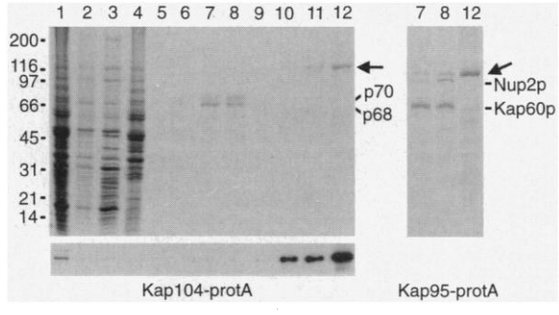

Fig. 3. Immunoisolation of Kap104– and Kap95–protein A complexes. Postnuclear cytosolic fractions from *KAP95-A* and *KAP104-A* cells were bound to IgG–Sepharose and eluted with a gradient of $MgCl_2$ (*32*). Lane 1, cytosolic extract; lane 2, low-speed pellet; lane 3, high-speed pellet; lane 4, pooled washes (one cell equivalent loaded); lanes 5 to 12, $MgCl_2$ stepped elutions (900 cell equivalents). The proteins of each fraction were separated by SDS-PAGE and stained with Coomassie blue. (Left) The elution profile for Kap104–protein A. The positions of p68, p70 (Nab2p and Nab4p, respectively), and Kap104–protein A (arrow) are shown. Fractions 7, 8, and 12 from the parallel experiment with Kap95–protein A are shown at the right. The positions of Kap60p, Nup2p, and Kap95–protein A (arrow) are shown. Immunoblotting for Kap104–protein A is shown at the bottom left and demonstrates the recovery of Kap104–protein A (lanes 1 to 4, 1 cell equivalent; lanes 5 to 12, 300 cell equivalents).

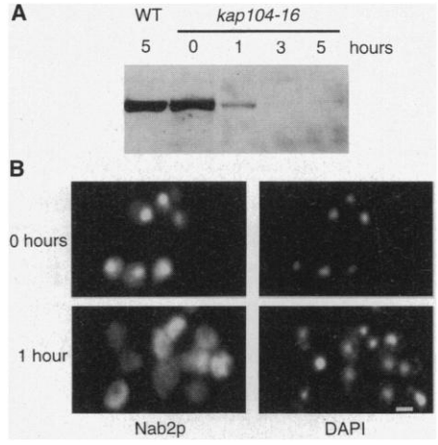

Fig. 4. Depletion of Kap104p causes mislocalization of Nab2p. (**A**) Immunoblotting of Kap104p in wild-type (WT) and *kap104-16* total cell lysates after a temperature shift to 37°C for the indicated length of time, demonstrating the rapid turnover of Kap104p in these cells after the temperature shift. (**B**) Immunofluorescent localization of Nab2p in *kap104-16* cells demonstrating that after the temperature shift to 37°C, Nab2p is rapidly redistributed from the nucleus to the cytoplasm. The length of time at 37°C is indicated. The nuclear DNA was visualized by coincident staining with DAPI. Bar, 2 μm. Other nuclear markers were not affected by the depletion of Kap104p in *kap104-16* cells (*29*).

port. The most straightforward interpretation of our results [and by analogy to hnRNPA1 (*19*)] is that Nab2p (and Nab4p) exit the nucleus as a major component of an hnRNP complex and upon release are reimported into the nucleus. The late-onset mRNA transport block we observed after the temperature shift in *kap104-16* cells may thus have been caused by depletion of the nuclear pool of essential mRNA binding proteins like Nab2p.

Thus, Kap104p represents a karyopherin that participates in a nuclear import pathway independent of the classical NLS-mediated pathway of Kap95p and Kap60p. It interacted directly both with its substrate and nucleoporins, bypassing the requirement of an NLS binding adapter like Kap60p. We suggest an import-export cycle in which Nab2p and Nab4p are imported into nuclei via Kap104p, are assembled with mRNA, and are exported to the cytoplasm as major components of hnRNPs, where they dissociate from the mRNA and upon rebinding of Kap104p, begin another cycle. A putative mammalian Kap104p homolog has recently been cloned (*37*), suggesting an evolutionary conservation of this transport system.

REFERENCES AND NOTES

1. S. A. Adam, R. Stern-Marr, L. Gerace, *J. Cell Biol.* **111**, 807 (1990); S. A. Adam and L. Gerace, *Cell* **66**, 837 (1991); M. S. Moore and G. Blobel, *ibid.* **69**, 939 (1992).
2. C. Dingwall and R. A. Laskey, *Trends Biochem. Sci.* **16**, 478 (1991).
3. Nomenclature: Karyopherin α: importin α/60, NLS receptor, SRP1, hSRP1α; karyopherin β: importin β/97, p97; p10: NTF2.
4. E. J. H. Adam and S. A. Adam, *J. Cell Biol.* **125**, 547 (1994); D. Görlich, S. Prehn, R. Laskey, E. Hartmann, *Cell* **79**, 767 (1994); N. C. Chi, E. J. H. Adam, S. A. Adam, *J. Cell Biol.* **130**, 265 (1995); D. Görlich *et al.*, *Curr. Biol.* **5**, 383 (1995); N. Imamoto, T. Tachibana, M. Matsubae, Y. Yoneda, *J. Biol. Chem.* **270**, 8859 (1995); J. Moroianu, G. Blobel, A. Radu, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2008 (1995); K. Weis, I. Mattaj, A. Lamond, *Science* **268**, 1049 (1995).
5. D. Görlich, F. Vogel, A. D. Mills, E. Hartmann, R. A. Laskey, *Nature* **377**, 246 (1995).
6. J. Moroianu, M. Hijikata, G. Blobel, A. Radu, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6532 (1995).
7. A. Radu, G. Blobel, M. S. Moore, *ibid.*, p. 1769.
8. M. Rexach and G. Blobel, *Cell* **83**, 683 (1995).
9. M. K. Iovine, J. L. Watkins, S. R. Wente, *J. Cell Biol.* **131**, 1699 (1995).
10. A. Radu, M. S. Moore, G. Blobel, *Cell* **81**, 215 (1995).
11. U. Nehrbass and G. Blobel, *Science* **272**, 120 (1996).
12. F. Melchior, J. Paschal, J. Evans, L. Geracė, *J. Cell Biol.* **123**, 1649 (1993); M. S. Moore and G. Blobel, *Nature* **365**, 661 (1993); *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10212 (1994); F. Melchior, T. L. Guan, N. Yokoyama, T. Nishimoto, L. Gerace, *J. Cell Biol.* **131**, 571 (1995); B. M. Paschal and L. Gerace, *ibid.* **129**, 925 (1995).
13. N. Michaud and D. S. Goldfarb, *J. Cell Biol.* **112**, 215 (1991); *ibid.* **116**, 851 (1992).
14. U. Fischer *et al.*, *ibid.* **113**, 705 (1991).
15. C. Kambach and I. W. Mattaj, *ibid.* **118**, 11 (1992).
16. N. Bataille, T. Helser, H. M. Fried, *ibid.* **111**, 1571 (1990); A. Jarmolowski, W. C. Boelens, E. Izaurralde, I. W. Mattaj, *ibid.* **124**, 627 (1994).
17. U. Fischer, J. Huber, W. C. Bolens, I. W. Mattaj, R. Luhrmann, *Cell* **82**, 475 (1995).
18. J. Hamm and I. W. Mattaj, *ibid.* **63**, 109 (1990); E. Izaurralde, J. Stepinski, E. Darzynkiewicz, I. W. Mattaj, *J. Cell Biol.* **118**, 1287 (1992); U. Fischer, V. Sumpter, M. Sekine, T. Satoh, R. Luhrmann, *EMBO J.* **12**, 573 (1993); W. Wen, J. L. Meinkoth, R. Y. Tsien, S. S. Taylor, *Cell* **82**, 463 (1995).
19. W. M. Michael, M. Choi, G. Dreyfuss, *Cell* **83**, 415 (1995); F. Weighardt, G. Biamonti, S. Riva, *J. Cell Sci.* **108**, 545 (1995).
20. R. Yano, M. Oakes, M. Yamaghishi, J. A. Dodd, M. Nomura, *Mol. Cell. Biol.* **12**, 5640 (1992); R. Yano, M. L. Oakes, M. M. Tabb, M. Nomura, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6880 (1994); K. D. Belanger, M. A. Kenna, S. Wei, L. I. Davis, *J. Cell Biol.* **126**, 619 (1994); C. Enenkel, G. Blobel, M. Rexach, *J. Biol. Chem.* **270**, 16499 (1995).
21. Kap104p: YBM7/YBR107c, position 270,905 to 273,658, chromosome II; Kap123p:YEU0/YER110c, position 376,750 to 380,088, chromosome V; Kap121p: PSE1/YMR308c, position 888,955 to 889,221, chromosome XIII.
22. T. Y. Chow, J. J. Ash, D. Dignard, D. Y. Thomas, *J. Cell Sci.* **101**, 709 (1992).
23. M. A. Andrade and P. Bork, *Nature Genet.* **11**, 115 (1995). These structural domains are predicted to be antiparallel, amphipathic α helices that form rod-like, tightly packed helical structures and present binding sites for additional proteins in loops between the packed helices. HEAT motifs have been predicted in mammalian karyopherin β (amino acids 122 to 482 and 600 to 725), Kap95p (amino acids 319 to 533 and 592 to 815), and Kap104p (amino acids 118 to 355 and 417 to 621), as well as in several other proteins, including Kap123p and Kap121p. The predicted exposed loops of four adjacent proposed HEAT repeats of karyopherin β provide overlapping binding sites for karyopherin α and RanGTP [J. Morianu, G. Blobel, A. Radu, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7059 (1996)]. Although the acidic amino acids of the central RanGTP binding site of karyopherin β (amino acids 333 to 340) are conserved in Kap95p (amino acids 338 to 344), the acidic stretch of Kap104p, which is aligned in the primary structure, is not present within its predicted HEAT motifs.
24. Deletion and disruption of the *KAP104* gene was accomplished by replacement of the *KAP104* open reading frame with the *URA3*-selectable marker in the diploid strain DF5 (*38*) by integrative transformation [R. Rothstein, *Methods Enzymol.* **194**, 281 (1991)] with modifications as described (*38*). Heterozygous diploids containing a wild-type and a disrupted copy of *KAP104* were sporulated, and tetrads were dissected on YPD plates at 23°C. Although all four spores could be recovered, haploid cells carrying a disrupted copy of *KAP104* failed to germinate at 30°C, were severely impaired in their growth at 23°C, and died when transferred to 30°C.
25. Indirect immunofluorescence was performed after 8-min fixation of yeast spheroplasts in 3.7% formaldehyde as described (*28*), with mouse antiserum to purified Kap104p or Nab2p, monoclonal antibodies to Nsr1p and Nop1p, or rabbit antiserum to Nsp1p. Double immunofluorescent labeling was done with donkey DTAF [(dichlorotriazinyl)aminofluorescein]–conjugated antiserum to rabbit IgG and donkey Cy3-conjugated antiserum to mouse IgG. Composite images were collected as described (*26*).
26. J. D. Aitchison, G. Blobel, M. P. Rout, *J. Cell Biol.* **131**, 1659 (1995).
27. U. Nehrbass *et al.*, *Cell* **61**, 979 (1990).
28. M. P. Rout and G. Blobel, *J. Cell Biol.* **123**, 771 (1993); M. P. Rout and J. V. Kilmartin, *ibid.* **111**, 1913 (1990).
29. J. D. Aitchison, G. Blobel, M. P. Rout, data not shown.
30. M. P. Rout, G. Blobel, J. Aitchison, in preparation.
31. The protein A gene was integrated into the genomic copy of each *KAP* gene, yielding functional chimeras as the only copy of each gene as described (*38*) (these strains are referred to as *KAP95-A* and *KAP104-A*). No growth defects were observed as a result of the presence of either tagged protein, and both subcellular fractionation and immunofluorescence microscopy of each of these constructs reflected the distribution observed with the wild-type proteins.
32. A postnuclear supernatant fraction containing cytosol was isolated as described (*28*). Cytosol (3. 2 ml, corresponding to ~30 mg of protein) was diluted with 12.0 ml of 20 mM Hepes, 110 mM KOAc, 2 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1% Tween-20 (pH 7.5), plus 50 μl of a protease inhibitor cocktail (*28*). This mixture was centrifuged at 2000*g* for 20 min (low-speed spin) at 4°C, and the supernatant was centrifuged at 260,000*g* for 1 hour (high-speed spin) at 4°C. The resulting supernatant was loaded onto 10 μl of prewashed, packed rabbit IgG–Sepharose (Cappel, Durham, NC) and incubated with gentle agitation for 12 hours at 4°C. After extensive washing, the bound proteins were eluted with a step gradient of 25 mM, 50 mM, 100 mM, 200 mM, 500 mM, 1 M, 2 M, and 4. 5 M $MgCl_2$ in 20 mM Hepes, 0. 05% Tween-20 (pH 7.5) plus protease inhibitor cocktail (1:1000 dilution) and precipitated with trichloroacetic acid for analysis by SDS–polyacrylamide gel electrophoresis (PAGE).
33. J. T. Anderson, S. M. Wilson, K. V. Datar, M. S. Swanson, *Mol. Cell. Biol.* **13**, 2730 (1993); M. Henry, C. Z. Borland, M. Bossie, P. A. Silver, *Genetics* **142**, 103 (1996).
34. The protein sequence of p68 and p70 yielded amino acids 295 to 312 of Nab2p and amino acids 245 to 257 of Nab4p or HRP1p (*33*). Nab2p YPD has the accession number YGL122c; HRP1p is also Nab4p (YPD accession number, YOL123w).
35. The *KAP104* gene was cloned into PRS314, PRS316, and PRS317 to yield p104-TRP, p104-URA, and p104-LYS. p104-URA was transformed into diploid DF5 cells (*38*), carrying a deletion or disruption of one copy of KAP104 (*kap104::ura3::HIS3*). These cells were sporulated, tetrads dissected, and *kap104::ura3::HIS3*, p104-URA haploids were selected. These cells were then transformed with p104-TRP that had been passaged through the mutagenizing *E. coli* strain XL-1 Red. Transformants were transferred to 5-fluoroorotic acid to select against p104-URA at 23°C and then replica-plated at 30° and 37°C to identify temperature-sensitive strains. Strains that maintained their temperature sensitivity on YPD, and were rescued when retransformed with p104-LYS, were selected.
36. W. C. Lee, Z. X. Xue, T. Melese, *J. Cell Biol.* **113**, 1 (1991); J. P. Aris and G. Blobel, *ibid.* **107**, 17 (1988); M. A. Bossie, C. DeHoratius, G. Barcelo, P. Silver. *Mol. Biol. Cell* **3**, 875 (1992).
37. N. Bonifaci and G. Blobel, personal communication.
38. J. D. Aitchison, M. P. Rout, M. Marelli, G. Blobel, R. W. Wozniak, *J. Cell Biol.* **131**, 1133 (1995).
39. D. M. Kraemer, C. Strambio-de-Castilla, G. Blobel, M. P. Rout, *J. Biol. Chem.* **270**, 19017 (1995).

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