

University of Massachusetts Medical School

eScholarship@UMMS

---

Open Access Articles

Open Access Publications by UMMS Authors

---

1998-12-29

## Functional analysis of Tpr: identification of nuclear pore complex association and nuclear localization domains and a role in mRNA export

Peter Lawrence Bangs

*University of Massachusetts Medical School*

*Et al.*

Let us know how access to this document benefits you.

Follow this and additional works at: <https://escholarship.umassmed.edu/oapubs>



Part of the [Cell Biology Commons](#)

---

### Repository Citation

Bangs PL, Burke B, Powers CM, Craig RW, Purohit A, Doxsey SJ. (1998). Functional analysis of Tpr: identification of nuclear pore complex association and nuclear localization domains and a role in mRNA export. Open Access Articles. Retrieved from <https://escholarship.umassmed.edu/oapubs/937>

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in Open Access Articles by an authorized administrator of eScholarship@UMMS. For more information, please contact [Lisa.Palmer@umassmed.edu](mailto:Lisa.Palmer@umassmed.edu).

# Functional Analysis of Tpr: Identification of Nuclear Pore Complex Association and Nuclear Localization Domains and a Role in mRNA Export

Peter Bangs,\* Brian Burke,§ Christine Powers,‡ Roger Craig,‡ Aruna Purohit,\* and Stephen Doxsey\*

\*Program in Molecular Medicine and ‡Department of Molecular Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605; and §Department of Cell Biology and Anatomy, University of Calgary, Calgary, Alberta, Canada T2N 4N1

**Abstract.** Tpr is a 270-kD coiled-coil protein localized to intranuclear filaments of the nuclear pore complex (NPC). The mechanism by which Tpr contributes to the structure and function of the nuclear pore is currently unknown. To gain insight into Tpr function, we expressed the full-length protein and several subdomains in mammalian cell lines and examined their effects on nuclear pore function. Through this analysis, we identified an NH<sub>2</sub>-terminal domain that was sufficient for association with the nucleoplasmic aspect of the NPC. In addition, we unexpectedly found that the acidic COOH terminus was efficiently transported into the nuclear interior, an event that was apparently mediated by a putative nuclear localization sequence. Ectopic expression

of the full-length Tpr caused a dramatic accumulation of poly(A)<sup>+</sup> RNA within the nucleus. Similar results were observed with domains that localized to the NPC and the nuclear interior. In contrast, expression of these proteins did not appear to affect nuclear import. These data are consistent with a model in which Tpr is tethered to intranuclear filaments of the NPC by its coiled coil domain leaving the acidic COOH terminus free to interact with soluble transport factors and mediate export of macromolecules from the nucleus.

**Key words:** Tpr • nuclear pore • nuclear localization sequence • nuclear export • mRNA

**T**HE cell nucleus is demarcated by the nuclear envelope, a double-membraned organelle that forms the interface between the nucleoplasmic and cytoplasmic compartments (reviewed in Goldberg and Allen, 1995). The inner and outer membranes of the nuclear envelope are connected at discrete structures, the nuclear pore complexes (NPCs)<sup>1</sup>, which mediate the flow of traffic between the nucleus and cytoplasm (for review see Gorch and Mattaj, 1996). The primary feature of the NPC is a symmetrical framework surrounding a central gated channel embedded within the double membranes of the nuclear envelope (Hinshaw et al., 1992; Akey and Radermacher, 1993; reviewed in Panté and Aebi, 1994). An extensive array of filamentous structures protrudes from

each side of the nuclear pore (Ris, 1991). On the cytoplasmic face, 8 fibrils extend from the framework to a distance of ~100 nm. These filaments are thought to have binding sites that serve as docking areas for proteins being transported into the nucleus (Newmeyer and Forbes, 1988; Richardson et al., 1988; Melchior et al., 1995; Wu et al., 1995; for review see Panté and Aebi, 1995). The nucleoplasmic face of the NPC has 8 shorter (50–100 nm) filaments that project into the nucleus and are joined at their distal ends by a ring-like structure with a diameter of 30–50 nm (Jarnik and Aebi, 1991; Ris, 1991). These filaments and their connecting ring form a structure with a basket-like appearance that is presumed to act in a manner analogous to the cytoplasmic fibrils and bind to substrates in the process of being exported through the nuclear pore (Bastos et al., 1996). A further series of intranuclear filaments extending in a cylindrical array as far as 300 nm from the basket structure has been described in amphibian oocytes (Ris and Malecki, 1993; Cordes et al., 1993, 1997).

A remarkable number of NPC proteins (collectively referred to as nucleoporins) have been identified and characterized in recent years (reviewed in Bastos et al., 1995; Doye and Hurt, 1997). A variety of approaches has been

Address correspondence to Stephen J. Doxsey, University of Massachusetts Medical Center, Program in Molecular Medicine, 373 Plantation Street, Worcester, MA 01605. Tel.: (508) 856-1613. Fax: (508) 856-4289. E-mail: stephen.doxsey@ummed.edu

1. *Abbreviations used in this paper:* GFP, green fluorescent protein; HA, hemagglutinin; NE, nuclear envelope; NLS, nuclear localization signal; NPC, nuclear pore complex.

taken to define the relative positions of nucleoporins within the NPC, to determine their interactions with one another, and to identify specific functions for individual components of the NPC. Many of the vertebrate nucleoporins have been localized by immunogold microscopy to specific substructures of the NPC (reviewed in Panté and Aebi, 1996). In yeast, a number of genetic approaches have established interactions between many NPC proteins and have identified NPC and nuclear envelope abnormalities induced by disruptions in genes encoding particular nucleoporins (for example see Aitchison et al., 1995; Heath et al., 1995; Pemberton et al., 1995; Nehrbass et al., 1996; for review see Doye and Hurt, 1995). Experiments such as these have defined interactions between subcomplexes of the NPC and have established a model for NPC architecture in which a number of nucleoporins interact to form the central core region of the NPC and serve as a framework to position and support the nucleoporins directly involved in nucleocytoplasmic transport (Kenna et al., 1996; Nehrbass et al., 1996; Zabel et al., 1996). The expression of nucleoporin cDNAs in mammalian cells has identified functional domains for individual proteins and has provided evidence for specific roles in nuclear transport and NPC assembly (Fornerod et al., 1995; Bastos et al., 1996, 1997). For instance, overexpression of the nucleoplasmic nup153 leads to disruption of the mRNA export pathway but does not appear to affect the import of proteins bearing a classical nuclear localization signal (NLS; Bastos et al., 1996). This approach should be useful in defining functional domains and specific roles for many of the vertebrate nucleoporins, particularly those which have not yet been directly implicated in the nuclear transport process.

Many nucleoporins belong to a family with highly repetitive motifs conforming to the single-letter amino acid consensus XFXFG or GLFG (see for example Davis and Blobel, 1986; Snow et al., 1987; Starr et al., 1990). FG repeat-containing nucleoporins have been localized to the cytoplasmic fibrils, the central channel region and the nucleoplasmic basket domain of the NPC (Panté et al., 1994; Hu et al., 1996), and they interact with soluble transport factors *in vitro* (Iovine et al., 1995; Radu et al., 1995; Rexach and Blobel, 1995). While the functions of these proteins have not been determined *in vivo*, their localization throughout the NPC and their ability to directly interact with soluble transport factors has led to suggestions that transport occurs by sequential binding reactions between the FG repeat domains and complexes transiting the NPC (Radu et al., 1995). Consistent with this model is the identification of a family of soluble receptors that mediate the transport of specific classes of macromolecules through the NPC. These receptors share distant homology to the shuttling carrier importin  $\beta$ , which is responsible for the import of substrates containing a classical NLS (Gorlich et al., 1995; Moroianu et al., 1995), and like importin  $\beta$ , all of these receptors have been shown to bind to various subsets of FG repeat nucleoporins *in vitro* (Pollard et al., 1996; Fornerod et al., 1997; Kutay et al., 1997; Pemberton et al., 1997; Rosenblum et al., 1997; Siomi et al., 1997; Stade et al., 1997). Recently, the use of *Xenopus* extracts to probe for molecular interactions between nucleoporins and importin  $\beta$  has shown that at least some of these interactions also occur *in vivo* (Shah, et al., 1998). These studies

have also indicated that nucleoporins that do not contain FG-repeat domains may also be binding sites for importin  $\beta$  and perhaps other nuclear transport receptors as well.

In this report, we present an analysis of the NPC-associated protein Tpr (for translocated promoter region), a protein originally identified as the oncogenic activator of the met, raf, and trk protooncogenes (Park et al., 1986; Soman et al., 1991; Greco et al., 1992). Tpr is a very large (~270 kD) protein with a bipartite structure consisting of an ~1,600-residue NH<sub>2</sub>-terminal domain that is almost entirely an  $\alpha$ -helical coiled-coil followed by a highly acidic noncoiled COOH terminus (Mitchell and Cooper, 1992). Chromosomal translocations resulting in the fusion of the ~140–230 NH<sub>2</sub>-terminal residues of Tpr with the protein kinase domains of the protooncogenes met, raf, and trk have been implicated in cellular transformations and human tumors (Park et al., 1986; Soman et al., 1991; Greco et al., 1992). Tpr was initially thought to be a component of the fibrils emanating from the cytoplasmic face of the NPC (Byrd et al., 1994; Bangs et al., 1996), but was subsequently shown to be localized exclusively to intranuclear filaments associated with the nucleoplasmic side of the NPC (Cordes et al., 1997), a finding in agreement with data from this laboratory (see below) and others (Shah, et al., 1998). A *Drosophila* homologue of Tpr, Bx34, has been identified and is localized to the nuclear interior (Zimowska et al., 1997). Recently, Tpr has been shown to exist *in vivo* complexed to importin  $\beta$ , suggesting that the protein may serve as a binding site for this NLS receptor. In this study, we identify a region of Tpr that is responsible for its association with the NPC. By doing so, we provide evidence that the mechanism of oncogenic transformation by Tpr fusion proteins probably does not involve interactions with the NPC. We also identify a region of Tpr that directs localization to the nuclear interior but does not associate with the NPC. Finally, we demonstrate that Tpr, when overexpressed in cultured mammalian cells, blocks the export of polyadenylated mRNA from the nucleus, but does not appear to affect protein import.

## Materials and Methods

### Cell Culture

BHK, COS, and HeLa cells were maintained at 37°C under 5% CO<sub>2</sub> in DMEM supplemented with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD).

### Antibodies

The monoclonal antibody 12CA5, which is specific for the influenza virus hemagglutinin (HA) epitope is commercially available (Berkeley Antibody Co., Inc., Richmond, CA). Rabbit polyclonal antibodies raised against the same epitope were a gift from Dr. Michael Czech (University of Massachusetts, Worcester, MA). The anti-Tpr monoclonal antibody 203.37 was obtained from Matritech, Inc. (Cambridge, MA). A rabbit anti-lamin B antibody was a gift from Dr. Howard Worman (National Institutes of Health, CHHD, Bethesda, MD). The rabbit anti-nup358 polyclonal antibodies were a gift from Dr. Jiang Wu (Rockefeller University, New York, NY; Wu et al., 1995). The monoclonal antibody QE5 is described by Panté et al. (1994).

### Plasmid Constructions

The expression vector pHA1 was a gift from Dr. Michael Green (University of Massachusetts, Worcester, MA) and contains a cDNA encoding the

13-amino acid influenza virus HA epitope (Field et al., 1988) just upstream of the HindIII site in the polylinker of pcDNA I (Invitrogen, Madison, WI). HA-tagged Tpr constructs were obtained by insertion of the appropriate Tpr cDNA fragments into the polylinker of pHA1. cDNA fragments encoding the desired Tpr sequences were obtained from either pT7Tpr, which contains the full-length human Tpr cDNA (a gift from Dr. Larry Gerace, Scripps Institute, La Jolla, CA; Byrd et al., 1994) or from Hela cDNA clones  $\lambda$ Z203-1 and  $\lambda$ Z203-5, which together encode all but the first 67 amino acids of Tpr (Bangs et al., 1996). Tpr fragments were obtained by restriction digests or PCR amplification of the desired domains. Primers used for amplification had restriction sites engineered into them to allow for in-frame insertion into pHA1. GFP-tagged constructs were obtained by inserting the appropriate cDNA fragments into pEGFP (CLONTECH Laboratories, Inc., Palo Alto, CA) such that Tpr domains were translated with a COOH-terminal GFP fusion.

## Transfections

Plasmids were prepared for transfections using QIAGEN Maxi Plasmid Kits (QIAGEN Inc., Chatsworth, CA). Transfections of HA-tagged Tpr cDNA constructs were done by electroporation using a Cell-Porator (GIBCO BRL, Gaithersburg, MD). Log phase COS cells were trypsinized to remove them from the tissue culture plates and pelleted at 300 g through complete media. Cells were resuspended in complete media to  $\sim 10^6$  cells per ml. 1 ml of cells were transferred to electroporation chambers, 2–5  $\mu$ g of the appropriate DNA was added to the cells, and the cells were electroporated using 300 V at a capacitance of 1,160  $\mu$ F. Cells were then immediately dispensed into fresh media and plated onto 150-mm tissue culture plates. Plates generally included glass coverslips to be used for immunofluorescence microscopy.

Transfection of BHK cells was by calcium phosphate precipitation (Graham and van der Eb, 1973).

## Immunofluorescence

Cells were processed 24–36 h after transfection when they were  $\sim 50\%$  confluent. In cases where cells were preextracted before fixation, coverslips were rinsed twice with PBS and cells were extracted in room temperature permeabilization buffer (80 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM MgCl<sub>2</sub>, 0.5% Triton X-100) two times for 2 min each. Cells were then fixed at room temperature for 15 min in 3.7% formaldehyde/PBS, washed three times with PBS, and blocked 15 min in PBS containing 1% BSA and 0.5% Triton X-100 (PBSAT) before incubation with primary antibodies. In cases where cells were fixed before being permeabilized, coverslips were rinsed twice in PBS, fixed 15 min in 3.7% formaldehyde/PBS at room temperature, and then extracted two times for 2 min in permeabilization buffer. Cells were then washed three times with PBS and blocked for 15 min in PBSAT. Cells were incubated with primary antibodies at room temperature for 45 min, washed with PBSAT, and incubated with fluorescently labeled secondary antibodies for 20 min. After three washes with PBSAT, cells were incubated with 25 ng/ml DAPI (30 s) to counterstain DNA and mounted on glass slides with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Primary antibodies were used at the following dilutions in PBSAT: anti-HA monoclonal antibody 12CA5, 1:2,000; anti-HA polyclonal antibodies, 1:2,000; anti-Nup358 polyclonal antibodies, 1:20,000; QE5 monoclonal antibody, 1:250. Secondary antibodies (diluted in PBSAT) were donkey anti-mouse IgG conjugated to CY3 and goat anti-rabbit IgG conjugated to FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

For differential permeabilization with digitonin, cells on coverslips were washed twice with ice-cold PBS and fixed with cold 3.7% formaldehyde. Hela cells were permeabilized in 0.004% digitonin in PBS for 10 min at 4°C. COS cells were permeabilized in 0.001% digitonin for 10 min at 4°C. Antibody incubations were as described above except that antibody diluent and washes were PBS with 1% BSA rather than PBSAT.

For the majority of experiments, cells were observed with a Zeiss Axioptan microscope (Carl Zeiss, Thornwood, NY) through either a 63 $\times$  Plan-Apochromat 1.4 NA objective or a 100 $\times$  Plan-Neofluar 1.3 NA objective. Images were obtained through a Xillix Technologies Microimager CCD camera (Richmond, BC, Canada) using BDI software (Biological Detection Incorporated, Pittsburgh, PA). For the import/export analyses, a Leica DMRB microscope equipped with 63 $\times$  PL Apo 1.4 NA and PL Fluotar 100 $\times$  1.3 NA objectives was used. In this case images were collected using a Princeton Instruments Micromax cooled CCD camera (Trenton NJ). Images were processed and prepared for figures using

Adobe PhotoShop (Adobe Systems, San Jose, CA) and Canvas (Deneba Systems, South Miami, FL) software.

## Immunoelectron Microscopy

Cells were transfected with the Tpr domain 400–649 as in immunofluorescence experiments (above). We found that the optimal fixation conditions for EM analysis were to prepermeabilize cells for 30 s with cold permeabilization buffer then fix in 4% formaldehyde, 0.05% glutaraldehyde in PBS for 5 min. While increasing the glutaraldehyde concentration improved the ultrastructure of pores, the reactivity of the HA antibody was diminished. Without prepermeabilization, penetration of the gold antibody was significantly impaired. Since the nuclear membranes are removed in processing, the pores appear as dense tufts of material that project from the edge of the nuclei into the cytoplasm. Staining for nup358 confirmed that these structures were nuclear pores. After fixation, cells were processed for gold labeling as previously described (Doxsey et al., 1994).

## Immunoprecipitations

Cells were lifted from tissue culture plates by trypsinization, pelleted through complete media and washed twice with 5 ml cold PBS. Triton X-100-soluble fractions were obtained by lysing cells in permeabilization buffer with proteinase inhibitors (40 mg/ml bestatin, 0.7 mg/ml pepstatin, 50 mg/ml phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 2 mg/ml leupeptin, and 1 mg/ml E64; Boehringer Mannheim Corp., Indianapolis, IN) for 10 min at 4°C and then centrifuging at top speed in a 4°C microfuge for 30 min. Supernatants were transferred to fresh tubes. Triton X-100-insoluble pellets were washed with permeabilization buffer, solubilized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing proteinase inhibitors for 10 min at 4°C and centrifuged as before. Supernatants were transferred to fresh tubes. HA-tagged proteins were immunoprecipitated by incubation with 1.2  $\mu$ g 12CA5 antibody while rotating for 2 h at 4°C. 30  $\mu$ l of a 50% slurry of protein G-Sepharose (Pharmacia, Uppsala, Sweden) in permeabilization buffer and proteinase inhibitors was added and rotated for 60 min. The beads were pelleted and washed three times with permeabilization buffer before SDS-PAGE.

## SDS-PAGE/Western Blots

Protein samples were resolved according to the method of Laemmli (Laemmli, 1970). Proteins were transferred onto PVDF membrane (Millipore, Bedford MA) using the semi-dry blotting technique of Kyhse-Anderson (Kyhse-Andersen, 1984). The membranes were blocked, incubated with antibodies (1:3,000 anti-HA polyclonal), and reactive bands were visualized, all according to the manufacturer's instructions for the Immuno-Lite chemiluminescent system (Bio-Rad Laboratories, Hercules, CA).

## In Situ Hybridization

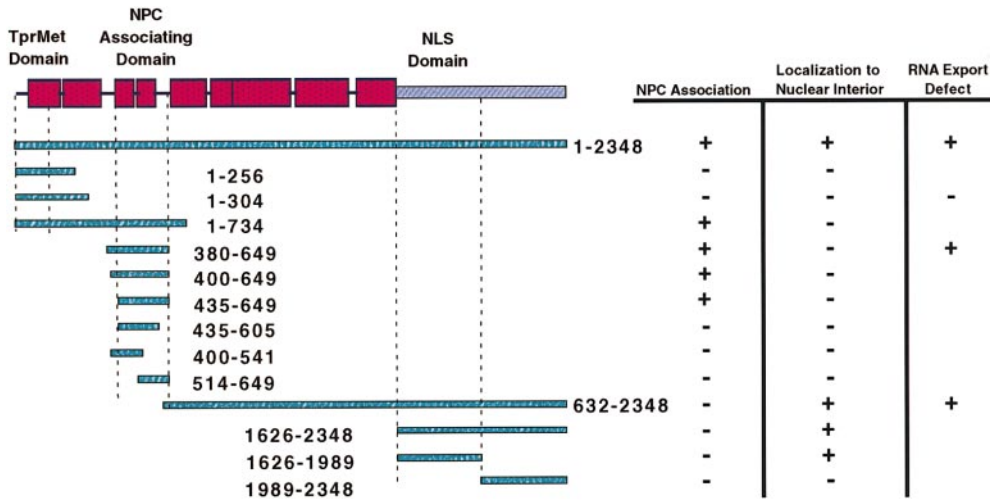
Fluorescence in situ hybridization with a biotinylated oligo-dT 45 mer (prepared with a 5' biotin by the University of Calgary Oligonucleotide Synthesis Facility, Calgary, Alberta, Canada) and subsequent double labeling with fluorescent antibodies was carried out on transfected and non-transfected BHK cells exactly as previously described (Bastos et al., 1996).

## Results

### HA-tagged Full-Length Tpr Localizes to the NPC

To identify functional domains of Tpr, a series of HA-tagged constructs of the human Tpr was developed and expressed in COS cells. Localization of Tpr proteins to specific cellular domains was determined by immunofluorescence microscopy. A summary of the relevant constructs and localizations of the expressed proteins is provided in Fig. 1.

Expression of the full-length HA-tagged Tpr (HA-Tpr) resulted in a fine punctate pattern at the surface of the nucleus as expected for a protein associated with NPCs (Fig.



**Figure 1.** Table of epitope-tagged Tpr constructs and expression phenotypes. Schematic diagrams of the Tpr constructs used for transfection studies. All constructs were tagged at the NH<sub>2</sub> termini with an HA-epitope. The localization phenotypes for all constructs are shown at right as are the RNA export phenotypes for the indicated proteins. The functional domains for Tpr and the Tpr-Met fusion domain are indicated by dotted lines.

2 A). At low to moderate levels of expression, HA-Tpr localized exclusively to the surface of the nucleus with an occasional focus of staining in the nuclear interior, a pattern indistinguishable from endogenous Tpr (Bangs et al., 1996; Cordes et al., 1997).

To determine whether HA-Tpr associated with the NPC, we used double-label immunofluorescence microscopy with antibodies to other components of the NPC. As shown in Fig. 2 A, the anti-HA staining pattern precisely overlapped with the staining pattern seen for the QE5 antibody that reacts with several nucleoporins (Panté et al., 1994), although the relative intensity of the two staining patterns differed at some locations. This result demonstrated that, within the limits of resolution of the light microscope, HA-Tpr colocalized with the NPC.

Next, we determined which side of the nuclear envelope HA-Tpr was on. To accomplish this, we expressed HA-Tpr at higher levels that caused accumulation of the protein within the nuclear interior and at cytoplasmic foci, in addition to the prominent NPC localization (Fig. 2, B and C). The presence of cytoplasmic foci allowed the detection of the full-length HA-Tpr in cells that had been differentially extracted with digitonin, as in Fig. 2. In brief, like the endogenous protein, HA-Tpr was not detected at the NPC when the plasma membrane was permeabilized with digitonin, but became accessible only after the nuclear membrane was permeabilized with Triton X-100, indicating that HA-Tpr was localized to the nuclear face of the NPC (Fig. 2).

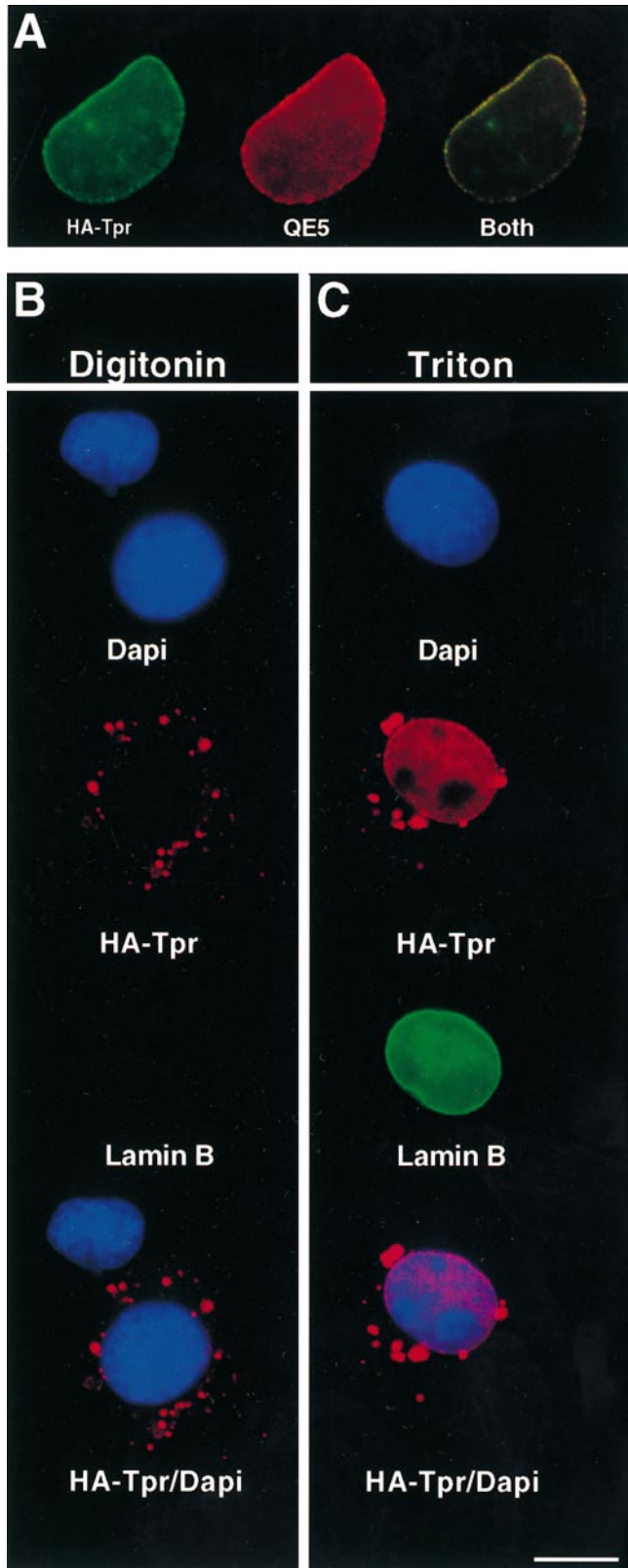
### The Tpr-Met Fusion Domain Does Not Localize to the NPC

Having shown that a full-length HA-tagged Tpr would localize to the NPC, we next constructed a series of HA-tagged truncations of Tpr to identify functional domains of the molecule. Since the NH<sub>2</sub>-terminal region of Tpr is involved in the activation of the Met oncogene (Mitchell and Cooper, 1992), we determined whether this domain was involved in the localization of Tpr to the NPC and thus in localization of the protein kinase domain of the Met oncoprotein to the NPC. Fig. 3 A shows a schematic diagram of

the clones used in the analysis of the NH<sub>2</sub>-terminus of Tpr and their relationship to the full-length molecule. Expression of the domain that included amino acids 1–734 resulted in a finely punctate labeling of the nuclear envelope indicating that the first 734 amino acids of Tpr were sufficient for NPC localization (Fig. 3 B). In contrast, Tpr domains consisting of amino acids 1–304 and smaller were scattered diffusely throughout both the cytoplasm and the nucleus and did not associate with the NPC (Fig. 3 B); at high levels of expression, the HA-Tpr1-304 domain sometimes formed long filaments in the cytoplasm (Fig. 3 D). While Triton X-100 extraction of cells expressing the HA-Tpr1-734 domain before fixation did not change the nuclear envelope staining (not shown), it completely abolished the staining seen with the HA-Tpr1-304 and HA-Tpr1-256 domains. Immunoprecipitation of these proteins from Triton X-100-soluble and -insoluble cellular extracts confirmed that the NPC-associated HA-Tpr1-734 domain was present in both soluble and insoluble fractions, while the nonlocalizing domains HA-Tpr1-256 and HA-Tpr1-304 were detectable only in the soluble fraction. These results suggest that the portion of Tpr involved in the activation of the Met oncogene (amino acids 1–140) is not sufficient to localize the Tpr-Met chimera to the NPC.

### Identification of a NPC Association Domain in Tpr

To further delineate the NPC association domain within amino acids 1–734 (Fig. 4), and to determine if other NPC association domains existed elsewhere in the molecule, a number of additional HA-tagged deletion mutants of Tpr were analyzed. The domain HA-Tpr400-649 localized exclusively to the nuclear envelope in a punctate pattern indistinguishable from that seen for the NPC. However, deletion of 34 amino acids from the COOH terminus of this clone abrogated NPC association but produced large spherical foci in the nuclear interior that were mostly resistant to extraction with Triton X-100 (Fig. 4, B and C). Deletion of 35 amino acids from the NH<sub>2</sub>-terminus of HA-Tpr400-649 had no effect on NPC association, but deletion of 149 amino acids completely eliminated NPC association and produced a diffuse staining pattern throughout both



**Figure 2.** HA-tagged full-length Tpr localizes to the NPC. (A) Double-label immunofluorescence of a COS cell expressing the full-length HA-Tpr. Anti-HA staining (green, left); staining by the antibody QE5, which recognizes a number of nucleoporins (red, center); superimposition of the two images showing overlap (yellow, right). (B) Two COS cells permeabilized with digitonin and colabeled with an anti-HA antibody and an anti-lamin B an-

the nucleus and the cytoplasm that was entirely soluble in Triton X-100 (Fig. 4, B and C). From these data, we conclude that the minimal domain necessary for association with the NPC lies within the region of Tpr bounded by the amino acids 435–649. As shown in the schematic diagram of this construct (Fig. 4 A), this domain includes two regions of heptad repeats that are predicted to form coiled-coils and two noncoiled stretches.

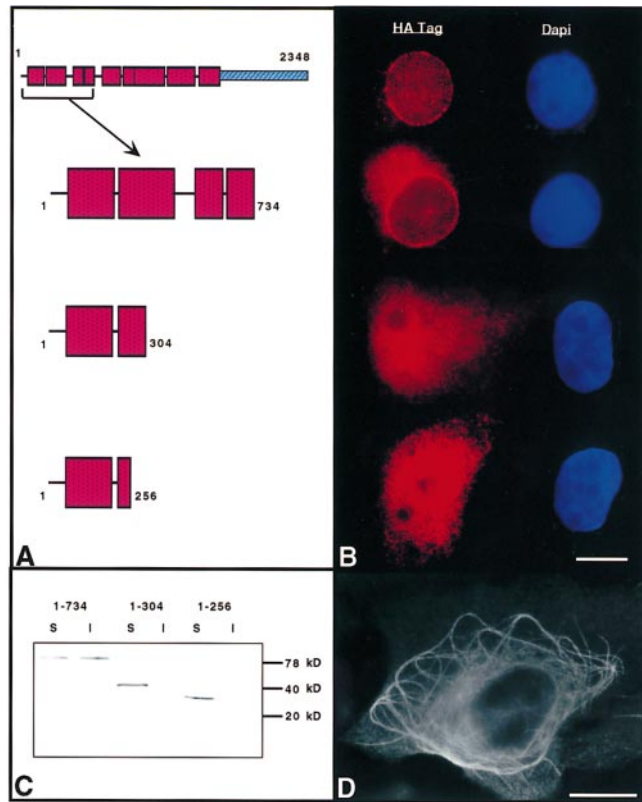
Immunogold analysis of the Tpr NPC associating domain confirmed that the protein was localized to the nucleoplasmic side of the pore (Fig. 5 A). Since the nuclear membranes are removed in processing (see Materials and Methods for details), the pores appear as dense tufts of material that project from the border of the nuclei into the cytoplasm. Quantitative analysis demonstrated that 83% of the nuclear gold particles were found within 200 nm of the nucleoplasmic side of pores (1,038/1,217,  $n = 3$  experiments), usually in clusters of three to five particles and consistent with labeling of the nucleoplasmic filaments in association with the NPC as previously described (Cordes et al., 1997). The remainder of the gold was distributed randomly along the cytoplasmic face of the nucleus and the nuclear interior. In parallel samples, immunogold labeling of nup358, a cytoplasmic nucleoporin (Wu et al., 1995), confirmed that the structures examined in this analysis were nuclear pores (Fig. 5 B). As expected, nup358 localized almost exclusively to the cytoplasmic face of nuclear pores further demonstrating the specificity of the HA antibody for structures on the nucleoplasmic side of the pore. No gold was detected if primary antibodies were omitted (Fig. 5 C).

To reinforce our finding that the Tpr peptide 435-649 was sufficient to convey NPC association, we fused the cDNA encoding this domain to green fluorescent protein (GFP) that does not localize to the NPC on its own. When fused with the NPC association domain and expressed in COS cells, the GFP-Tpr435-649 fusion protein produced a nuclear pattern with punctate staining at the nuclear rim, demonstrating that this peptide was sufficient for targeting to the nuclear rim (Fig. 6).

#### *The COOH Terminus of Tpr Contains a Nuclear Localization Domain*

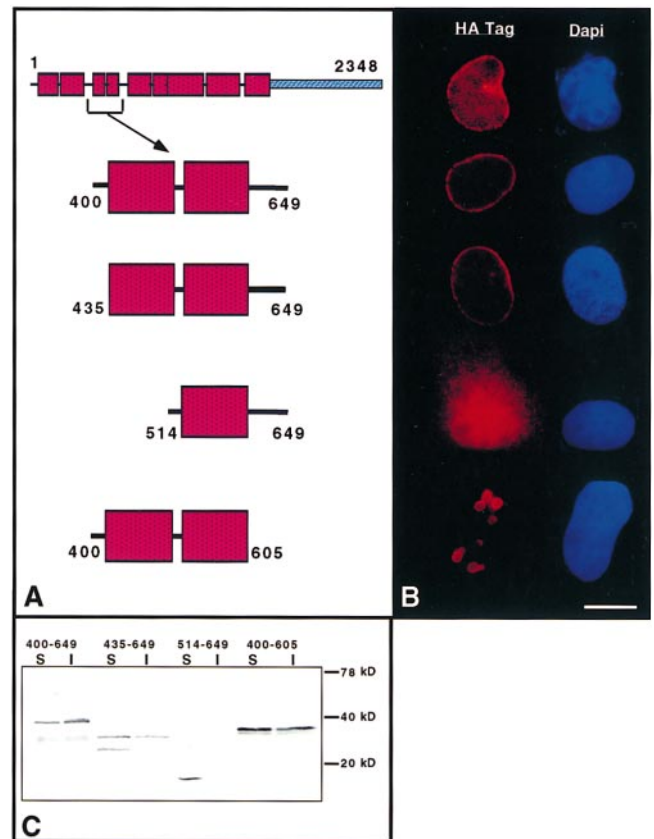
We used another set of HA-tagged deletion constructs to determine the localization of the COOH-terminal domain of Tpr. To our surprise, removal of the NH<sub>2</sub>-terminal NPC association domain (above) resulted in the accumulation of the remainder of the protein within the nuclear interior,

tibody. Only one of the cells is expressing HA-Tpr. Vertical series are of the same cell: DAPI staining of nuclei; HA-staining to show cytoplasmic foci of HA-Tpr but no labeling of the nuclear envelope; lamin B staining to show that the nuclear membrane is intact after digitonin extraction; and DAPI and anti-HA staining to demonstrate the lack of HA-Tpr labeling at the nuclear envelope. (C) A cell expressing HA-Tpr permeabilized with Triton X-100. The cell is depicted as in B to show: DAPI staining of the nucleus; anti-HA staining of HA-Tpr at the nuclear envelope and cytoplasmic foci; lamin B staining to show that the nuclear membrane has been permeabilized; and DAPI and anti-HA staining to show HA-Tpr labeling of the nuclear envelope. Bar, 10  $\mu$ m.



**Figure 3.** The NH<sub>2</sub> terminus of Tpr does not localize to the nuclear envelope. (A) A schematic diagram of the HA-tagged Tpr deletion mutants used to analyze the NH<sub>2</sub> terminus of Tpr. The bracket under HA-Tpr indicates the portion of the full-length protein that has been expanded and shown beneath. The numbers shown in the diagram indicate amino acids of the Tpr domains that are expressed. The large stippled boxes indicate areas predicted to be in a coiled-coil motif. (B) Immunofluorescence of cells expressing truncated forms of HA-Tpr. The cells are aligned directly to the right of the schematic diagram of the construct being expressed. Images of the anti-HA staining pattern (*left*) and DAPI stains (*right*) of the same cells. While HA-Tpr and HA-Tpr1-734 show labeling of the nuclear envelope, HA-Tpr1-304 and HA-Tpr1-256 show diffuse localization in the cytoplasm and nucleus. The cells were fixed before permeabilization with Triton X-100. (C) A composite of Western blot analyses of HA-Tpr proteins immunoprecipitated from Triton X-100-soluble and -insoluble cell extracts. While all three proteins are in soluble fractions (S), only HA-Tpr1-734 is in the insoluble fractions (I). Molecular mass markers on right ( $\times 10^3$ ). (D) Immunofluorescence of a BHK cell expressing high levels of HA-Tpr1-304 and showing long filaments in the cytoplasm. Bars, 10  $\mu$ m.

although not in association with the NPC (Fig. 7). This domain, HA-Tpr632-2348, although far too large (190 kD) to diffuse through the nuclear pore, accumulated to high levels throughout the nucleus and was detected only at low levels, if at all, in the cytoplasm. This result suggested that localization to the nucleus was an active process (Fig. 7 B). This domain was also retained within the nucleus after Triton X-100 extraction of cells before fixation as shown by morphological (not shown) and biochemical analysis (Fig. 7 C). Deletion of a 994-amino acid coiled-coil region from this domain did not have any effect on lo-

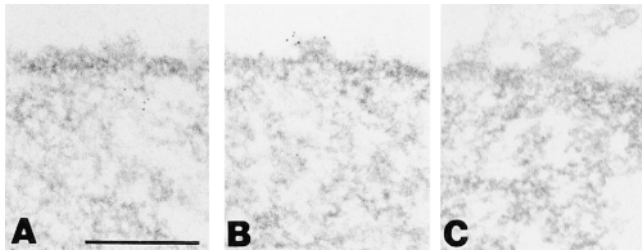


**Figure 4.** Identification of a NPC association domain. (A) Schematic diagram of the HA-Tpr clones used for the identification of an NPC association domain in Tpr. See Fig. 3 for details. (B) Immunofluorescence of COS cells expressing HA-tagged Tpr fragments. HA-Tpr400-649 and HA-Tpr435-649 localize to the NPC; HA-Tpr514-649 is diffusely scattered throughout the nucleus and cytoplasm; HA-Tpr400-605 forms large spherical foci located within the nucleus but not associated with the nuclear envelope. (C) A composite of Western blot analyses of HA-Tpr domains immunoprecipitated from Triton X-100-soluble and -insoluble cellular extracts. All proteins are present in both soluble and insoluble fractions (I) except HA-Tpr514-649, which is only detected in the soluble fraction (S). Molecular mass markers on right ( $\times 10^3$ ). Bar, 10  $\mu$ m.

calization to the nuclear interior, although the resulting domain (HA-Tpr1626-2348, see Fig. 7 A) was rendered completely soluble in Triton X-100 (Fig. 7 C).

Accumulation of the non-coiled COOH terminus of Tpr in the nucleus suggested the presence of a nuclear localization signal (NLS). Close inspection of the amino acid sequence revealed a potential bipartite NLS based on a consensus of two basic amino acids, followed by a spacer of variable length, followed by a region of five amino acids of which three are basic (Dingwall, 1991). The amino acid sequence of Tpr located between amino acids 1,829 and 1,860 (KR-X<sub>25</sub>-KCLK, where X is any amino acid) fits the consensus, although the 25-amino acid spacer is atypically long.

To further define the region of Tpr responsible for targeting to the nuclear interior, we divided the noncoiled

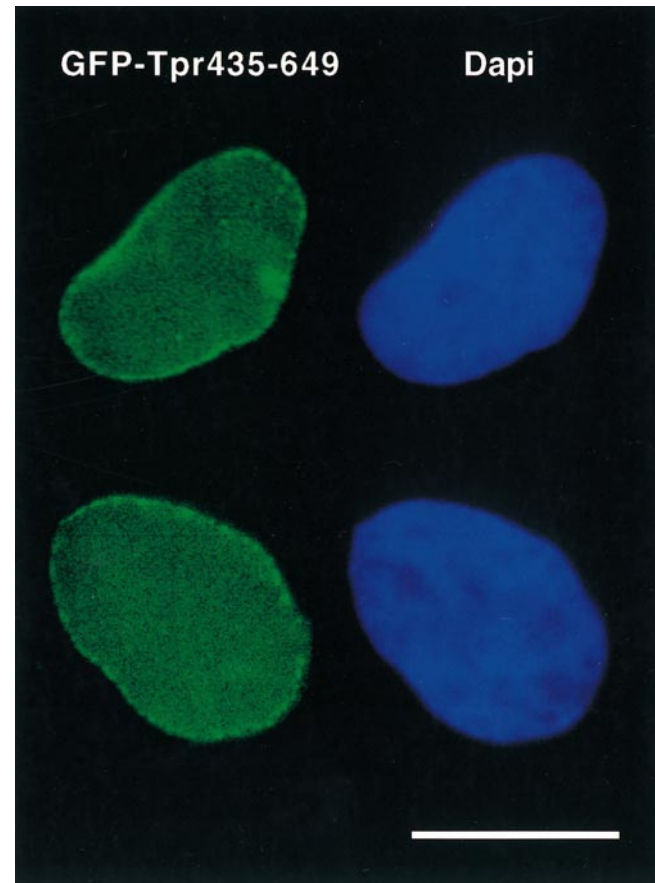


**Figure 5.** Immunogold localization of the HA-tagged, NPC-associated domain of Tpr overexpressed in COS cells. HA-Tpr400-649 was detected with polyclonal HA antibodies and 5-nm gold goat-rabbit IgG in cells permeabilized with detergent (see Materials and Methods for details). Gold particles were found predominantly within 200 nm of the nucleoplasmic face of nuclear pores (A). Quantitative analysis revealed that the majority of the gold was localized to the nucleoplasmic aspect of the pore (see Results). In contrast, nup358 was localized to the cytoplasmic aspect of the pore in cells processed in parallel (B). No gold was detected if primary antibodies were omitted (C). Bar, 250 nm.

COOH terminus roughly in half, making the 363-amino acid domain HA-Tpr1626-1989 (which contains the putative NLS) and the 359-amino acid domain HA-Tpr1989-2348 (Fig. 7 A). The domain containing the putative bipartite NLS accumulated within the nucleus, whereas the domain lacking the putative NLS was diffusely localized throughout the cytoplasm and nucleus (Fig. 7 C). Thus, while both domains were small enough to diffuse into and out of the nucleus (~40 kD), only the one with the putative NLS was preferentially localized to the nuclear interior. To confirm that a nuclear localization sequence was contained within amino acids 1,626–1,989, we fused this domain to GFP and expressed the chimeric protein in COS cells. As shown in Fig. 8, the GFP-Tpr1626-1989 protein was confined to the nucleus when expressed in COS demonstrating that this Tpr domain was sufficient for localization to the nuclear interior.

#### **Overexpression of Tpr Leads to Apparent Disruption of a Nuclear Export Pathway but Does Not Affect Protein Import**

As an initial test of Tpr function, we examined two important functions of the nuclear pore, protein import and polyadenylated RNA export, in the presence of various HA-Tpr fusions. To study protein import, we used the BHK cell line BHKgr $\beta$  (Bastos et al., 1996) that constitutively expresses a glucocorticoid receptor- $\beta$ -galactosidase fusion protein. This chimeric protein acts as an inducible reporter for nuclear import as it transits from the cytoplasm into the nucleus upon the addition of dexamethasone (Picard and Yamamoto, 1987). Using this assay, we examined nuclear import in cells expressing: the full-length Tpr (HA-Tpr), the nuclear localizing COOH-terminal domain (HA-Tpr632-2348), the soluble NH<sub>2</sub> terminus (HA-Tpr1-304), and the NPC-associating domain (HA-Tpr380-649). None of these HA-Tpr fusion proteins had a detectable effect on nuclear transport of the glucocorticoid receptor- $\beta$ -galactosidase fusion protein (Figs. 9 and 10). However, at very high levels we could observe a par-

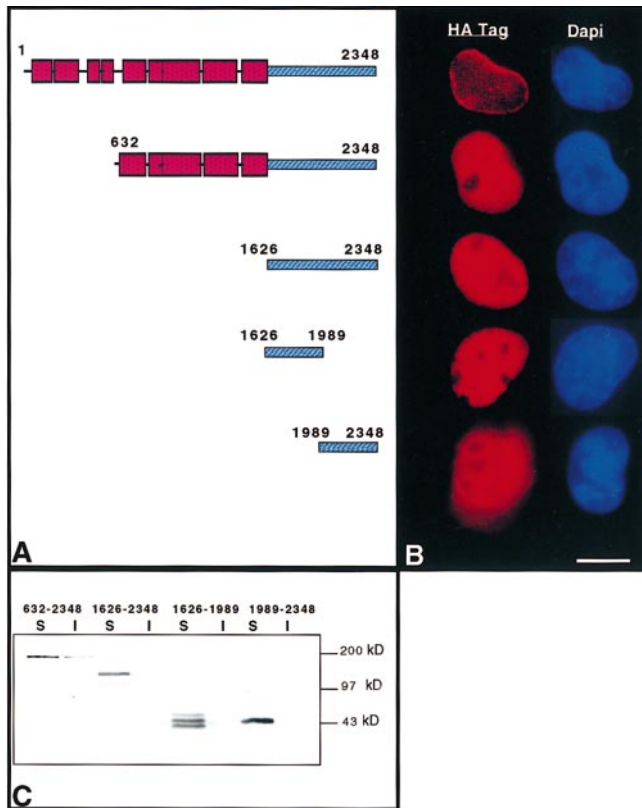


**Figure 6.** The NPC association domain of Tpr is sufficient to localize green fluorescent protein to the nuclear envelope. Two COS cells expressing GFP-Tpr435-649 (left) and the corresponding DAPI stains (right). Localization of GFP to the nuclear envelope is evident in both cells that were permeabilized with Triton X-100 before fixation to remove soluble protein that obscures the nuclear envelope signal. Bar, 10  $\mu$ m.

tial redistribution of  $\alpha$ -importin in cells expressing the full-length HA-Tpr (data not shown, see Discussion).

In contrast to the results observed for protein import, the full-length, NPC-associating and nuclear localizing Tpr domains had a marked effect on the distribution of poly(A)<sup>+</sup> RNA. This was examined by *in situ* hybridization with a biotinylated oligo-dT 45-mer (Bastos et al., 1996). In nontransfected cells, poly(A)<sup>+</sup> RNA was distributed roughly evenly between the nucleus and the cytoplasm with nuclear poly(A)<sup>+</sup> RNA restricted to a punctate reticulum throughout the nucleoplasm and excluded from the nucleoli (Huang et al., 1994; Bastos et al., 1996). Overexpression of the full-length, NPC-associating and nuclear localizing Tpr domains led to significant increases in nuclear poly(A)<sup>+</sup> RNA, and accompanying decreases in cytoplasmic poly(A)<sup>+</sup> RNA (Fig. 11, A, C, D, E, G, and H). The percentage of cells that exhibited these elevated levels of nuclear poly(A)<sup>+</sup> RNA were found to be increased by factors of 3.3 (full-length Tpr), 4.6 (HA-Tpr380-649), and 5.5 (HA-Tpr632-2348) over a background level that is always observed in either mock transfected cells or cells



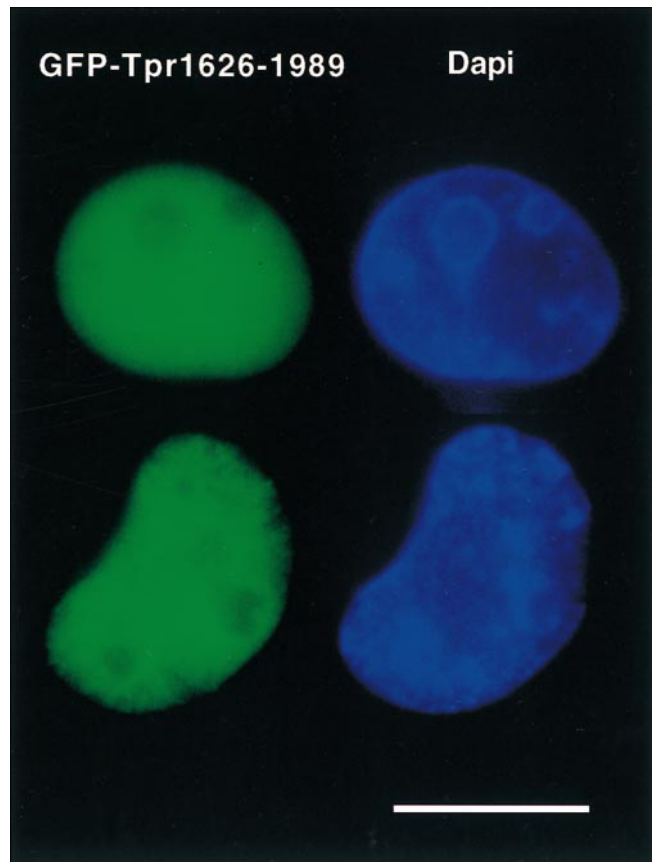


**Figure 7.** The COOH terminus of Tpr contains a nuclear localization domain. (A) Schematic diagrams of the HA-tagged Tpr truncations used for identification of a nuclear localization domain. See Fig. 3 for details. (B) Immunofluorescence of COS cells expressing the HA-tagged Tpr domains. Full-length HA-Tpr decorates the nuclear envelope while HA-Tpr632-2348, HA-Tpr1626-2348, and HA-Tpr1626-1989 all accumulate to high levels within the nuclear interior. HA-Tpr1989-2348 is diffusely localized throughout the cytoplasm and nucleus. (C) A composite of Western blot analyses of HA-Tpr proteins immunoprecipitated from Triton X-100-soluble and -insoluble cellular fractions. HA-Tpr632-2348 is detected in soluble and insoluble fractions and all others are detected only in soluble fractions. Molecular mass markers on right ( $\times 10^3$ ). Bar, 10  $\mu\text{m}$ .

transfected with irrelevant DNA. In contrast, overexpression of the NH<sub>2</sub>-terminal peptide HA-Tpr1-304 did not appear to markedly affect the distribution of poly(A)<sup>+</sup> RNA (Fig. 11, B and F) in BHK cells. It should be noted that the relatively harsh conditions (50% formamide) used for *in situ* hybridization experiments are known to cause a loss or redistribution of immunofluorescent signals when the two methods are used in combination. Thus, the images for HA-Tpr constructs in these experiments do not show a nuclear envelope localization as clearly as those seen in Figs. 3, 4, and 6.

## Discussion

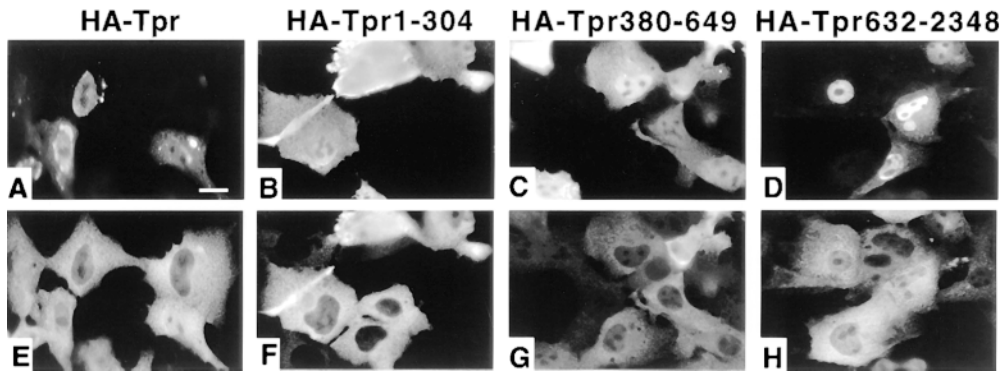
In this study, we have examined the function of the Tpr protein through a molecular dissection of its various domains. In particular, we have identified distinct regions of



**Figure 8.** The NLS domain of Tpr localizes GFP to the nuclear interior. Two COS cells expressing GFP-Tpr1626-1989 fixed as in Fig. 3 to show that virtually all of the protein accumulates within the nuclear interior. Bar, 10  $\mu\text{m}$ .

the molecule that can confer localization either to the nuclear face of NPCs or to the nuclear interior. Since Tpr was first identified in NH<sub>2</sub>-terminal oncogenic fusions with certain protein kinases, we were especially interested in the role that the Tpr NH<sub>2</sub>-terminal domain might play in intracellular targeting. Finally, we have identified regions of the Tpr protein that when expressed in mammalian cells cause an accumulation of nuclear poly(A)<sup>+</sup> RNA. Through this analysis, we have been able to implicate Tpr in the process of mRNA export from the nucleus.

Previous studies have shown that Tpr is often fused with the protein kinase domains of several protooncogenes (met, raf, and trk) in human tumors (Park et al., 1986; Soman et al., 1991; Greco et al., 1992), and that the Tpr-Met chimera dimerizes *in vivo* inducing autophosphorylation and constitutive activation of the protein kinase domain (Rodrigues and Park, 1993). Although it was possible that Tpr also directed localization to the NPC, thereby tethering an active protein kinase at the nuclear periphery, we found that the NH<sub>2</sub>-terminal region of Tpr implicated in oncogenesis does not in fact contain such a targeting function. While this NH<sub>2</sub>-terminal domain does occasionally form striking filamentous arrays extending throughout the cytoplasm, this only occurs at extremely high expression levels and it is doubtful that this phenomenon contributes



**Figure 9.** Overexpression of HA-Tpr proteins does not effect the distribution of a glucocorticoid receptor- $\beta$ -galactosidase fusion protein. Immunofluorescence of BH-Kgr $\beta$  cells expressing (A and E) HA-Tpr, (B and F) HA-Tpr1-304, (C and G) HA-Tpr380-649, and (D and H) HA-Tpr632-2348. Cells in A-D show staining with anti-HA. Cells in E-H are the corresponding fields stained with an anti- $\beta$ -galactosidase antibody.

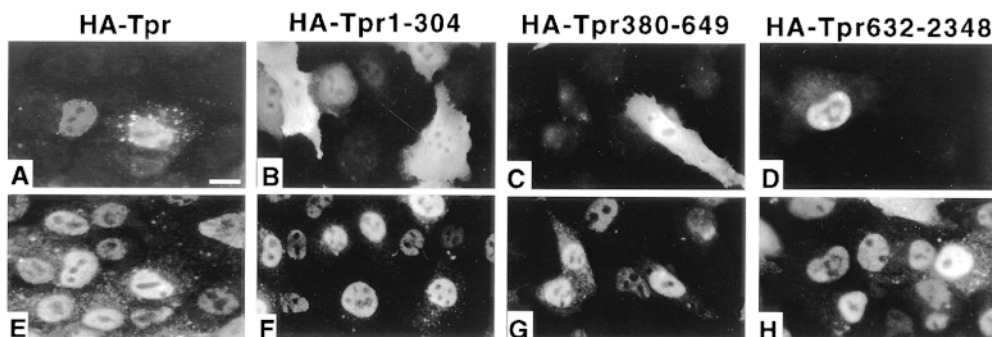
In the absence of dexamethasone, the glucocorticoid receptor- $\beta$ -galactosidase fusion is largely cytoplasmic for all HA-Tpr proteins and nontransfected cells alike. Bars, 10  $\mu$ m.

to the transforming activity of Tpr-Met chimeras. Based on these observations, it appears that the role of Tpr in the activation of protooncogenes is unrelated to NPC localization but instead is most likely the promotion of dimerization or higher-order oligomerization (Rodrigues and Park, 1993).

A minimal sequence lying between residues 435 and 649 of the Tpr protein can confer localization to the nucleoplasmic region of NPCs in transient transfection assays. This domain appears to be essential for NPC association of the Tpr molecule since both NH<sub>2</sub>- and COOH-terminal deletions that extend into the sequence abolish NPC localization. Domains containing NH<sub>2</sub>-terminal truncations do, however, accumulate within the nucleoplasm. At present, we cannot rule out the possibility that, in the context of the full-length molecule, other regions of Tpr may be involved in interactions with the NPC. Furthermore, the minimal 225-residue NPC targeting or association region that we have identified could actually represent a domain within the molecule that functions in self association. In this way, NPC targeting by this minimal sequence could be by virtue of interaction with endogenous Tpr. This is not an issue that can be immediately resolved in normal cells that constitutively express the full-length protein. However, if this segment of Tpr does have a direct function in NPC-association it must logically interact with another component of the NPC or the nuclear envelope. Identification of such

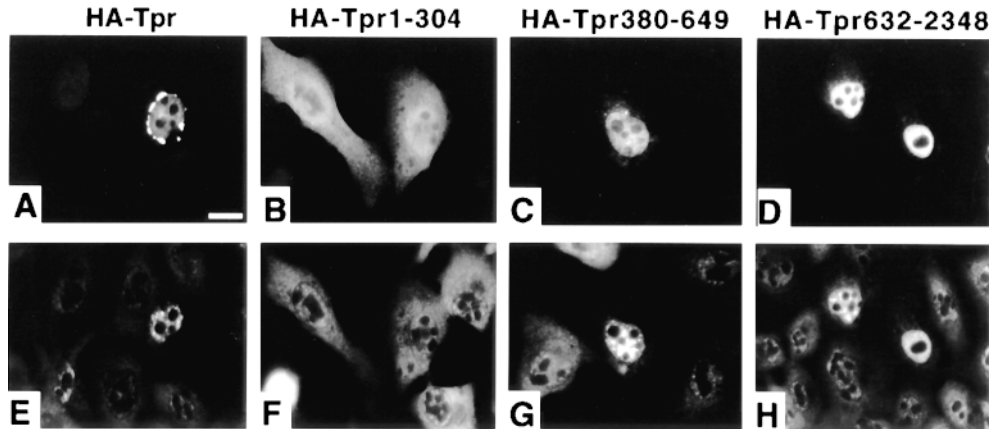
protein(s), by two hybrid analysis or more conventional *in vitro* binding assays for instance, would lend support for a direct role in NPC targeting. Such findings would also provide further insight into the role of Tpr in the maintenance of the normal architectures of both the NPC and nucleus.

A COOH-terminal domain of Tpr (amino acids 1,626–1,989) localizes to the nucleus and also imparts nuclear localization to GFP suggesting that this domain contains a nuclear localization signal. A sequence closely matching the consensus NLS for import via the importin  $\alpha/\beta$  pathway (Dingwall, 1991) is contained within this domain, but as we have not shown that this sequence is responsible for nuclear localization, it is also possible that the nuclear localization domain contains a nuclear import signal unrelated to the classical basic domain NLS, (as recently described by Pollard et al., 1996; Pemberton et al., 1997; Rosenblum et al., 1997; Rout et al., 1997). A signal-mediated transport mechanism could explain how a protein as large as Tpr becomes associated with the nucleoplasmic face of the NPC, since it is far too large to diffuse through the NPC on its own. Alternatively, Tpr may “piggy-back” its way into the nucleus via binding to another protein that is itself transported into the nucleus. This possibility has been suggested for the nucleoporins Nup153 and Nup98, which also associate with the nucleoplasmic face of the NPC but at the same time do not appear to use a recognizable NLS (Bastos et al., 1996). This scheme, however,



**Figure 10.** Overexpression of Tpr proteins does not effect nuclear import of a glucocorticoid receptor- $\beta$ -galactosidase fusion protein. Immunofluorescence of BHKgr $\beta$  cells expressing (A and E) HA-Tpr, (B and F) HA-Tpr1-304, (C and G) HA-Tpr380-649, and (D and H) HA-Tpr632-2348 as described in Fig. 8. Cells were treated with 10  $\mu$ g/ml dexamethasone for 30 min before fixation.

to induce nuclear import of the receptor. In all cases, nuclei of cells expressing the Tpr proteins accumulate the glucocorticoid receptor- $\beta$ -galactosidase fusion protein to the same levels as nontransfected cells. Bar, 10  $\mu$ m.



**Figure 11.** Overexpression of full-length Tpr, the NPC association domain and the nuclear localization domain interfere with poly(A)<sup>+</sup> RNA trafficking. Poly(A)<sup>+</sup> RNA distribution in BHK cells expressing (A and E) HA-Tpr, (B and F) HA-Tpr1-304, (C and G) HA-Tpr380-649, and (D and H) HA-Tpr632-2348. A–D Show anti-HA staining patterns for the respective domains. E–H show the corresponding fields after in situ hybridization with a biotinylated oligo-dT 45 mer. Poly(A)<sup>+</sup> RNA accumulates in cells expressing HA-Tpr, HA-Tpr380-649, and HA-Tpr632-2348, but not HA-Tpr1-304. Bar, 10 µm.

seems unlikely for Tpr since it is almost entirely localized to the nucleus even at expression levels that might be expected to saturate endogenous levels of a non-shuttling carrier protein, another NPC protein for example.

While it is clear that the COOH-terminal portion of Tpr contains a nuclear localization domain, it is also possible that an additional nuclear localization signal exists within the NH<sub>2</sub>-terminal coiled-coil domain. The HA-Tpr1-734 domain localizes to the NPC (Fig. 4) as does a larger construct HA-Tpr1-1387 (not shown), although both of these constructs are too large to diffuse through the NPC. As suggested above, it is possible that these molecules enter the nucleus by association with another nuclear protein, but a second nuclear localization sequence between the amino acids 304 and 734 cannot be ruled out.

Our data indicate that Tpr has a potential role in the export of mRNA. Moderate overexpression of Tpr domains that localize to the NPC or to the nuclear interior cause the dramatic accumulation of poly(A)<sup>+</sup> RNA within the nucleus. In contrast, NH<sub>2</sub>-terminal domains that do not associate with the NPC have no such effect, even at extremely high expression levels. A recent publication by Shah et al. (Shah et al., 1998) has provided compelling evidence that Tpr also interacts with components of the nuclear import machinery, in particular importin  $\alpha/\beta$  heterodimers. Whether this reflects a role in import or a function in the recycling of the NLS receptor subunits to the cytoplasm has yet to be satisfactorily resolved. However, the observation that interaction of importin  $\alpha/\beta$  with Tpr occurs only in the absence of binding of an NLS-bearing transport substrate would perhaps be more consistent with the latter interpretation. We have not been able to detect any inhibition of nuclear protein import at Tpr expression levels that have a clear effect on poly(A)<sup>+</sup> RNA distribution. Nevertheless, at very high Tpr expression levels we observe a partial redistribution of importin  $\alpha$ . However, the abundance of this and other soluble transport factors is sufficiently great that the overall impact of excess Tpr on their availability may be only marginally de-

tectable in terms of distribution of nuclear import substrates. Thus, the results of Tpr overexpression are not inconsistent with the results of Shah et al. (Shah et al., 1998). In any case, the fact that the nuclear import of the glucocorticoid receptor- $\beta$ -galactosidase fusion protein (which probably exists as a ~750 kD tetramer) is largely unaffected by Tpr overexpression, suggests that the effect on RNA distribution is not due simply to a loss or occlusion of NPCs.

The mechanism by which Tpr over expression inhibits poly(A)<sup>+</sup> RNA export is still a matter of speculation, particularly since two separate domains that localize to either the NPC or the nuclear interior both disturb the normal distribution of poly(A)<sup>+</sup> RNA. Although it is possible that the NPC-binding region domains might displace the full-length Tpr from NPCs, there are likely additional reasons since immunofluorescence observations on cells overexpressing such domains reveal no major alterations in the localization of the endogenous molecule (Bangs, P., unpublished results). As discussed above, it is possible that this NPC targeting region could actually function at least in part by virtue of association with the endogenous protein as its coiled coil structure predicts. Such an interaction would most likely be formed in the cytoplasm soon after synthesis. The hybrid full-length/mutant Tpr dimer would then be imported into the nucleus (full-length provides the NLS) followed by localization to the NPC. The prediction of course is that this heterodimer would be nonfunctional (or would have reduced function) in terms of mediating RNA export. This model would explain why we do not see any major redistribution of endogenous Tpr from the NPC, since a 50% reduction (the maximum that could be achieved in this dimerization scheme) might not be immediately apparent by immunofluorescence microscopy. An alternative to the self association that would explain the poly(A)<sup>+</sup> RNA effect is that a large excess of the NPC association region of Tpr might potentially block assembly of one or more additional nucleoporins that are essential for normal RNA trafficking. The merits of either model

will be better evaluated once we have more information on the identities of proteins with which the Tpr NPC targeting region (435–649) interacts.

A somewhat different scenario is likely to account for the block in poly(A)<sup>+</sup> RNA export caused by overexpression of the Tpr COOH-terminal domain. Since this truncated protein exhibits no preferential association with the NPC and instead localizes uniformly throughout the nucleus, the resulting accumulation of poly(A)<sup>+</sup> RNA is probably not due to a disruption of activity at the site of the NPC. It is more likely the result of titration of factors necessary for efficient RNA export. These could include any of the recently identified carriers that mediate export from the nucleus (Fornerod et al., 1997; Kutay et al., 1997; Stade et al., 1997), as well as RNA-binding proteins necessary for the assembly of transportable RNA-protein complexes, a possibility suggested previously (Cordes et al., 1997).

Based on the identification of distinct Tpr domains that localize to the NPC and the nuclear interior and the ability of these domains to inhibit mRNA export but not nuclear import, we conclude that Tpr is involved in the export of macromolecules from the nucleus. Our data are consistent with a previously suggested model in which Tpr, by virtue of its extensive coiled-coil structure, is an integral component of the intranuclear filaments that extend from the NPC and that the acidic COOH terminus of this protein interacts with soluble transport factors to facilitate the passage of RNA-protein complexes to the transport machinery of the NPC (Cordes et al., 1997). Discovery of the NPC association domain of Tpr will facilitate the identification of proteins that localize Tpr to the NPC and subsequently provide a better understanding of the role of Tpr in nuclear export.

The authors wish to thank Cindy Sparks and Aaron Young for their helpful discussions and comments on the manuscript.

This work was supported by grants to S.J. Doxsey from the National Institute of General Medical Sciences of the National Institutes of Health (R01-GM51994), the American Cancer Society, and the American Heart Association (Established Investigator Award).

Received for publication 21 May 1998 and in revised form 24 November 1998.

## References

- Akey, C.W., and M. Radermacher. 1993. Architecture of the *Xenopus* nuclear pore complex revealed by three-dimensional cryo-electron microscopy. *J. Cell Biol.* 122:1–19.
- Aitchison, J.D., M.P. Rout, M. Marelli, G. Blobel, and R.W. Wozniak. 1995. Two novel related yeast nucleoporins Nup170p and Nup157p: complementation with the vertebrate homologue Nup155p and functional interactions with the yeast nuclear pore-membrane protein Pom152p. *J. Cell Biol.* 131:1133–1148.
- Bangs, P.L., C.A. Sparks, P.R. Odgren, and E.G. Fey. 1996. Product of the oncogene-activating gene Tpr is a phosphorylated protein of the nuclear pore complex. *J. Cell. Biochem.* 61:48–60.
- Bastos, R., N. Panté, and B. Burke. 1995. Nuclear pore complex proteins. *Int. Rev. Cytol.* 162:257–302.
- Bastos, R., A. Lin, M. Enarson, and B. Burke. 1996. Targeting and function in mRNA export of nuclear pore complex protein Nup153. *J. Cell Biol.* 134:1141–1156.
- Bastos, R., L. Ribas de Pouplana, M. Enarson, K. Bodoor, and B. Burke. 1997. Nup84, a novel nucleoporin that is associated with CAN/Nup214 on the cytoplasmic face of the nuclear pore complex. *J. Cell Biol.* 137:989–1000.
- Byrd, D.A., D.J. Sweet, N. Panté, K.N. Konstantinov, T. Guan, A.C.S. Saphire, P.J. Mitchell, C.S. Cooper, U. Aebi, and L. Gerace. 1994. Tpr, a large coiled coil protein whose NH<sub>2</sub> terminus is involved in the activation of oncogenic kinases, is localized to the cytoplasmic face of the nuclear pore complex. *J. Cell Biol.* 127:1515–1526.
- Cordes, V.C., S. Reidenbach, A. Kohler, N. Stuurman, R. van Driel, and W.W. Franke. 1993. Intranuclear filaments containing a nuclear pore complex protein. *J. Cell Biol.* 123:1333–1344.
- Cordes, V.C., S. Reidenbach, H.R. Rackwitz, and W.W. Franke. 1997. Identification of protein p270/Tpr as a constitutive component of the nuclear pore complex-attached intranuclear filaments. *J. Cell Biol.* 136:515–529.
- Davis, L.I., and G. Blobel. 1986. Identification and characterization of a nuclear pore complex protein. *Cell.* 45:699–709.
- Dingwall, C. 1991. Transport across the nuclear envelope: enigmas and explanations. *Bioessays.* 13:213–218.
- Doxsey, S.J., P. Stein, L. Evans, P. Calarco, and M.W. Kirschner. 1994. Pericentriolar, a highly conserved centrosome protein involved in microtubule organization. *Cell.* 76:639–650.
- Doye, V., and E.C. Hurt. 1995. Genetic approaches to nuclear pore structure and function. *Trends Genet.* 11:235–241.
- Doye, V., and E.C. Hurt. 1997. From nucleoporins to nuclear pore complexes. *Curr. Opin. Cell Biol.* 9:401–411.
- 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.
- Fornerod, M., J. Boer, S. van Baal, M. Jaegle, M. von Lindern, K.G. Murty, 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.
- Fornerod, M., M. Ohno, M. Yoshida, and I.W. Mattaj. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell.* 90:1051–1060.
- Goldberg, M.W., and T.D. Allen. 1995. Structural and functional organization of the nuclear envelope. *Curr. Opin. Cell Biol.* 7:301–309.
- Gorlich, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, and S. Prehn. 1995. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.* 5:383–392.
- Gorlich, D., and I.W. Mattaj. 1996. Nucleocytoplasmic transport. *Science.* 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.
- Greco, A., M.A. Pierotti, I. Bongarzone, S. Pagliardini, C. Lanzi, and G. Della Porta. 1992. TRK-T1 is a novel oncogene formed by the fusion of TPR and TRK genes in human papillary thyroid carcinomas. *Oncogene.* 7:237–242.
- 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.
- Hu, T., T. Guan, and L. Gerace. 1996. Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins. *J. Cell Biol.* 134:589–601.
- 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.
- Iovine, M.K., J.L. Watkins, and S.R. Wente. 1995. The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. *J. Cell Biol.* 131:1699–1713.
- Jarnik, M., and U. Aebi. 1991. Toward a more complete 3-D structure of the nuclear pore complex. *J. Struct. Biol.* 107:291–308.
- Kenna, M.A., J.G. Petranka, J.L. Reilly, and L.I. Davis. 1996. Yeast N1e3p/Nup170p is required for normal stoichiometry of FG nucleoporins within the nuclear pore complex. *Mol. Cell Biol.* 16:2025–2036.
- Kutay, U., E. Izaurralde, F.R. Bischoff, I.W. Mattaj, and D. Gorlich. 1997. Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:1153–1163.
- Kyhse-Andersen, J. 1984. Electrophoretic transfer of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods.* 10:203–209.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.
- Melchior, F., T. Guan, N. Yokoyama, T. Nishimoto, and L. Gerace. 1995. GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. *J. Cell Biol.* 131:571–581.
- Mitchell, P.J., and C.S. Cooper. 1992. The human tpr gene encodes a protein of 2094 amino acids that has extensive coiled-coil regions and an acidic C-terminal domain. *Oncogene.* 7:2329–2333.
- Moroianu, J., M. Hijikata, G. Blobel, and A. Radu. 1995. Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92:6532–6536.
- Nehrbass, U., M.P. Rout, S. Maguire, G. Blobel, and R.W. Wozniak. 1996. The yeast nucleoporin Nup188p interacts genetically and physically with the core structures of the nuclear pore complex. *J. Cell Biol.* 133:1153–1162.
- Newmeyer, D.D., and D.J. Forbes. 1988. Nuclear import can be separated into distinct steps *in vitro*: nuclear pore binding and translocation. *Cell.* 52:641–653.

- Panté, N., and U. Aebi. 1994. Toward the molecular details of the nuclear pore complex. *J. Struct. Biol.* 113:179–189.
- 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.
- Panté, N., and U. Aebi. 1995. Toward a molecular understanding of the structure and function of the nuclear pore complex. *Int. Rev. Cytol.* 126B:225–255.
- Panté, N., and U. Aebi. 1996. Toward the molecular dissection of protein import into nuclei. *Curr. Opin. Cell Biol.* 8:397–406.
- Park, M., M. Dean, C.S. Cooper, M. Schmidt, S.J. O'Brien, D.G. Blair, and G.F. Van de Woude. 1986. Mechanism of met oncogene activation. *Cell.* 45:895–904.
- 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.
- Pemberton, L.F., J.S. Rosenblum, and G. Blobel. 1997. A distinct and parallel pathway for the nuclear import of an mRNA-binding protein. *J. Cell Biol.* 139:1645–1653.
- Picard, D., and K.R. Yamamoto. 1987. Two signals mediate hormone-dependent nuclear localization of the gluco-corticoid receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3333–3340.
- Pollard, V.W., W.M. Michael, S. Nakielny, N.C. Siomi, F. Wang, and G. Dreyfuss. 1996. A novel receptor-mediated nuclear protein import pathway. *Cell.* 86:985–994.
- 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.
- 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 nuclear pores. *Cell.* 52:655–664.
- Ris, H. 1991. The three dimensional structure of the nuclear pore complex as seen by high voltage electron microscopy and high resolution low voltage scanning electron microscopy. *EMSA Bulletin.* 21:54–56.
- Ris, H., and M. Malecki. 1993. High-resolution field emission scanning electron microscope imaging of internal cell structures after Epon extraction from sections: a new approach to correlative ultrastructural and immunocytochemical studies. *J. Struct. Biol.* 111:148–157.
- Rodrigues, G.A., and M. Park. 1993. Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. *Mol. Cell Biol.* 13:6711–6722.
- Rosenblum, J.S., L.F. Pemberton, and G. Blobel. 1997. A nuclear import pathway for a protein involved in tRNA maturation. *J. Cell Biol.* 139:1655–1661.
- Rout, M.P., G. Blobel, and J.D. Aitchison. 1997. A distinct nuclear import pathway used by ribosomal proteins. *Cell.* 89:715–725.
- Shah, S., S. Tugendreich, and D. Forbes. 1998. Major binding sites for the nuclear import receptor are the internal nucleoporin nup153 and the adjacent nuclear filament protein Tpr. *J. Cell Biol.* 141:31–49.
- Siomi, M.C., P.S. Eder, N. Kataoka, L. Wan, Q. Liu, and G. Dreyfuss. 1997. Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. *J. Cell Biol.* 138:1181–1192.
- Snow, C.M., A. Senior, and L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J. Cell Biol.* 104:1143–1156.
- Soman, N.R., P. Correa, B.A. Ruiz, and G.N. Wogan. 1991. The TPR-MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor lesions. *Proc. Natl. Acad. Sci. USA.* 88:4892–4896.
- Stade, K., C.S. Ford, C. Guthrie, and K. Weis. 1997. Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell.* 90:1041–1050.
- Starr, C.M., M. D'Onofrio, M.K. Park, and J.A. Hanover. 1990. Primary sequence and heterologous expression of nuclear pore glycoprotein p62. *J. Cell Biol.* 110:1861–1871.
- 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.
- Zabel, U., V. Doye, H. Tekotte, R. Wepf, P. Grandi, and E.C. Hurt. 1996. Nic96p is required for nuclear pore formation and functionally interacts with a novel nucleoporin, Nup188p. *J. Cell Biol.* 133:1141–1152.
- Zimowska, G., J.P. Aris, and M.R. Paddy. 1997. A *Drosophila* Tpr protein homolog is localized both in the extrachromosomal channel network and to nuclear pore complexes. *J. Cell Sci.* 110:927–944.