

# Targeting and Function in mRNA Export of Nuclear Pore Complex Protein Nup153

Ricardo Bastos,\* Amy Lin,\* Mark Enarson,‡ and Brian Burke\*‡

\*Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115; and ‡Department of Anatomy, The University of Calgary, Calgary, Alberta, Canada T2N 4N1

**Abstract.** Nup153 is a large (153 kD) O-linked glycoprotein which is a component of the basket structure located on the nucleoplasmic face of nuclear pore complexes. This protein exhibits a tripartite structure consisting of a zinc finger domain flanked by large (60–70 kD) NH<sub>2</sub>- and COOH-terminal domains. When full-length human Nup153 is expressed in BHK cells, it accumulates appropriately at the nucleoplasmic face of the nuclear envelope. Targeting information for Nup153 resides in the NH<sub>2</sub>-terminal domain since this region of the molecule can direct an ordinarily cytoplasmic protein, pyruvate kinase, to the nuclear face of the nuclear pore complex. Overexpression of Nup153 results in the dramatic accumulation of nuclear poly (A)<sup>+</sup> RNA, suggesting an inhibition of RNA export

from the nucleus. This is not due to a general decline in nucleocytoplasmic transport or to occlusion or loss of nuclear pore complexes since nuclear protein import is unaffected. While overexpression of certain Nup153 constructs was found to result in the formation of unusual intranuclear membrane arrays, this structural phenotype could not be correlated with the effects on poly (A)<sup>+</sup> RNA distribution. The RNA trafficking defect was, however, dependent upon the Nup153 COOH-terminal domain which contains most of the XFXFG repeats. It is proposed that this region of Nup153, lying within the distal ring of the nuclear basket, represents a docking site for mRNA molecules exiting the nucleus.

THE bidirectional movement of macromolecules across the nuclear envelope is a multi-step process mediated by nuclear pore complexes (NPCs)<sup>1</sup> (Feldherr et al., 1984; Melchior and Gerace, 1995). These are massive multiprotein assemblies which penetrate the nuclear envelope in regions where both the inner and outer nuclear membranes are joined (Panté and Aebi, 1994). Ultrastructural analyses of amphibian oocyte NPCs have revealed that they are extremely intricate objects organized about a symmetrical framework lying in the plane of the nuclear membranes (Hinshaw et al., 1992; Akey and Radermacher, 1993; Akey, 1995). This structure, roughly 130 nm in diameter and 70 nm deep, appears as a circle of eight spokes radiating from a central gated channel com-

plex or transporter. The spokes are joined towards their distal ends by two stacked rings, each composed of eight large subunits. One ring faces the nucleoplasm, and the other faces the cytoplasm. Towards the periphery of this ring-spoke assembly, in the vicinity of the nuclear membranes, are eight additional channels with diameters of approximately 9 nm (Hinshaw et al., 1992). It is thought that these provide free passage for small molecules across the nuclear envelope. This current view of the NPC framework with both central and peripheral channels provides a structural basis for the sieve-like properties observed for the nuclear envelope as a whole (Paine, 1975; Paine et al., 1975).

Associated with the central framework are peripheral filamentous structures (Panté and Aebi, 1994). On the cytoplasmic face, there are eight short, kinky filaments which extend about 100 nm into the cytoplasm. It is thought that these contain the sites at which nuclear proteins initially dock, as complexes with soluble nuclear localization signal receptors, before energy-dependent translocation through the central channel of the NPC (Richardson et al., 1988). On the nucleoplasmic face, there are eight seemingly rigid filaments joined at their distal ends by a 50-nm-diam ring. The appearance is of a basket or fish trap-like structure extending about 100 nm into the nucleus (Ris, 1991). The function of the basket is still a

Address all correspondence to Brian Burke, Department of Anatomy, The University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta Canada T2N 4N1. Tel.: (403) 220-7287. Fax: (403) 270-0979. E-mail: bburke@acs.ucalgary.ca

Ricardo Bastos' current address is Dept. de Biologie Cellulaire, Institute Jacques Monod, CNRS, Université Paris 7, Tour 43, 2 Place Jussieu, 75251 Paris Cedex 05, France.

1. *Abbreviations used in this paper:* CMPK, chicken muscle pyruvate kinase; HA, influenza virus hemagglutinin; NLS, nuclear localization signal; NPC, nuclear pore complex; NRK, normal rat kidney.

matter of speculation, although it is conceivable that it might, like the cytoplasmic filaments, have a docking role for transport substrates.

With a total mass of about 125 MD, the vertebrate NPC is about 30 times as large as a ribosome (Reichelt et al., 1990). It has been suggested on this basis that it may be composed of as many as 100 different protein subunits (Reichelt et al., 1990). This figure is given some credence by studies on isolated yeast NPCs (Rout and Blobel, 1993), which, at a little less than two thirds the size of their vertebrate counterparts, appear to contain 60–80 distinct polypeptides. To date, only a dozen or so vertebrate NPC proteins or nucleoporins have been characterized (Rout and Wentz, 1994), which, even assuming very generous stoichiometries, can account for no more than 30–50% of the NPC mass. Several of these NPC proteins are members of rapidly expanding nucleoporin families (Rout and Wentz, 1994) distinguished on the basis of the repetitive sequence motifs XFXFG or GLFG (single letter code, where X is a residue with a small or polar side chain).

While considerable progress has been made in describing the roles of soluble factors in nucleocytoplasmic transport (Sweet and Gerace, 1995), there is scant knowledge of how the protein components of the NPC itself might affect the translocation of macromolecules. Since the NPC operates vectorially, it seems obvious that the function of individual nucleoporins can only be fully understood in the context of their location and interactions within the NPC. So far, none of the vertebrate nucleoporins have been assigned any specific function, and while the majority have been located in fairly general terms (for instance, exposed either to the nucleoplasm or to the cytoplasm), only three (CAN/Nup214, Nup153, and p62) have been localized with any degree of precision to distinct substructures within the NPC (Panté et al., 1994; Guan et al., 1995). These three proteins are all members of the XFXFG family (Rout and Wentz, 1994) and are extensively modified with O-linked *N*-acetyl glucosamine (Holt et al., 1987). p62, a very abundant NPC protein associated with three other nucleoporins, p45, p54, and p58 (Finlay et al., 1991; Kita et al., 1993; Guan et al., 1995), is located within the central channel complex (Guan et al., 1995). In contrast, both CAN/Nup214 and Nup153 are found in peripheral structures (Panté et al., 1994). The former, in association with another nucleoporin Nup84 (Bastos, R., and B. Burke, manuscript in preparation), is a component of the short cytoplasmic filaments (Panté et al., 1994). Nup153, which can be eluted from the NPC as a large homooligomer, is a component of the distal ring of the nuclear basket (Panté et al., 1994).

In this paper, we have focused on the structure, interactions, and possible functions of Nup153 (Sukegawa and Blobel, 1993). This protein is one of only two nucleoporins (the other being Nup358; Wu et al., 1995; Yokoyama et al., 1995) known to contain a series of zinc fingers, which at least in vitro are capable of binding DNA (Sukegawa and Blobel, 1993). The sequence of Nup153 reveals three distinct domains (Sukegawa and Blobel, 1993; McMorrow et al., 1994): the zinc finger region of ~220–amino acid residues (Z-domain) flanked by a ~600–residue NH<sub>2</sub>-terminal domain (N-domain) and a similarly sized COOH-terminal domain (NP-domain). The latter contains the majority (26

out of 33) of the XFXFG repeats and consequently bears the strongest similarity to other nucleoporins from a variety of species, including yeast (Rout and Wentz, 1994). Our intention has been to define regions of Nup153 which contain independent and separable functions and in this way lay the foundations for further experiments to identify interacting proteins using both biochemical and genetic approaches. In addition, we envisaged that these studies may provide a rational basis for the design of dominant interfering mutants, which may in turn lead to new understanding of the role of Nup153 in nucleocytoplasmic transport. As will be described in this paper, we have indeed been able to dissect Nup153 into functional domains. In particular, we have identified a discrete region of the Nup153 molecule that contains information required for appropriate association with the NPC. Furthermore, overexpression of a second region of the Nup153 molecule leads to dramatic changes in RNA trafficking, leading to the suggestion that Nup153 may have a primary role in RNA export from the nucleus.

## Materials and Methods

### Cell Culture

BHK, normal rat kidney (NRK), and HeLa cells were maintained in DMEM containing 10% fetal calf serum (Hyclone Labs, Logan, UT) and penicillin/streptomycin (GIBCO BRL, Gaithersburg, MD). *Xenopus laevis* A6 cells were grown in medium containing (by volume) 75% NCTC109 (GIBCO BRL), 10% fetal calf serum, and 15% deionized water. All cells were grown in a humidified incubator with a 7.5% CO<sub>2</sub> and 92.5% air atmosphere. The mammalian cells were maintained at 37°C, whereas the amphibian cells were kept at 26.5°C.

### Antibodies

The monoclonal antibody QE5 against NPC glycoproteins, as well as polyclonal antibodies against CAN/Nup214 (referred to in our previous publication as p250), and Nup153 are described by Panté et al. (1994). An antipeptide rabbit antibody specific for lamin A has also been previously described (Burke, 1990). The human autoantibody against lamin B1 was a gift from Dr. Marvin Fritzler (University of Calgary, Calgary, Canada). The monoclonal antibody 12CA5 (Wilson et al., 1984; Field et al., 1988) against the influenza virus hemagglutinin (HA) epitope was obtained from the Berkeley Antibody Company (BAbCo, Berkeley, CA). A rabbit antibody against the same HA epitope was obtained from MBL Co., Ltd. (Nagoya, Japan). A monoclonal antibody against  $\beta$ -galactosidase was obtained from Promega Corp. (Madison, WI), while a polyclonal anti- $\beta$ -galactosidase antibody raised in rabbits was purchased from Cappel Inc. (Milvern, PA).

### DNA Constructs

The expression vector pCMV-HA, based on a plasmid designed by Morrie Birnbaum (Harvard Medical School, Cambridge, MA), was obtained from Roydon Price and Frank McKeon (Harvard Medical School, Cambridge, MA). The polylinker region of this vector was modified by ligating a double-stranded synthetic oligonucleotide adapter (Ausubel et al., 1987) between the unique XhoI and XbaI sites to generate pCMV-HA(XB). The adapter contained NotI and BglII sites and was designed such that the XbaI site was destroyed upon ligation. Full-length human Nup153 cloned in pBluescript II SK between the NotI and XhoI sites was cut with NotI and XbaI, thus removing a 1.8-kb fragment at the 5' end of Nup153. It was replaced with a similarly cut fragment of DNA that had been amplified, from the same plasmid, between the two primers P<sub>1</sub> (ATATGCGGC-CGCTCGAGTCAGGAGCCGGAGGAGTCCGAGGG) and P<sub>2</sub> (CGCGATGCGAAACCAGG). P<sub>1</sub> was designed in such a way that codons 1 and 2 from Nup153 were replaced by the six base XhoI recognition sequence. The 5' end of this primer contained a NotI site. The resultant plasmid was pBSNup153(NX). Finally, a 4,554-bp Nup153 fragment (which in-

cludes the termination codon) was cut from this plasmid using XhoI and BglII and was ligated into pCMV-HA(XB) so that the Nup153 coding sequence was in frame with the HA epitope. Specific deletions were made between the unique internal XbaI, EcoRV, and BstBI sites and between the terminal XhoI and BglII sites using double-stranded oligonucleotide adapters. For 3' end truncations, oligonucleotides containing an in frame stop codon were used. For chicken muscle pyruvate kinase (CMPK) fusions, CMPK cDNA was amplified by PCR from the vector pJ3 $\Omega$ -CMPK (Frangioni et al., 1992) between codons 20 and 477 using pairs of primers incorporating combinations of the restriction enzyme sites above. PCR products were digested as appropriate and ligated into similarly cut pCMV-HA-Nup153. The authenticity of the various constructs was verified by double-stranded sequencing of the appropriate regions using Sequenase<sup>TM</sup> (United States Biochem Corp., Cleveland OH) as recommended by the manufacturer.

## Transfections

The various constructs were purified by centrifugation through a CsCl gradient containing ethidium bromide (Ausubel et al., 1987). Transfections were carried out using the calcium phosphate method (Graham and van der Eb, 1973) or using the Lipofectamine reagent (GIBCO BRL) exactly as described by the manufacturer. For each DNA construct, the two procedures yielded equivalent results. However, where possible we preferred to use the calcium phosphate method for immunofluorescence experiments since it generally gave a cleaner background.

To construct the cell line BHKgr $\beta$ , BHK cells were transfected (using the calcium phosphate method) with pSV2neo, which confers resistance to the antibiotic G418, and a second plasmid containing a glucocorticoid receptor- $\beta$ -galactosidase cDNA (generously provided by Drs. Keith Yamamoto [University of California, San Diego] and Didier Picard [University of Geneva, CH]). Stable transfectants were selected in medium containing 800  $\mu$ g/ml G418 (GIBCO BRL). Single clones were isolated and screened for glucocorticoid receptor- $\beta$ -galactosidase fusion protein expression as well as dexamethasone responsiveness by immunofluorescence microscopy using antibodies against  $\beta$ -galactosidase.

## Immunofluorescence and Confocal Microscopy

Cells grown on glass coverslips were fixed with formaldehyde and labeled with antibodies according to previously described procedures (Ash et al., 1977). In short, cells were fixed for 20 min at room temperature in 3% formaldehyde (prepared from paraformaldehyde dissolved at 80°C in phosphate-buffered saline). After PBS washes, the fixed cells were permeabilized for 5 min at room temperature with 0.2% Triton X-100 in PBS and labeled with appropriate primary and secondary antibodies. For differential permeabilization experiments (in which only the cytoplasmic face of the nuclear envelope is exposed to antibody), cells were instead treated for 15 min at 4°C with 0.004% digitonin in PBS (Panté et al., 1994). The digitonin was prepared from a 10% stock solution in dimethyl sulfoxide. Integrity of the nuclear membranes in digitonin permeabilized cells was always confirmed using a rabbit anti-lamin A antibody (Burke, 1990). Rhodamine- and fluorescein-conjugated secondary antibodies were obtained from Tago, Inc. (Burlingame, CA). In addition, all samples were stained with the DNA-specific Hoechst dye (No. 33258) to reveal the cell nuclei. Specimens were observed and photographed under appropriate illumination with either a Zeiss Axiophot microscope (Thornwood, NY) equipped with a  $\times 63$  PlanApo objective lens or a Leica DMRB microscope (Deerfield, IL) equipped with a  $\times 63$  PL APO NA1.4 objective. For confocal microscopy and digital image acquisition, specimens were observed at an Optovar setting of 2.0 using a Zeiss Photomicroscope III equipped with  $\times 63$  PlanApo NA1.4 and  $\times 100$  Neofluor NA 1.32 objectives lenses and a Kodak MegaPlus model 1.6 camera (Rochester, NY) linked to a Touch TX4664, Mississauga, Ontario computer. Series of images acquired at 0.5- $\mu$ m focal intervals were digitally processed using Micro-Tome version 3.1 (VayTek, Inc., Fairfield, IA) to yield stacks of confocal slices.

## In Situ Hybridization

Fluorescence in situ hybridization of BHK cells employing a biotinylated oligo-dT 45 mer (prepared with a 5' biotin by the University of Calgary Oligonucleotide Synthesis Facility, Calgary, Alberta, Canada) was carried out according to the general procedures described by Spector and co-workers (Huang et al., 1994). Certain modifications were introduced, however, so that cells could be double labeled with fluorescent antibodies.

BHK cells (transfected or otherwise), grown on glass coverslips, were fixed for 15 min at room temperature in 3% formaldehyde (from paraformaldehyde) in PBS. After three 15 min PBS washes, the cells were permeabilized for 5 min at 4°C in sterile PBS containing 0.5% Triton X-100. They were then labeled for 30 min at room temperature with the primary antibody (usually 12CA5 culture supernatant at a dilution of 1:50) in sterile PBS containing 0.2% Triton X-100, 1 mM DTT, and 200 U/ml RNasin (Promega Corp.). After three 15 min washes in PBS/0.2% Triton X-100, the labeled cells were refixed as described above. Fixative was removed with three 15-min PBS washes, and the cells were then equilibrated in 2 $\times$  SSC for 10 min at 42°C (Huang et al., 1994). Hybridization was carried out by inverting the coverslips onto 100  $\mu$ l of hybridization mix and incubating overnight at 42°C in a humidified chamber. The hybridization mix consisted of 2 $\times$  SSC containing 1 mg/ml tRNA, 10% dextran sulfate, 25% formamide, and 50  $\mu$ g/ml biotin-oligo dT<sub>(45)</sub>. After hybridization, the cells were washed twice for 15 min at 42°C in 2 $\times$  SSC and once, also for 15 min at 42°C, in 0.5 $\times$  SSC. Immediately upon completion of this wash, the cells were again refixed as described above. After three washes in PBS, they were incubated for 30 min at room temperature with FITC-streptavidin (Sigma Immunochemicals, St Louis, MO) and rhodamine-goat anti-mouse IgG, each diluted appropriately into PBS/0.2% Triton X-100. Unbound fluorescent probes were removed with three washes in PBS/0.2% Triton X-100 and two washes in PBS. Finally, the coverslips were mounted in Slow Fade<sup>TM</sup> (Molecular Probes Inc., Eugene, OR) and examined by fluorescence microscopy.

## Immunoprecipitations and Immunoblotting

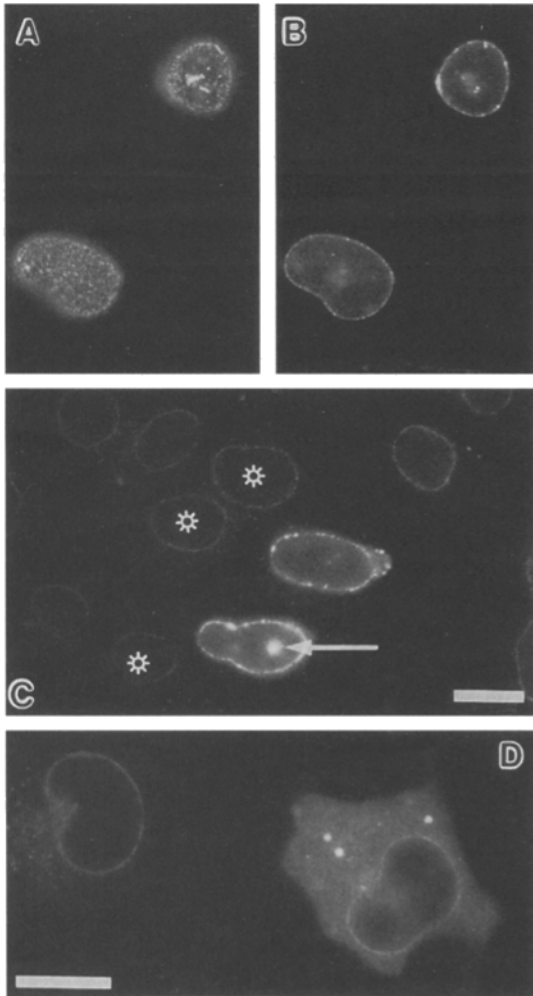
Transfected or mock transfected cells grown in 35-mm tissue culture dishes were washed once in PBS and then lysed in a buffer containing 50 mM triethanolamine (TEA), 500 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1:1,000 CLAP (10 mg/ml in DMSO of each of the following: chymostatin, leupeptin, antipain, and pepstatin). The lysate was centrifuged for 5 min in an Eppendorf centrifuge (Fremont, CA) at 4°C. 20  $\mu$ l of a QE5 affinity matrix, consisting of a 50% suspension of protein G-Sepharose to which QE5 IgG had been chemically crosslinked using dimethyl pimelimidate, (Harlow and Lane, 1988) was added to the supernatant. The mixture was then rotated overnight at 4°C. The following morning, the QE5-beads were washed five times in either the same buffer or in a higher stringency buffer containing 50 mM TEA, 100 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mM DTT, 1 mM PMSF, and 1:1,000 CLAP (Panté et al., 1994). After two final washes in 50 mM Tris, pH 7.4, the QE5 beads were suspended in SDS-polyacrylamide gel sample buffer and fractionated by electrophoresis (Laemmli, 1970). On completion of electrophoresis, gels were blotted onto nitrocellulose filters (model BA85; Schleicher & Schuell Inc., Keene, NH; Burnette, 1981) using a semi-dry blotting apparatus manufactured by Hoefer Scientific Instruments Inc. (San Francisco, CA). Filters were blocked, labeled with primary antibodies, and then developed with peroxidase-conjugated secondary antibodies exactly as previously described (Burke et al., 1982).

## Electron Microscopy

Cells grown in 35-mm tissue culture dishes were fixed in 1% glutaraldehyde in 100 mM PIPES, pH 7.0, washed, and then postfixed in 2% reduced OsO<sub>4</sub> in 100 mM cacodylate, pH 7.3. The samples were dehydrated in ethanol and embedded in Epon 812. Sections, cut with a diamond knife, were contrasted with uranyl acetate and lead citrate.

## Results

We have expressed a series of epitope-tagged (with HA) human Nup153 constructs in BHK cells and followed their distribution by immunofluorescence microscopy. Fig. 1, (A and B) shows that at moderate expression levels, full-length HA-tagged Nup153 (HA-Nup153) associates exclusively with the nuclear envelope. Labeling with the 12CA5 (anti-HA) monoclonal antibody reveals a finely punctate distribution across the nuclear surface consistent with assembly of HA-Nup153 into NPCs. This view is reinforced by differential permeabilization of cells using either 0.2% Triton X-100 or 0.004% digitonin (Panté et al., 1994),



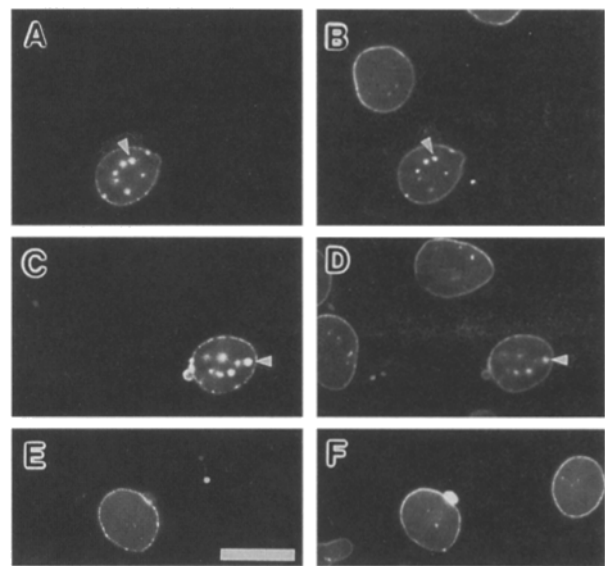
**Figure 1.** Indirect immunofluorescence microscopy of BHK cells expressing HA-tagged human Nup153 (HA-Nup153, *A–C*) and HA-tagged human CAN/Nup214 (*D*). In *A* and *B*, detection of HA-Nup153 is with a monoclonal anti-HA (12CA5). Two different focal planes of the same cells are shown. In *A* the microscope is focused at the nuclear surface, whereas in *B* it is focused at the equator. The punctate labeling pattern revealed in *A* is consistent with association of HA-Nup153 with NPCs. Massive concentration of HA-Nup153 in the nuclear envelope is documented in *C*. In this case, the cells were labeled with the antinucleoporin antibody QE5, which provides a comparison of the nuclei of transfected versus nontransfected cells (*asterisks*). Note the elongation of the nuclei containing high levels of HA-Nup153 as well as the appearance of brightly staining inclusions (*arrow*). Two cells expressing HA-CAN/Nup214 and labeled with 12CA5 are evident in *D*. In the cell on the left, expressing HA-CAN/Nup214 at a low level, the heterologous protein is restricted largely to the nuclear envelope. At a higher level of expression seen in the cell on the right, HA-CAN/Nup214 accumulates in the cytoplasm. Bar, 10  $\mu$ m.

which indicates that all of the HA-Nup153 is localized appropriately on the nucleoplasmic face of the nuclear envelope (not shown). This same distribution of heterologous HA-Nup153 has been observed in a variety of vertebrate cells, including HeLa cells and *X. laevis* A6 cells (Bastos, R. and B. Burke, unpublished observations).

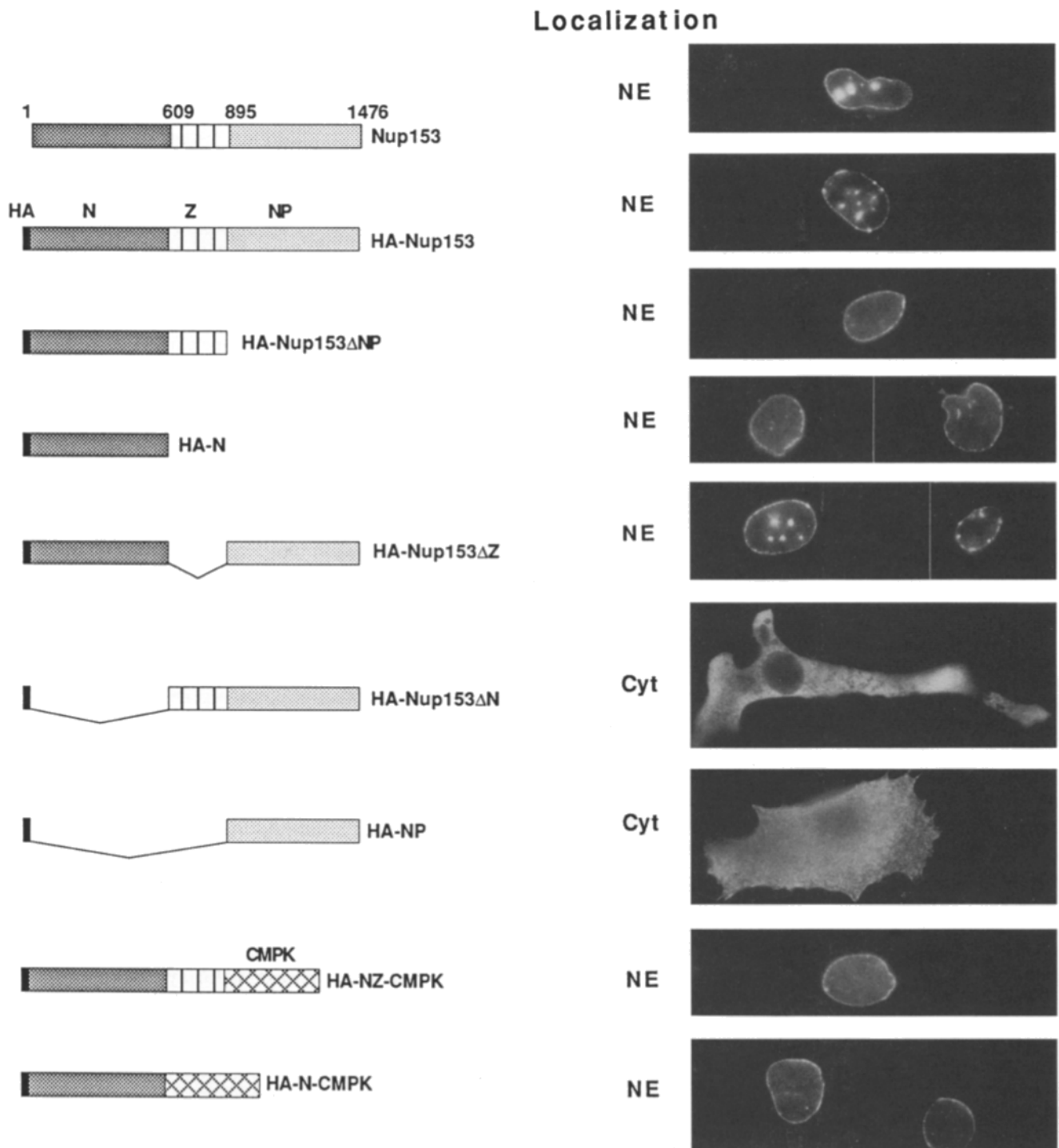
At higher levels of expression, the HA-Nup153 continues to accumulate at the nucleoplasmic surface of the nu-

clear envelope (Fig. 1 *C*). This observation was quite contrary to expectations since it was assumed that given an invariant number of NPCs, each with a precisely defined complement of protein subunits, the capacity of the nuclear envelope to accommodate heterologous Nup153 would be rapidly saturated, leading to an accretion elsewhere in the cell, probably the nucleus. With another nucleoporin, CAN/Nup214 (von Lindern et al., 1992; Kraemer et al., 1994; Fornerod et al., 1995), this is precisely what is seen. Accumulation of HA-CAN/Nup214 at the nuclear periphery increases to a maximum level, which is identical in all cells within a given population, after which it spills over into the cytoplasm (Fig. 1 *D*). With Nup153 (or HA-Nup153), however, no such saturation is observed. Instead, as expression level increases, so does the labeling of the nuclear envelope. Ultimately, changes in nuclear morphology are observed involving elongation of the originally spherical BHK nuclei concomitant with the appearance of micron-scale nuclear inclusions (Fig. 1 *C*) that contain nuclear lamins in addition to HA-Nup153 (Fig. 2).

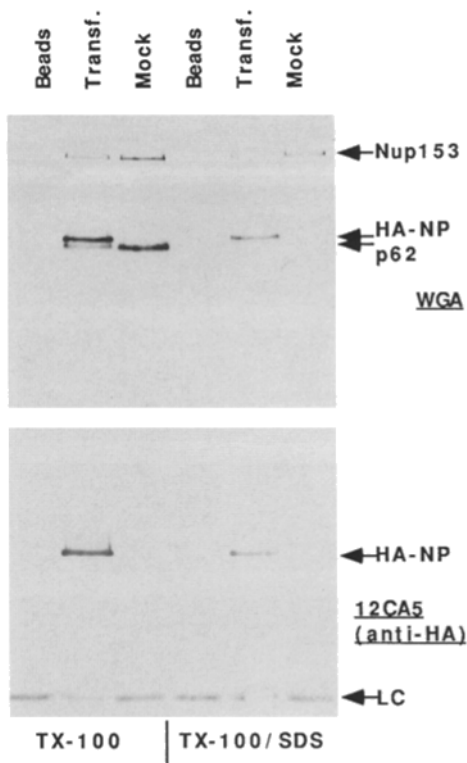
To broadly define regions of the Nup153 molecule involved in delivery to the inner face of the nuclear envelope, we constructed a series of HA-tagged deletion mutants and chimeric proteins, which we proceeded to express in BHK cells. The identity and subcellular localization of each construct is summarized in Fig. 3. It is immediately evident from this figure that the only region of the heterologous Nup153 molecule essential for association with the nuclear envelope is contained within the NH<sub>2</sub>-terminal domain. Indeed, this segment of the molecule can direct an ordinarily soluble protein, CMPK, to the nuclear envelope. In terms of targeting, the NP- and Z-domains are dispensable. While all molecules lacking the N-domain remain cytoplasmic (HA-Nup153 $\Delta$ N, HA-NP), they do acquire the capacity to bind wheat germ agglutinin, indicating the addition of *N*-acetyl glucosamine



**Figure 2.** Double label immunofluorescence microscopy of BHK cells expressing HA-Nup153 (*A* and *B*), HA-Nup153 $\Delta$ Z (*C* and *D*), and HA-Nup153 $\Delta$ NP (*E* and *F*). (*A*, *C*, and *E*) Anti-HA. (*B*, *D*, and *F*) Anti-lamin A. Note the lack of inclusions in the HA-Nup153 $\Delta$ NP images (*E* and *F*). Bar, 10  $\mu$ m.



*Figure 3.* A series of Nup153 deletions and chimeric molecules which were expressed in BHK cells. The subcellular localization of each is shown on the right hand side. HA indicates the location of the influenza hemagglutinin epitope. N, Z, and NP refer to the Nup153 NH<sub>2</sub>-terminal domain, Zinc finger domain, and COOH-terminal (or Nucleoporin XFXFG repeat) domain. All of the constructs were detected with the anti-HA antibody 12CA5, with the exception of Nup153, which was detected with QE5, an antinucleoporin antibody. NE indicates a nuclear envelope localization while Cyt indicates cytoplasmic localization. CMPK corresponds to residues 20-477 of chicken muscle pyruvate kinase, a soluble cytoplasmic protein. It is clear that only sequences within the N-domain of Nup153 are required for delivery to the nuclear envelope and that this region of the molecule can confer a nuclear envelope localization on CMPK. The presence of the HA epitope tag at the NH<sub>2</sub> terminus has no discernible effect on the behavior of Nup153. All panels are shown at the same magnification.



**Figure 4.** The Nup153 NP-domain is modified with *N*-acetyl glucosamine. BHK cells expressing HA-NP were processed for immunoprecipitation using the QE5 affinity matrix. Immunoprecipitates were washed in either low stringency (TX-100) or high stringency (TX-100/SDS) buffer. Only endogenous Nup153 and HA-NP (which contains the QE5 epitope) are recovered following high stringency washes. After low stringency washes, p62, CAN/Nup214, and their associated proteins (p45, 54, 58, and Nup84) are also recovered. Immunoprecipitates from both transfected (Transf.) and mock-transfected (Mock) cells, as well as QE5 beads alone (Beads), were run on duplicate gels. Both were transferred to nitrocellulose filters. One filter was labeled with peroxidase-wheat germ agglutinin (WGA) to detect *N*-acetyl glucosamine-containing proteins. The other was probed with 12CA5 to identify HA-NP. It is evident that Nup153, p62, and HA-NP all bind WGA. CAN/Nup214 transfers only very poorly and is therefore barely detectable on these blots.

(Fig. 4). Furthermore, as will be shown below, they do possess at least some biological activity. Of those constructs which are associated with the nuclear envelope, differential permeabilization using Triton X-100 versus digitonin indicates that all are appropriately localized on the nucleoplasmic face (data not shown).

Do all of these Nup153 constructs associate with the NPC? At present, this can not be answered definitively since the HA epitope in the context of the Nup153 NH<sub>2</sub> terminus does not function well for EM-immunocytochemistry. However, in the case of HA-N-CMPK, which localizes almost exclusively to the nuclear envelope (Figs. 3 and 5), immunofluorescence microscopy reveals a punctate labeling pattern of the nuclear surface identical to that observed with other antibodies against endogenous NPC proteins. In fact, double label experiments (Fig. 5) demonstrate a precise overlap of HA-N-CMPK with the nucle-

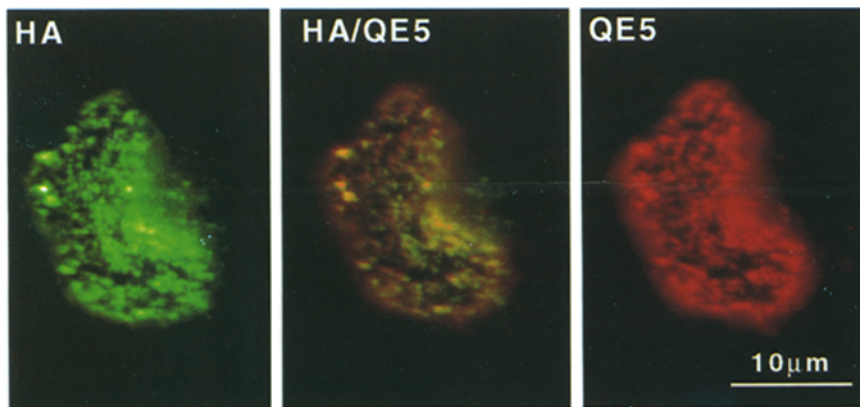
oporins recognized by QE5 (HA-N-CMPK lacks the QE5 epitope). These experiments indicate that, at the very least, the distribution of HA-N-CMPK is linked to that of endogenous nucleoporins. This similarity of labeling patterns is not observed when other nuclear envelope proteins, such as lamins or lamina-associated membrane proteins, are compared with nucleoporins (not shown).

While all of the constructs containing the N-domain become nuclear envelope-associated, subtle differences between them are nevertheless evident. HA-Nup153 lacking the entire zinc finger domain (HA-Nup153ΔZ) behaves in a similar fashion to the full-length molecule in that it accumulates to a very high level at the inner nuclear membrane and ultimately gives rise to brightly staining nuclear inclusions (Figs. 2 and 3) containing nuclear lamins (Fig. 2). In this case, however, the inclusions are, if anything, larger and frequently clustered at the nuclear periphery, often in direct contact with the inner face of the nuclear envelope (Figs. 2, 3, and 10), a finding which has been confirmed by confocal microscopy (not shown). These inclusions appear quite distinct, in both size and number, from invaginations or folds in the nuclear envelope that are frequently observed in both transfected and nontransfected BHK cells. EM observations reveal that the occurrence of the nuclear inclusions in cells overexpressing either HA-Nup153 or HA-Nup153ΔZ coincides with the appearance of extensive bundles and arrays of intranuclear membrane tubules and cisternae (Figs. 6 and 7). These ultrastructural perturbations are altogether unlike those associated with mutations in certain yeast nucleoporin genes (Wente and Blobel, 1993; Bogerd et al., 1994; Doye et al., 1994; Wente and Blobel, 1994; Pemberton et al., 1995; ). The concomitant appearance and similar spatial distributions suggest, although do not prove, that the inclusions observed in the light microscope and the intranuclear membrane arrays represent different views of the same structures.

Both the HA-Nup153 NH<sub>2</sub>-terminal domain (HA-N) and the HA-Nup153 construct lacking the NP-domain (HA-Nup153ΔNP) become highly concentrated at the nuclear periphery, but in neither case have we clearly observed the large micron-scale lamin-positive nuclear inclusions or intranuclear membrane arrays characteristic of HA-Nup153 and HA-Nup153ΔZ expression (Figs. 2, 3, and 11). The only immediately obvious structural phenotype associated with these two constructs is that as expression level rises, nuclei may become increasingly misshapen and may feature numerous invaginations of the nuclear envelope (Fig. 3, HA-N; Fig. 11). Similar results were obtained with the chimeric constructs, HA-NZ-CMPK and HA-N-CMPK, which also lack the NP-domain.

To determine whether the phenotypes associated with Nup153 overexpression include changes in nucleocytoplasmic transport kinetics, we have examined both import and export pathways before and after transfection with the various Nup153 constructs described above. To follow import, we constructed a BHK cell line, BHKgrβ, which constitutively expresses a glucocorticoid receptor-β-galactosidase fusion protein (Picard and Yamamoto, 1987). This fusion protein resides exclusively in the cytoplasm until addition of dexamethasone (10 μg/ml), whereupon it moves rapidly (within 30 min) and quantitatively into the nucleus. It therefore provides us with an inducible nuclear





*Figure 5.* BHK cell expressing the HA-N-CMPK chimera, double labeled with QE5 (anti-nucleoporin) and anti-HA. For these images, the microscope was focused on the nuclear surface. The QE5 epitope, which is contained within the Nup153 NP domain, is absent from HA-N-CMPK. While the relative intensities of individual spots vary between the two rhodamine and fluorescein images, their spatial overlap is precise. The spots probably represent single NPCs or clusters of NPCs. The 8-bit digital images shown here were given false colors and then superimposed employing Adobe Photoshop 3.0.

import reporter (Fig. 8, *A* and *B*). To inspect at least one aspect of nuclear export, we examined cellular poly (A)<sup>+</sup> RNA localization by in situ hybridization employing a biotinylated oligo-dT 45 mer. Changes in RNA trafficking might therefore be manifested as variations in global distribution of poly (A)<sup>+</sup> RNA. As shown in Fig. 8 *C*, poly (A)<sup>+</sup> RNA is found largely in the cytoplasm of nontransfected BHK cells. Labeling within the nucleus is restricted to a punctate reticulum excluded from nucleoli. This labeling pattern, which may be abolished by incubation with RNase A before hybridization or RNase H following hybridization (not shown), is identical to that reported by Spector and colleagues for the poly (A)<sup>+</sup> RNA distribution in HeLa cells (Huang et al., 1994).

Hybridization analysis of BHK cells 24 h after transfection with HA-Nup153 revealed a dramatic change in poly (A)<sup>+</sup> RNA distribution over mock transfected cells. In particular, a large increase in nuclear labeling with a concomitant decrease in cytoplasmic labeling was evident (Fig. 9). This change in poly (A)<sup>+</sup> RNA distribution was not observed in cells expressing high levels of two other NPC proteins, Nup84 or CAN/Nup214 (Fig. 11), or of the glucocorticoid receptor- $\beta$ -galactosidase construct (not shown). It is not, therefore, a trivial consequence of overexpression per se, but rather it is specific to Nup153. These observations point to a Nup153-dependent inhibition of poly (A)<sup>+</sup> RNA export. Analysis of nuclear protein import in BHKgr $\beta$  cells overexpressing HA-Nup153 revealed no discernible effect (Fig. 9), a result which indicates that the altered distribution of poly (A)<sup>+</sup> RNA cannot be due simply to loss or occlusion of NPCs. A second Nup153 construct, HA-Nup153 $\Delta$ Z, was found to yield identical results; poly (A)<sup>+</sup> RNA accumulated in the nucleus with no measurable effect on nuclear protein import (Fig. 10).

Like HA-Nup153, HA-Nup153 $\Delta$ Z causes the formation of nuclear inclusions at high expression levels, and an obvious inference is that these structures may be linked to the apparent nuclear export defect. To begin to address this issue, we examined the phenotype of cells expressing HA-Nup153 $\Delta$ NP, a construct which, as we have already described, does not appear to induce inclusion formation. This truncated form of Nup153, in contrast to HA-Nup153 and HANup153 $\Delta$ Z, exhibits a marginal effect, if any, on poly (A)<sup>+</sup> RNA distribution (Fig. 11), even at the highest expression levels. This is also true of several additional

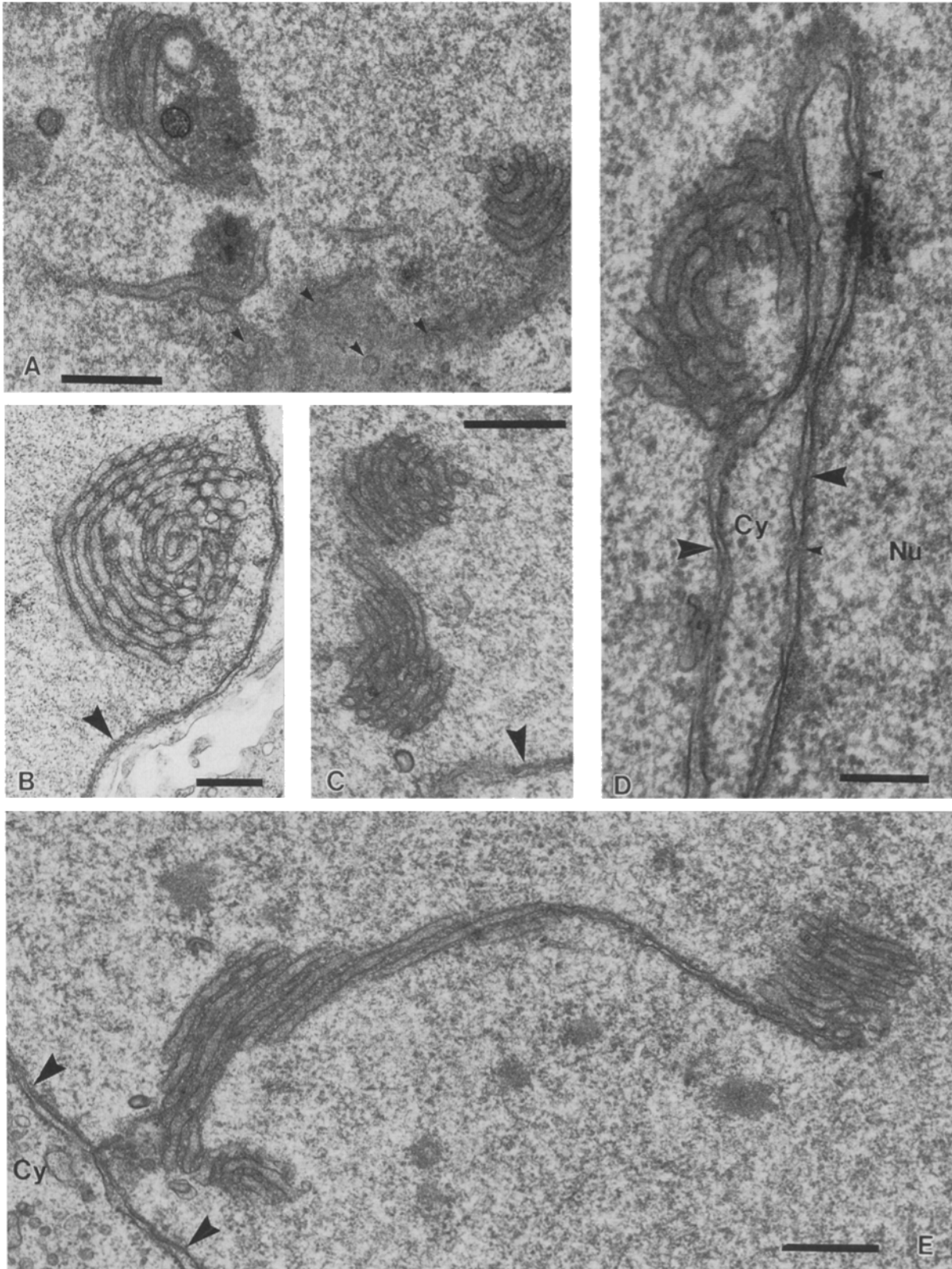
HA-Nup153 deletion constructs and fusion proteins (summarized in Table I) that lack the NP-domain. Taken together, these results indicate that the NP-domain of Nup153 is essential for the observed apparent block in poly (A)<sup>+</sup> RNA export from the nucleus.

The question that immediately arises is whether the NP-domain alone is sufficient to induce this block. To answer this, we examined cells overexpressing HA-NP. As described above, this construct, which becomes modified with *N*-acetyl glucosamine, remains uniformly distributed throughout the cytoplasm and does not accumulate in the nucleus or in the nuclear envelope. Furthermore, it does not induce the formation of the nuclear inclusions and intranuclear membrane arrays associated with HA-Nup153 and HA-Nup153 $\Delta$ Z overexpression. As revealed in Fig. 12, BHK cells expressing HA-NP exhibit a clear accumulation of poly (A)<sup>+</sup> RNA in the nucleus, with a corresponding decline in the cytoplasm. The overall effect is, if anything, even more dramatic than that observed with HA-Nup153 and HA-Nup153 $\Delta$ Z. As with all of the other Nup153 constructs, there is no effect on nuclear protein import. Similar results were obtained with a second cytoplasmic construct, HA-Nup153 $\Delta$ N (Table I). From these results, it is possible to conclude that changes in RNA distribution are unrelated to the nuclear envelope structural alterations associated with overexpression of HA-Nup153 and HA-Nup153 $\Delta$ Z. Furthermore, it is evident that it is the Nup153 NP-domain that is necessary and sufficient to produce these alterations in RNA trafficking.

## Discussion

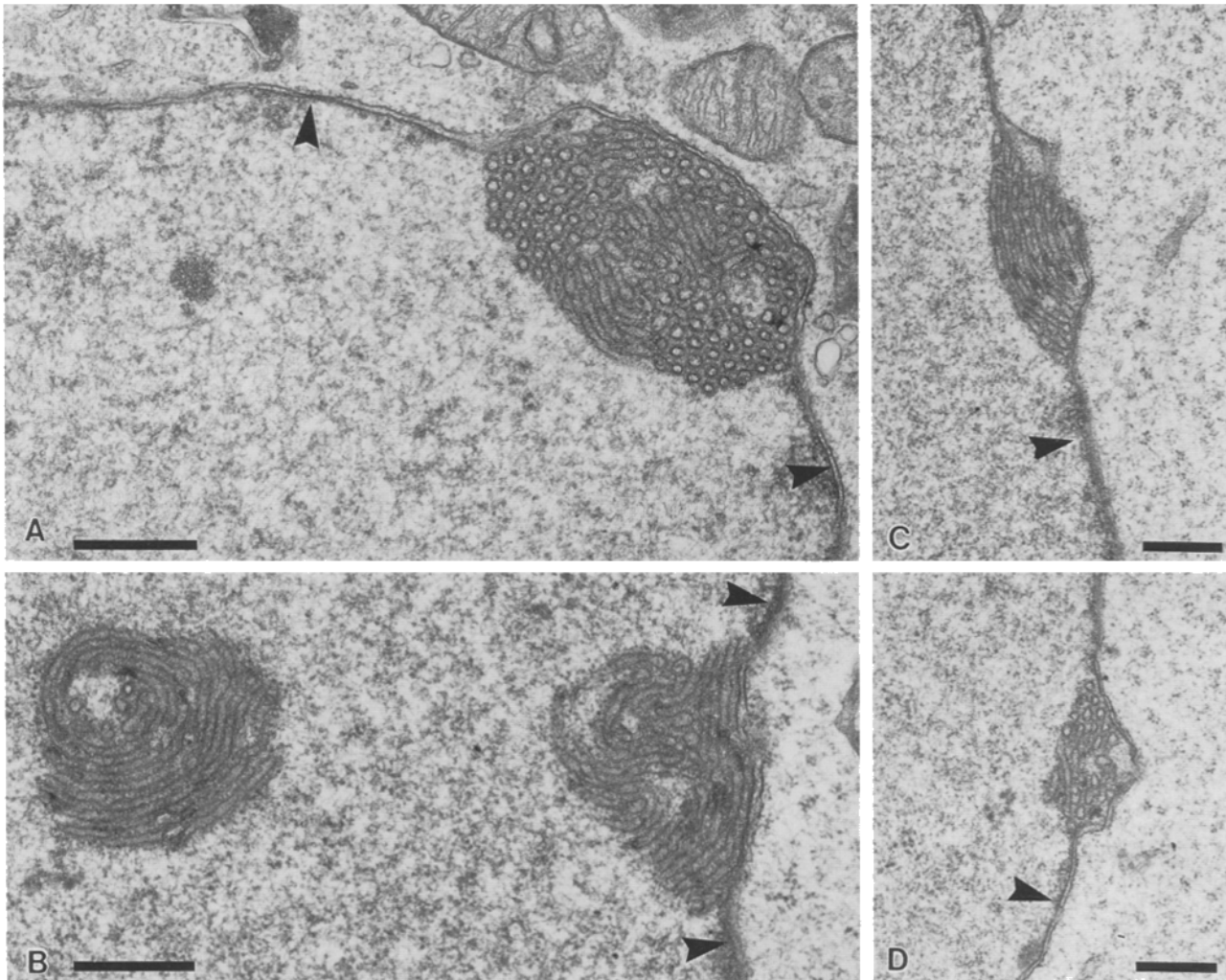
While considerable progress has recently been made in the characterization of molecules involved in the process of nuclear protein import, little detailed information is available concerning nuclear export mechanisms (Görlich and Mattaj, 1996). In contrast to the ubiquitous basic domain nuclear localization sequences found on numerous nuclear proteins, no comparable general export signal has so far been identified. Instead, it has become increasingly clear that different RNA classes, as well as proteins returning to the cytoplasm, possess distinct export signals that in turn may access kinetically separable export pathways (Jarmolowski et al., 1994; Pokrywka and Goldfarb, 1995).

A number of proteins of diverse functions have been identified, in both higher and lower cells, whose activities



**Figure 6.** Thin section electron micrographs of several membrane arrays from the nuclei of BHK cells expressing high levels of HA-Nup153. The small arrowheads in *A* indicate en face views of NPCs where a segment of the nuclear envelope has been cut tangentially, while in *D* they indicate NPCs in transverse section. The large arrowheads indicate the inner face of the nuclear envelope. Note that few, if any authentic NPCs are evident in these intranuclear membranes. *Cy*, cytoplasm; *Nu*, nucleus. (*A–C* and *E*) Bars, 0.5  $\mu\text{m}$ . *D* Bar, 0.2  $\mu\text{m}$ .



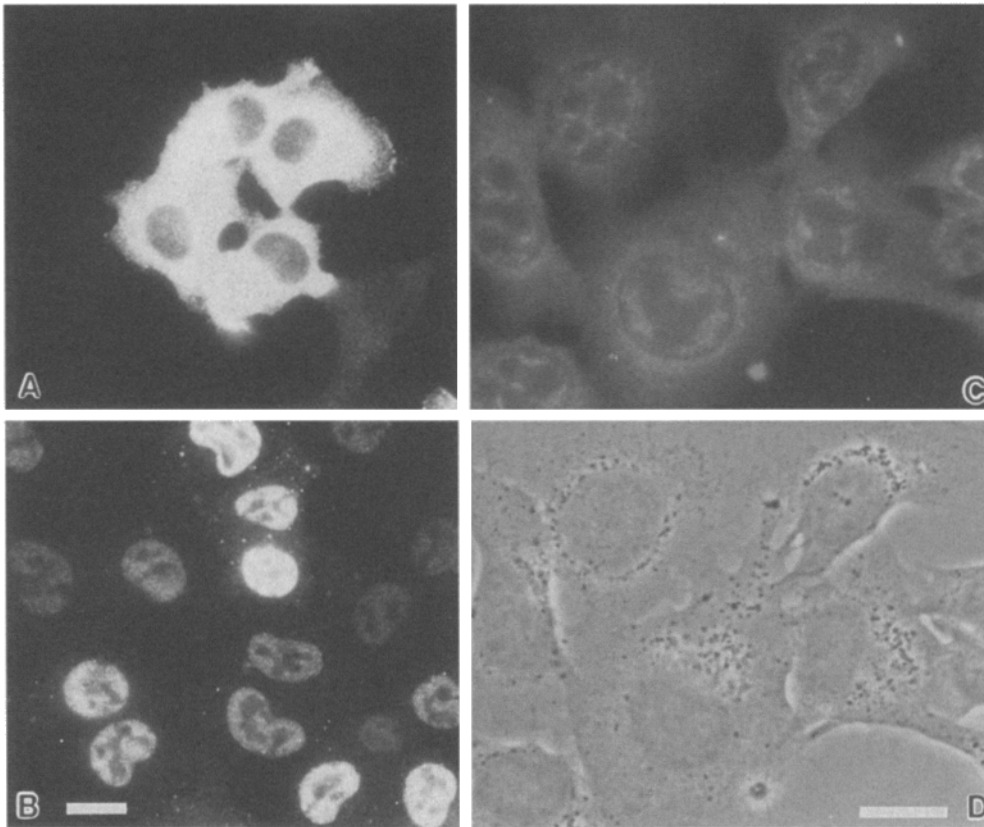


**Figure 7.** Thin section electron micrographs of membrane arrays formed within BHK cell nuclei in response to HA-Nup153ΔZ overexpression. If anything, these are more elaborate than the similar structures induced by full-length Nup153 (Fig. 6). Arrowheads indicate the nuclear face of the nuclear envelope. Bars, 0.5  $\mu\text{m}$ .

are required for the normal transit to the cytoplasm of various molecular species (Izaurrealde and Mattaj, 1995; Görlich and Mattaj, 1996). These include cap binding proteins (CBP80 and CBP20, in the case of U snRNA and mRNA; Izaurrealde et al., 1992; Izaurrealde et al., 1994), hnRNP A1 (mRNA; Piñol-Roma and Dreyfuss, 1991, 1992), Rev-interacting protein (RIP, in the case of HIV Rev protein; Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1995), as well as several yeast nucleoporins, including both GLFG and XFXFG family members (Wente et al., 1992; Wimmer et al., 1992; Bogerd et al., 1994; Doye et al., 1994; Fabre et al., 1994; Wente and Blobel, 1994; Aitchison et al., 1995; Grandi et al., 1995; Heath et al., 1995; Hurwitz and Blobel, 1995; Siniossoglou et al., 1996). In addition, constituents of the Ran GTPase cycle have also been implicated in RNA export in both yeast and in higher eukaryotes (Amberg et al., 1993; Kadowaki et al., 1993; Cheng et al., 1995). For instance, tsBN2 cells, a BHK derivative containing temperature-sensitive RCC1 (a Ran guanine nucleotide exchange protein), accumulate both UsnRNAs and poly (A)<sup>+</sup> RNA within the nucleus when grown at their non-

permissive temperature (Amberg et al., 1993; Kadowaki et al., 1993). Indeed, the reported distribution of poly (A)<sup>+</sup> RNA in these cells is indistinguishable from that induced by the Nup153 overexpression described in the present study (Amberg et al., 1993; Kadowaki et al., 1993).

To date, only one nucleoporin from higher cells has been implicated in RNA export. p62, which is a component of the central channel complex (Guan et al., 1995) and which is essential for nuclear protein import (Finlay et al., 1991), was found in cross-linking experiments to contact mRNA en route to the cytoplasm (Dargemont et al., 1995). These observations extend earlier antibody microinjection experiments that suggested a function for XFXFG nucleoporins in tRNA export (Featherstone et al., 1988). In the present study, we have provided evidence that Nup153, which like p62 is a member of the XFXFG family, may also have a role in RNA export. This is based upon the observation that a number of Nup153 constructs cause the dramatic accumulation of nuclear poly (A)<sup>+</sup> RNA when expressed in BHK cells, an effect which was not seen when several other proteins (including nucleoporins) were



**Figure 8.** Immunofluorescence detection of the glucocorticoid receptor- $\beta$ -galactosidase fusion protein in BHKgr $\beta$  cells before *A* and 30 min after *B* the addition of 10  $\mu$ g/ml dexamethasone. *C* shows the distribution of poly (A)<sup>+</sup> RNA in nontransfected BHK cells revealed by in situ hybridization with a biotinylated oligo-dT 45 mer. The same field viewed under phase contrast optics is shown in *D*. Bar, 10  $\mu$ m.

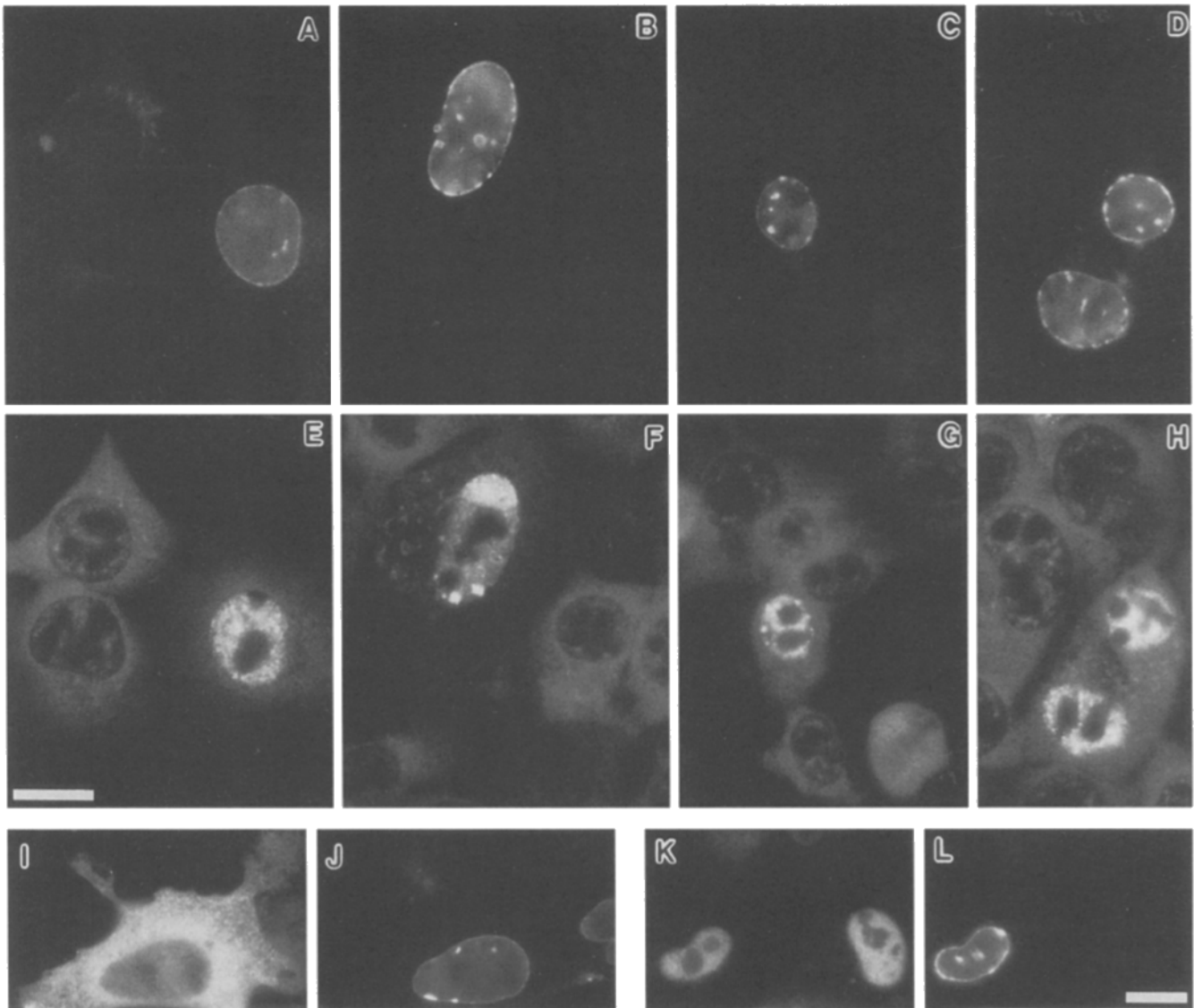
expressed at similar levels. At the same time, we could find no indication that the Nup153 constructs had an influence on nuclear protein import. This led us to conclude that whatever the effects of Nup153 overexpression, they did not involve either a loss or occlusion of NPCs leading to a general decline in nucleocytoplasmic transport.

Analysis of a series of Nup153 deletion constructs and chimeras indicate that it is the carboxy-terminal NP-domain, containing most of the XFXFG repeats, which is responsible for the observed nuclear poly (A)<sup>+</sup> RNA accumulation. Since certain of these NP-domain constructs do not associate with the NPC, indeed they are actually cytoplasmic, an interaction with some essential soluble factor, rather than a direct effect on NPCs, seems likely. This cannot, however, be a property of all XFXFG domain proteins since CAN/Nup214 overexpression has no discernible effect on poly (A)<sup>+</sup> RNA distribution. Clearly not all XFXFG domains are equivalent, a conclusion also reached with respect to GLFG domain proteins (Iovine et al., 1995).

It could be argued that the effects of the Nup153 NP-domain on poly (A)<sup>+</sup> RNA distribution could be a result of either impaired processing leading to a secondary decline in export or enhanced polyadenylation. The latter might in turn be a result of either increased poly (A) polymerase activity or reduced nuclear poly (A) trimming. While none of these mechanisms can be ruled out at present, we believe a more likely explanation is the titration, by the NP-domain, of an essential export factor. This proposition is lent credence by the fact that both nuclear and cytoplasmic NP-domain constructs have similar phenotypes with respect to poly (A)<sup>+</sup> RNA distribution, im-

plying that if they are associating with a soluble factor, it must be distributed between both compartments. This is precisely what would be expected of an export factor, at least by analogy with known import factors (Melchior and Gerace, 1995; Sweet and Gerace, 1995) such as Ran and the nuclear localization signal (NLS) receptor (importin- $\alpha$ , karyopherin- $\alpha$ ), and could potentially involve a protein such as hnRNP A1, which is believed to have a role in mRNA export and does indeed shuttle between the nucleus and cytoplasm (Piñol-Roma and Dreyfuss, 1991, 1992). Taking these conjectures further, the NP-domain of Nup153 may represent a docking site for certain RNA classes during translocation across the NPC to the cytoplasm, much as has been suggested as a role for the repeat domain proteins in nuclear protein import (Rexach and Blobel, 1995). Consistent with the existence of multiple distinct export pathways, we could find no evidence for an effect of Nup153 overexpression on protein export (Bastos, R., and B. Burke, unpublished results). This conclusion is based upon observations of the transcription factor NF-AT, which cycles between the nucleus and the cytoplasm as a function of intracellular calcium levels (Shibasaki et al., 1996). It is also in accord with the finding that the export of HIV Rev protein from the nucleus involves a pathway that is distinct from that utilized by mRNAs (Fischer et al., 1995).

This putative role for the NP-domain in poly (A)<sup>+</sup> RNA export contrasts with the targeting function which we have documented for the N-domain. At least when expressed against a background of wild-type protein, only the NH<sub>2</sub>-terminal region of Nup153 is required for delivery to the inner surface of the nuclear envelope. This finding indi-

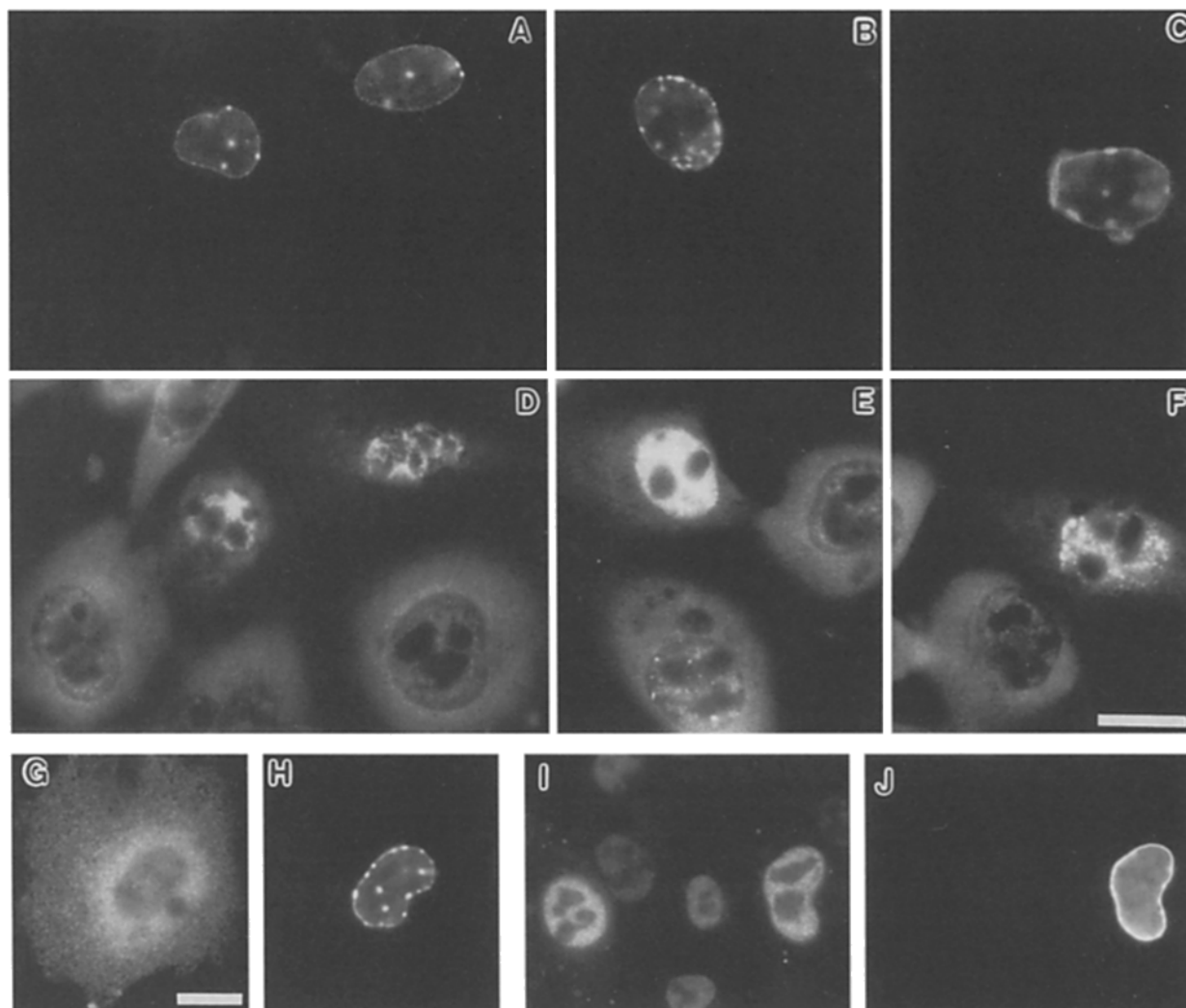


**Figure 9.** Poly (A)<sup>+</sup> RNA distribution in BHK cells expressing HA-Nup153. (A–D) A series of transfected cells revealed by indirect immunofluorescence microscopy employing the 12CA5 monoclonal antibody. (E–H) The corresponding fields following in situ hybridization with a biotinylated oligo-dT 45mer. The accumulation of poly (A)<sup>+</sup> RNA within the nuclei of cells expressing HA-Nup153 is quite clear. The lack of effect of HA-Nup153 overexpression on nuclear protein import in BHKgrβ cells is documented in I–L. Distribution of the glucocorticoid receptor–β-galactosidase fusion protein (I and K) and HA-Nup153 (J and L) revealed by double label indirect immunofluorescence using rabbit anti-β-galactosidase and 12CA5 either before (I and J) or 30 min after (K and L) the addition of 10 μg/ml dexamethasone. Bars, 10 μm.

cates that this segment of Nup153 must interact with other NPC or nuclear envelope components. The fact that the Nup153 N-domain does not contain any recognizable NLS implies that such interaction might initially occur in the cytoplasm, shortly after synthesis. In this way, the N-domain constructs may gain access to the interphase nucleus on the back of a “carrier” protein containing a conventional NLS. Since we already know that Nup153 can be eluted from the NPC in the form of a ~1 MD homooligomer (Panté et al., 1994), at least some of the data presented here could be explained in terms of interaction between the N-domain constructs and the endogenous full-length Nup153. In short, the N-domain might represent a site of self-association. To begin to address this issue, we tried to detect association

between HA-N and endogenous Nup153. However, in a series of immunoprecipitation experiments performed under conditions which preserve full-length Nup153 homooligomers, we could find no evidence for such an interaction (Burke, B., unpublished observations).

Even had we been successful in detecting association between HA-N and endogenous Nup153, this would only bring to the forefront the issue of how newly synthesized Nup153 itself enters the nucleus before assembly into an NPC. This question arises because the only conventional basic-domain NLS-like sequence contained within the Nup153 molecule is located at the COOH terminus (...RKIKTAVRRRK·COOH). However, we can find no evidence that this sequence actually has a targeting func-

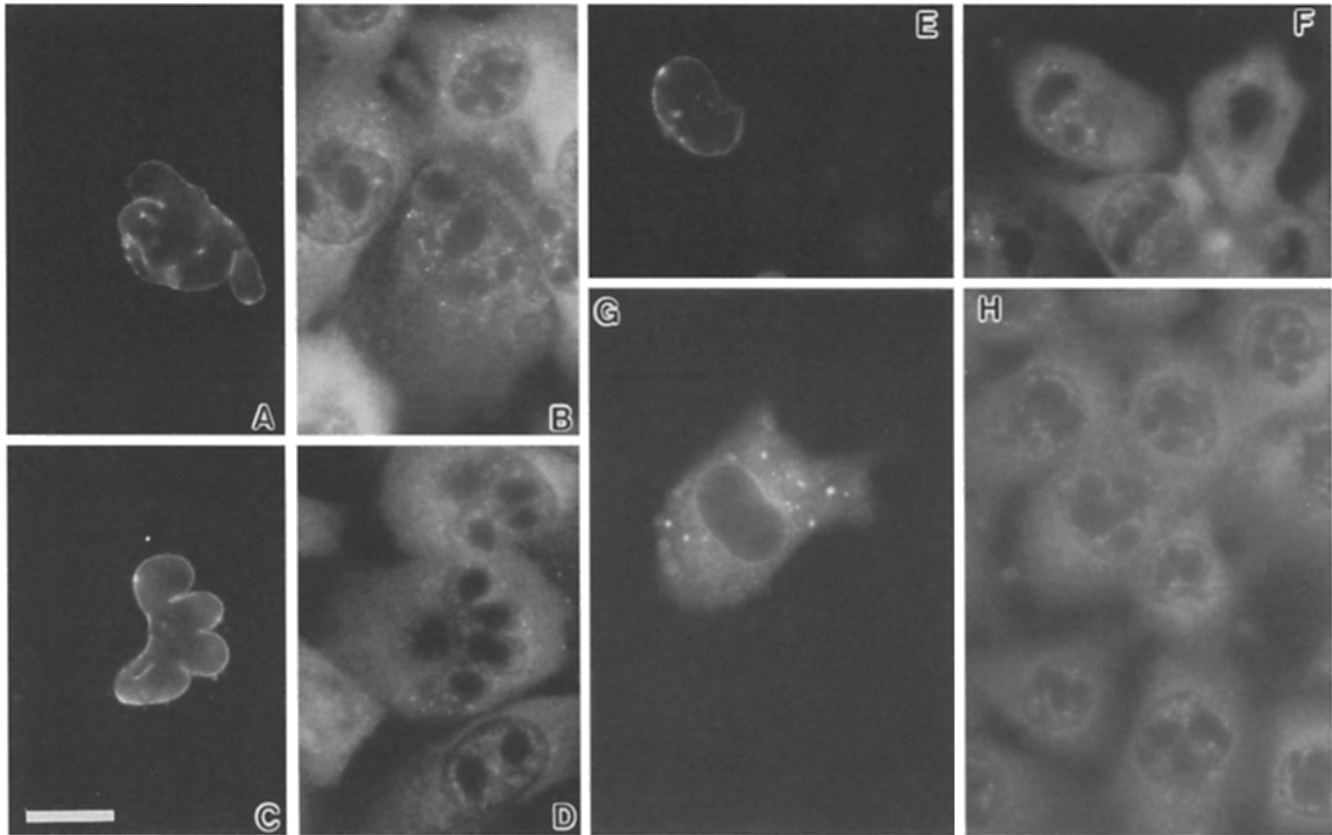


**Figure 10.** Poly (A)<sup>+</sup> RNA distribution in BHK cells expressing HA-Nup153ΔZ. (A–C) A series of transfected cells revealed by indirect immunofluorescence microscopy employing the 12CA5 monoclonal antibody. (D–F) The corresponding fields following in situ hybridization with a biotinylated oligo-dT 45 mer. The accumulation of poly (A)<sup>+</sup> RNA within the nuclei of cells expressing HA-Nup153ΔZ is quite clear. The lack of effect of HA-Nup153ΔZ overexpression on nuclear protein import in BHKgrβ cells is documented in G–J. Distribution of the glucocorticoid receptor-β-galactosidase fusion protein (G and I) and HA-Nup153 (H and J) revealed by double label indirect immunofluorescence using rabbit anti-β-galactosidase and 12CA5 either before (G and H) or 30 min after (I and J) the addition of 10 μg/ml dexamethasone. Bars, 10 μm.

tion. Indeed, the cytoplasmic localization of NH<sub>2</sub>-terminal deletions (which are biologically active insofar as they interfere with RNA trafficking) would speak against such a role. We are therefore faced with a situation in which Nup153 itself might obligatorily associate with an as yet unidentified NLS-containing protein in order to gain access to the nucleus. The only alternatives to this type of carrier model for Nup153 nuclear import are that its assembly into NPCs occurs only at the end of mitosis before nuclear reformation or that Nup153 contains an unconventional NLS. The former is certainly not the case since this model would predict, erroneously, that HA-Nup153 should accumulate in the cytoplasm during interphase. However, a precedent for the latter has been recently set by hnRNP A1, which does indeed appear to contain an unusual NLS within a 38-amino acid domain (Michael et al., 1995). An additional entry mechanism exclusive of NLS-

mediated transport and involving a translocation event coupled to de novo NPC formation seems unlikely since Nup153 continues to accumulate within the nucleus even when expressed at a level one to two orders of magnitude higher than that of endogenous NPC proteins. In this way, the rate of accumulation of Nup153 would appear to far outstrip the rate of new NPC assembly. It is worth pointing out that a second nucleoporin, Nup98, which is located on the nuclear face of the NPC, also seems to lack a conventional NLS (Radu et al., 1995).

The N-domain cannot be the only region of Nup153 in contact with other NPC or nuclear envelope molecules. While it is true that the NP-domain, containing most of the XFXFG repeats, is dispensable in terms of delivery to the inner nuclear membrane (a similar finding was reported for the yeast nucleoporin NSP1p; Nehrbass et al., 1990), appearance of the intranuclear membrane arrays seems to



**Figure 11.** Poly (A)<sup>+</sup> RNA distribution in BHK cells expressing HA-Nup153ΔNP (A–F) and HA-CAN/Nup214 (G and H). (A, C, E, and G) A series of transfected cells revealed by indirect immunofluorescence microscopy employing the 12CA5 monoclonal antibody. (B, D, F, and H) The corresponding fields following in situ hybridization with a biotinylated oligo-dT 45 mer. The distribution of poly (A)<sup>+</sup> RNA within cells is largely unaffected by overexpression of either HA-Nup153ΔNP or HA-CAN/Nup214. In A and C, cells expressing extremely high levels of HA-Nup153ΔNP are shown, as evidenced by the abnormal shapes of the nuclei. The high intensity of the labeling was compensated for during the photographic process. Bars, 10 μm.

be contingent upon the presence of this segment of the molecule. This would suggest that the NP-domain is not free but instead must interact, directly or indirectly, with other envelope components. Were this not the case, it would be difficult to imagine how its presence might otherwise be required to induce these structural rearrangements. It should also be emphasized that high resolution localization of Nup153 has been carried out exclusively with antibodies against NP-domain epitopes (Panté et al., 1994). Consequently, we can be fairly certain that this region of the molecule contributes to the structure of the distal ring of the nuclear basket. The distal ring in turn has

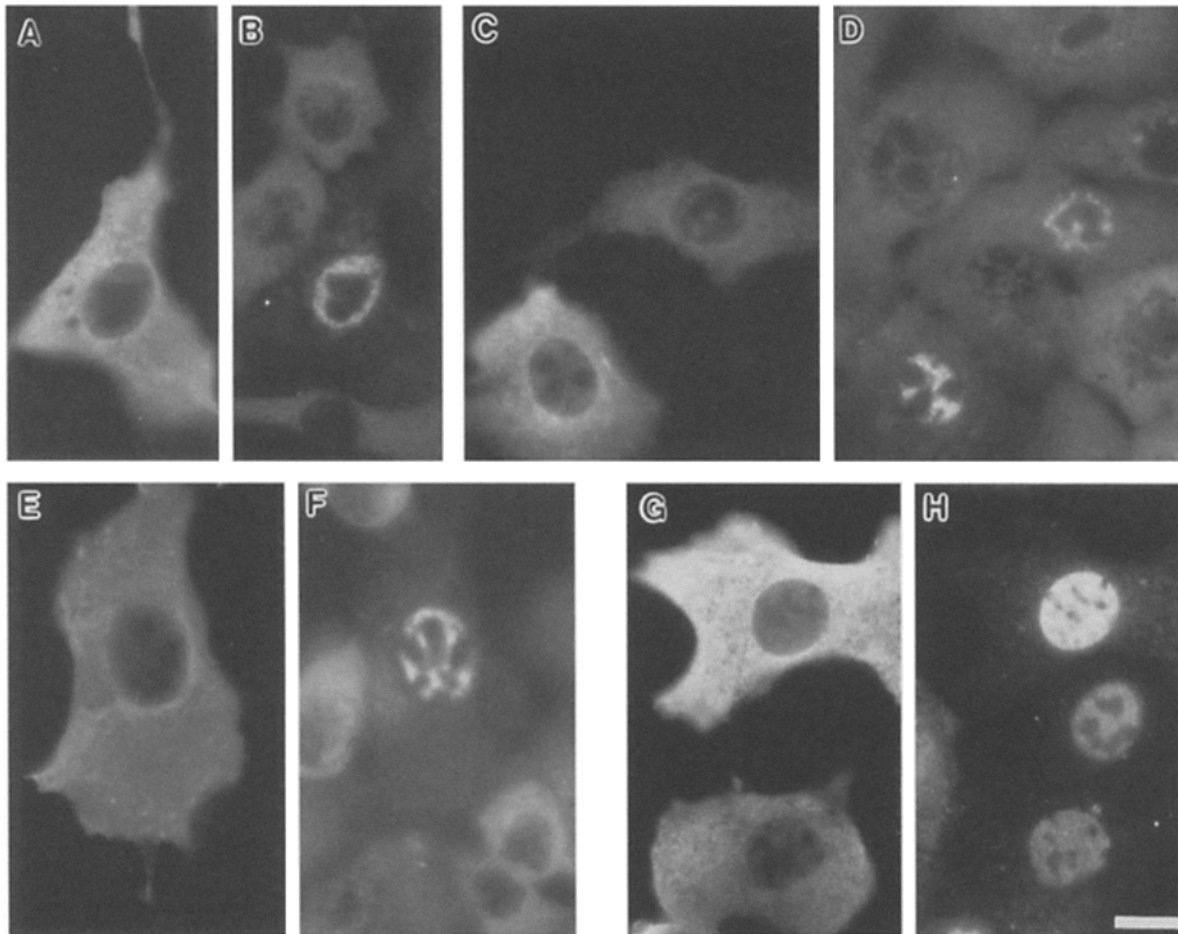
been shown to be the site of attachment of the nuclear envelope lattice, a filamentous meshwork distinct from the nuclear lamina that interconnects NPCs and that was originally identified in amphibian oocytes (Goldberg and Allen, 1992). This obviously begs the question of whether the NP-domain might interact directly with the nuclear envelope lattice. Despite the fact that it is the N-domain only that can direct delivery to the nuclear envelope, we have no direct knowledge of how this segment of Nup153 might be disposed within the NPC.

A very surprising feature of Nup153 is its ability to accumulate in the nuclear envelope to extremely high levels. This is in marked contrast to another nucleoporin, CAN/Nup214, which rapidly saturates all available binding sites at the nuclear periphery (Fornerod et al., 1995). Since NPCs are likely to have a precisely defined composition, they should be capable of accommodating only a fixed number of Nup153 molecules. How then can the behavior of Nup153 be rationalized? There are at least two possibilities, neither of which can be eliminated at present. The first is that at elevated expression levels, Nup153 might form higher order oligomers “seeded” off of NPCs. In favor of this model is the finding that Nup153 can be eluted from NPCs as a large (~1 MD) homooligomer, so it can certainly self-associate. In addition, it would be consistent

**Table I. Effects of Nup153 Constructs on Poly (A)<sup>+</sup> RNA Distribution**

Nup153 construct	Location	Increased nuclear poly (A) <sup>+</sup> RNA
HA-Nup153	NE	+
HA-Nup153ΔNP	NE	–
HA-N	NE	–
HA-Nup153ΔZ	NE	+
HA-Nup153ΔN	Cyt	+
HA-NP	Cyt	+
HA-NZ-CMPK	NE	–
HA-N-CMPK	NE	–





**Figure 12.** Poly (A)<sup>+</sup> RNA distribution in BHK cells expressing HA-NP. (A, C, and E) A series of transfected cells revealed by indirect immunofluorescence microscopy employing the 12CA5 monoclonal antibody. (B, D, and F) The corresponding fields following in situ hybridization with a biotinylated oligo-dT 45 mer. The accumulation of poly (A)<sup>+</sup> RNA within the nuclei of cells expressing HA-NP is quite clear. The lack of effect of HA-NP overexpression on nuclear protein import in BHKgr $\beta$  cells is documented in G and H. Distribution of the glucocorticoid receptor- $\beta$ -galactosidase fusion protein (H) and HA-Nup153 (G) revealed by double label indirect immunofluorescence using rabbit anti- $\beta$ -galactosidase and 12CA5 30 min after the addition of 10  $\mu$ g/ml dexamethasone. Bars, 10  $\mu$ m.

with observations that at least under some circumstances, Nup153 can be detected in extended filamentous structures (Cordes et al., 1993). The second possibility is that the nuclear envelope contains large numbers of Nup153-binding sites in addition to the limited number available within each NPC.

In summary, we have defined an NH<sub>2</sub>-terminal region of the Nup153 molecule required for association with the NPC. Logically, this segment of the molecule must interact with at least one other NPC subunit, although the identity of this remains unknown. Similarly, whether the N-domain of Nup153 might also be involved in self-association is also still an open question. The NP-domain, containing most of the XFXFG repeats and at least some of the O-linked N-acetyl glucosamine, is apparently dispensable in terms of delivery of the molecule to the inner nuclear membrane. However, overexpression of this region of the molecule leads to large scale accumulation of intranuclear poly (A)<sup>+</sup> RNA, suggesting that Nup153 may play a role in RNA export. The location of the NP-domain within the distal ring of the nuclear basket raises the possibility that

this structure may represent a docking site for molecules exiting the nucleus. The future application of biochemical and genetic approaches combined with ultrastructural analyses should provide the means to test this proposition.

We would like to thank Drs. Morrie Birnbaum, Frank McKeon, Roydon Price, and Manfred Lohka for helpful discussions during the course of this work. Drs. Keith Yamamoto and Didier Picard kindly provided the glucocorticoid receptor- $\beta$ -galactosidase construct, while the HA-CAN/Nup214 expression plasmid was a generous gift from Dr. Gerard Grosveld. We are also particularly grateful to Dr. Michael Schoel for considerable assistance and advice on the confocal and digital microscopy.

This work was supported by grants to B. Burke from the National Institutes of Health, the Medical Research Council of Canada, and the Alberta Heritage Foundation for Medical Research.

Received for publication 29 January 1996 and in revised form 17 June 1996.

#### References

- Aitchison, J.D., G. Blobel, and M.P. Rout. 1995. Nup120p: a yeast nucleoporin required for NPC distribution and mRNA transport. *J. Cell Biol.* 131:1659-1675.

- Akey, C.W. 1995. Structural plasticity of the nuclear pore complex. *J. Mol. Biol.* 248:273-293.
- Akey, C.W., and M. Radermacher. 1993. Architecture of the *Xenopus* nuclear pore complex revealed by three dimensional cryo-electron microscopy. *J. Cell Biol.* 122:1-20.
- Amberg, D.C., M. Fleischman, I. Stajlar, C.N. Cole, and M. Aebi. 1993. Nuclear PRP20 protein is required for mRNA export. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:233-241.
- Ash, J.F., D. Louvard, and S.J. Singer. 1977. Antibody-induced linkages of plasma membrane proteins to intracellular actomyosin-containing filaments in cultured fibroblasts. *Proc. Natl. Acad. Sci. USA.* 74:5584-5588.
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1987. *Current Protocols in Molecular Biology*. John Wiley & Sons, New York. pp. 3.01-3.19.8.
- Boger, A.M., J.A. Hoffman, D.C. Amberg, G.R. Fink, and L.I. Davis. 1994. nup1 mutants exhibit pleiotropic defects in nuclear pore complex function. *J. Cell Biol.* 127:319-332.
- Boger, H.P., R.A. Fridell, S. Madore, and B.R. Cullen. 1995. Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell.* 82:485-494.
- Burke, B. 1990. On the cell-free association of lamins A and C with metaphase chromosomes. *Exp. Cell Res.* 186:169-176.
- Burke, B., G. Griffiths, H. Reggio, D. Louvard, and G. Warren. 1982. A monoclonal antibody against a 135k Golgi membrane protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1621-1628.
- Cheng, Y., J.E. Dahlberg, and E. Lund. 1995. Diverse effects of the guanine nucleotide exchange factor RCC1 on RNA transport. *Science (Wash. DC).* 267:1807-1810.
- Cordes, V., S. Reidenbach, A. Köhler, N. Stuurman, R. van Driel, and W.W. Franke. 1993. Intracellular filaments containing a nuclear pore complex protein. *J. Cell Biol.* 123:1333-1344.
- Dargemont, C., M.S. Schmidt-Zachmann, and L.C. Kühn. 1995. Direct interaction of nucleoporin p62 with mRNA during its export from the nucleus. *J. Cell Sci.* 108:257-263.
- Doye, V., R. Wepf, and E.C. Hurt. 1994. A novel nuclear pore protein Nup133p with distinct roles in poly(A)<sup>+</sup> RNA transport and nuclear pore distribution. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:6062-6075.
- Fabre, E., W.C. Boelens, C. Wimmer, I.W. Mattaj, and E.C. Hurt. 1994. Nup145p is required for nuclear export of mRNA and binds homopolymeric RNA in vitro via a novel conserved motif. *Cell.* 78:275-289.
- Featherstone, C., M.K. Darby, and L. Gerace. 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. *J. Cell Biol.* 107:1289-1287.
- Feldherr, C., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* 99:2216-2222.
- Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I.A. Wilson, R.A. Lerner, and M. Wigler. 1988. Purification of a RAS-responsive adenyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell Biol.* 8:2159-2165.
- Finlay, D., E. Meier, P. Bradley, J. Horecka, and D.J. Forbes. 1991. A complex of nuclear pore proteins required for pore function. *J. Cell Biol.* 114:169-183.
- Fischer, U., J. Huber, W.C. Boelens, I.W. Mattaj, and R. Lührmann. 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell.* 82:475-483.
- Fornerod, M., J. Boer, S. van Baal, M. Jaegle, M. von Lindern, K.G. Murti, D. Davis, J. Bonten, A. Buijs, and G. Grosveld. 1995. Relocation of the carboxy-terminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene.* 10:1739-1748.
- Frangioni, J.V., P.H. Beahm, V. Shifrin, C.A. Jost, and B.G. Neel. 1992. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell.* 68:545-560.
- Fritz, C.C., M.L. Zapp, and M.R. Green. 1995. A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature (Lond.)* 376:530-533.
- Goldberg, M.W., and T.D. Allen. 1992. High resolution scanning electron microscopy of the nuclear envelope: demonstration of a new, regular, fibrous lattice attached to the baskets of the nucleoplasmic face of the nuclear pores. *J. Cell Biol.* 119:1429-1440.
- Görllich, D., and I.W. Mattaj. 1996. Nucleocytoplasmic transport. *Science (Wash. DC).* 271:1513-1518.
- Graham, F.L., and A.J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5. *Virology.* 52:456-467.
- Grandi, P., S. Emig, C. Weise, F. Hucho, T. Pohl, and E.C. Hurt. 1995. A novel nuclear pore protein Nup82p which specifically binds to a fraction of Nsp1p. *J. Cell Biol.* 130:1263-1273.
- Guan, T., S. Müller, G. Klier, N. Panté, J.M. Blevitt, M. Haner, B. Paschal, U. Aebi, and L. Gerace. 1995. Structural analysis of the p62 complex, an assembly of O-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex. *Mol. Biol. Cell.* 6:1591-1603.
- Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 511-552.
- Heath, C.V., C.S. Copeland, D.C. Amberg, V. Del Priore, M. Snyder, and C.N. Cole. 1995. Nuclear pore complex clustering and nuclear accumulation of poly(A)<sup>+</sup> RNA associated with mutation of the *Saccharomyces cerevisiae* RAT2/NUP120 gene. *J. Cell Biol.* 131:1677-1697.
- Hinshaw, J.E., B.O. Carragher, and R.A. Milligan. 1992. Architecture and design of the nuclear pore complex. *Cell.* 69:1133-1141.
- Holt, G.D., C.M. Snow, A. Senior, R.S. Haltiwanger, L. Gerace, and G.W. Hart. 1987. Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetyl glucosamine. *J. Cell Biol.* 104:1157-1164.
- Huang, S., T.J. Deerinck, M.H. Ellisman, and D.L. Spector. 1994. In vivo analysis of the stability and transport of nuclear poly(A)<sup>+</sup> RNA. *J. Cell Biol.* 126:877-899.
- Hurwitz, M.E., and G. Blobel. 1995. NUP82 is an essential nucleoporin required for poly(A)<sup>+</sup> RNA export. *J. Cell Biol.* 130:1275-1281.
- Iovine, M.K., J.L. Watkins, and S.R. Wente. 1995. The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap97p, an essential yeast nuclear import factor. *J. Cell Biol.* 131:1699-1713.
- Izaurre, E., and I.W. Mattaj. 1995. RNA export. *Cell.* 81:153-159.
- Izaurre, E., J. Stepinski, E. Darzynkiewicz, and I.W. Mattaj. 1992. A cap-binding protein that may mediate export of RNA polymerase II-transcribed RNAs. *J. Cell Biol.* 118:1287-1295.
- Izaurre, E., J. Lewis, C. McGuigan, M. Jankowska, E. Darzynkiewicz, and I.W. Mattaj. 1994. A nuclear cap binding protein complex involved in pre-mRNA splicing. *Cell.* 78:657-668.
- Jarmolowski, A., W.C. Boelens, E. Izaurre, and I.W. Mattaj. 1994. Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.* 124:627-635.
- Kadowaki, T., D. Goldfarb, L.M. Spitz, A.M. Tartakoff, and M. Ohno. 1993. Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the ras superfamily. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2929-2937.
- Kita, K., S. Omata, and T. Horigome. 1993. Purification and characterization of a nuclear pore glycoprotein complex containing p62. *J. Biochem. (Tokyo).* 113:377-382.
- Kraemer, D., R.W. Wozniak, G. Blobel, and A. Radu. 1994. The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc. Natl. Acad. Sci. USA.* 91:1519-1523.
- Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- McMorrow, I.M., R. Bastos, H. Horton, and B. Burke. 1994. Sequence analysis of a cDNA encoding a human nuclear pore complex protein, hnup153. *Biochim. Biophys. Acta.* 1217:219-223.
- Melchior, F., and L. Gerace. 1995. Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.* 7:310-318.
- Michael, W.M., M. Choi, and G. Dreyfuss. 1995. A nuclear export signal in hnRNP A1: a signal mediated, temperature-dependent nuclear protein export pathway. *Cell.* 83:415-422.
- Nehrbass, U., H. Kern, A. Mutvei, H. Horstmann, B. Marshallsay, and E.C. Hurt. 1990. NSP1: a yeast nuclear envelope protein localized at the nuclear pores exerts its essential function by its carboxy-terminal domain. *Cell.* 61:979-989.
- Paine, P.L. 1975. Nucleocytoplasmic movement of fluorescent tracers microinjected into living salivary gland cells. *J. Cell Biol.* 66:652-657.
- Paine, P.L., L.C. Moore, and S.B. Horowitz. 1975. Nuclear envelope permeability. *Nature (Lond.)* 254:109-114.
- Panté, N., and U. Aebi. 1994. Towards understanding the three-dimensional structure of the nuclear pore complex at the molecular level. *Curr. Opin. Struct. Biol.* 4:187-196.
- Panté, N., R. Bastos, I. McMorrow, B. Burke, and U. Aebi. 1994. Interactions and three-dimensional localization of a group of nuclear pore complex proteins. *J. Cell Biol.* 126:603-617.
- Pemberton, L.F., M.P. Rout, and G. Blobel. 1995. Disruption of the nucleoporin gene NUP133 results in clustering of nuclear pore complexes. *Proc. Natl. Acad. Sci. USA.* 92:1187-1191.
- Picard, R., and K.R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3333-3340.
- Piñol-Roma, S., and G. Dreyfuss. 1991. Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins. *Science (Wash. DC).* 253:312-314.
- Piñol-Roma, S., and G. Dreyfuss. 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature (Lond.)* 355:730-732.
- Pokrywka, N.J., and D.S. Goldfarb. 1995. Nuclear export pathways of tRNA and 40S ribosomes include both common and specific intermediates. *J. Biol. Chem.* 270:3619-3624.
- Radu, A., M.S. Moore, and G. Blobel. 1995. The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell.* 81:215-222.
- Reichelt, R., A. Holzenburg, E.L. Buhle, M. Jarnik, A. Engel, and U. Aebi. 1990. Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore components. *J. Cell Biol.* 110:883-894.
- Rexach, M., and G. Blobel. 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors and nucleoporins. *Cell.* 83:683-692.
- Richardson, W.D., A.D. Mills, S.M. Dilworth, R.A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through the nuclear pores. *Cell.* 52:655-664.
- Ris, H. 1991. The 3-D structure of the nuclear pore complex as seen by high

- voltage electron microscopy and high resolution low voltage scanning electron microscopy. *Electron Microscopy Society of America (EMSA) Bull.* 21: 54-56.
- Rout, M.P., and G. Blobel. 1993. Isolation of the yeast nuclear pore complex. *J. Cell Biol.* 123:771-783.
- Rout, M.P., and S.R. Wente. 1994. Pores for thought: nuclear pore complex proteins. *Trends Cell Biol.* 4:357-365.
- Shibasaki, F., R.E. Price, D. Milan, and F. McKeon. 1996. Role of calcineurin and kinases in NF-AT4 nuclear shuttling during calcium signalling. *Nature (Lond.)*. In press.
- Siniossoglou, S., C. Wimmer, M. Rieger, V. Doye, H. Tekotte, C. Weise, S. Emig, A. Segref, and E.C. Hurt. 1996. A novel complex of nucleoporins, which includes Sec13p and a Sec13p homolog, is essential for normal nuclear pores. *Cell.* 84:265-275.
- Stutz, F., M. Neville, and M. Rosbash. 1995. Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell.* 82:495-506.
- Sukegawa, J., and G. Blobel. 1993. A nuclear pore complex protein that contains zinc fingers and faces the nucleoplasm. *Cell.* 72:29-38.
- Sweet, D.J., and L. Gerace. 1995. Taking from the cytoplasm and giving to the pore: soluble transport factors in nuclear protein import. *Trends Cell Biol.* 5: 444-447.
- von Lindern, M., S. van Baal, J. Wiegant, A. Raap, A. Hagemijer, and G. Grosveld. 1992. *can*, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. *Mol. Cell. Biol.* 12:3346-3355.
- Wente, S.R., and G. Blobel. 1993. A temperature-sensitive NUP116 null mutant forms a nuclear envelope seal over the nuclear pore complex thereby blocking nucleocytoplasmic traffic. *J. Cell Biol.* 123:275-284.
- Wente, S.R., and G. Blobel. 1994. NUP145 encodes a novel yeast glycine-leucine-phenylalanine-glycine (GLFG) nucleoporin required for nuclear envelope structure. *J. Cell Biol.* 125:955-969.
- Wente, S.R., M.P. Rout, and G. Blobel. 1992. A new family of yeast nuclear pore complex proteins. *J. Cell Biol.* 119:705-723.
- Wilson, I.A., H.L. Niman, R.A. Houghten, A.R. Cherenon, M.L. Connolly, and R.A. Lerner. 1984. The structure of an antigenic determinant in a protein. *Cell.* 37:767-778.
- Wimmer, C., V. Doye, P. Grandi, U. Nehrass, and E.C. Hurt. 1992. A new subclass of nucleoporins that functionally interact with nuclear pore protein NSP1. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:5051-5061.
- Wu, J., M.J. Matunis, D. Kraemer, G. Blobel, and E. Coutavas. 1995. Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J. Biol. Chem.* 270:14209-14213.
- Yokoyama, N., N. Hayashi, T. Seki, N. Pante, T. Ohba, K. Nishii, K. Kuma, T. Hayashida, T. Miyata, U. Aebi and et al., 1995. A giant nucleopore protein that binds Ran/TC4. *Nature (Lond.)*. 376:184-188.