Characterization of the Nuclear Export Signal of Polypyrimidine Tract-binding Protein*

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The poly(pyrimidin) tract-binding protein (PTB) is a nuclear protein that regulates alternative splicing. In addition, it plays a role in the cytoplasm during infection by some viruses and functions as a positive effector of hepatitis B virus RNA export. Thus, it presumably contains a nuclear export signal (NES). Using a heterokaryon export assay in transfected cultured cells, we have shown that the N-terminal 25 amino acid residues of PTB function as an autonomous NES, with residues 11–16 being important for its activity. Unlike the heteronuclear ribonucleoprotein A1 NES, this NES is separable from the nuclear localization signal, which spans the entire N-terminal 60 residues of PTB. The PTB NES cannot be shown to bind to CAS or Crm1, cellular receptors known to export proteins from the nucleus, and it functions in the presence of leptomycin B, a specific inhibitor of Crm1-dependent export. PTB deleted of its NES, unlike wild type PTB, does not stimulate the export of hepatitis B virus RNA. Therefore, the PTB NES is a functionally important domain of this multifunctional protein that utilizes an unknown export receptor.

The presence of a nucleus physically separated from the remainder of the cell by the nuclear envelope is the defining characteristic of eukaryotes. This compartmentalization is thought to allow greater cellular complexity. However, it also presents a challenge in requiring the controlled trafficking of various macromolecules between the nucleus and cytoplasm. All such movement takes place through nuclear pores that are protein-lined aqueous channels penetrating the double membranes of the nuclear envelope (1–4). Because of the size of the pores, large macromolecules must be actively transported through them, and even many small macromolecules appear to cross the nuclear envelope by active transport rather than simple diffusion.

In recent years the molecular details of the import and export processes have been partly elucidated. It is now clear that most cargoes (proteins and RNA) are ferried across nuclear pores by a family of receptor proteins (for simplicity hereon collectively called the transportins, although many other names are used in the literature, including importins, exportins, karyopherins, and Ran-binding proteins) (see Refs. 1–4, and references therein). These receptors bind to their cargoes via motifs on the cargo proteins known as nuclear localization signals (NLS) or nuclear export signals (NES). These receptors also interact with components of the nuclear pore complex, thereby accounting for their ability to carry cargoes across the pore. To provide directionality to cargo movement, transportins also bind to a small GTPase called Ran. Because of the cytosolic localization of proteins that activate the GTPase activity of Ran but the nuclear localization of proteins that stimulate exchange of GDP for GTP on Ran, Ran is thought to exist largely in the GDP-bound state in the cytosol but in the GTP-bound state in the nucleus. Export receptors such as Crm1 bind to their cargoes only as a ternary complex with RanGTP, i.e. in the nucleus. When the complex reaches the cytosol, the presence of GTPase stimulating factors causes the conversion of RanGTP to RanGDP and the subsequent release of both cargo and Crm1 from Ran. The transportin-type receptors are responsible for the import and export of most RNA and protein molecules in the cell. However, a few non-transportin family proteins apparently interact directly with nuclear pore proteins to cross the nuclear envelope. Examples include NTF2, which reimports RanGDP into the nucleus (5, 6), and TAP, which exports simian retroviral mRNA and probably other cargoes out of the nucleus (7).

The export receptors responsible for the nuclear export of some proteins are now known. Most are exported by Crm1 (1, 2), which recognizes a small leucine-rich NES such as the one present in the human immunodeficiency virus type 1 (HIV 1) Rev protein (8–11), although at least one protein with a large NES spread over 150 residues also binds to Crm1 (12). Another receptor known to export proteins is CAS, which is a transportin family member that binds to a large NES in the importin α family of proteins (13, 14). Thus, the export receptors for many other proteins, including hnRNP proteins and SR proteins, remain unknown, although NES have been localized in some of these proteins.

The poly(pyrimidin) tract-binding protein (PTB, also called hnRNP I) is an RNA-binding protein that is involved in regulation of splicing and is nuclear in localization at steady state (15). However, it also appears to be an accessory factor in the cap-independent translation of some RNA viruses (16), which takes place in the cytoplasm. PTB has been shown to shuttle rapidly between the nucleus and cytoplasm and thus has also been postulated to be involved in mRNA export (17). Indeed, we have shown that PTB binds to the RNA export element of hepatitis B virus (HBV) and acts as a positive effector of HBV...
RNA export (18). However, because PTB shows avid RNA binding, it is also possible that PTB exits the nucleus passively by “piggy-backing” on RNA molecules that are exported by other protein factors. To resolve this issue, we have analyzed PTB to look for a putative NES. Our results show that PTB has an autonomous NES distinct from its RNA binding domains. This NES is relatively short and contains two critical basic residues. Yeast two-hybrid experiments suggest that the PTB NES does not bind to either Crm1 or CAS, two transportin family members known to export proteins from the nucleus, nor is its NES function blocked by leptomyxin B, a specific inhibitor of Crm1-mediated export. Deletion of the NES renders PTB incapable of activating the export of HIV RNA. Therefore, the PTB NES is a functionally important domain of PTB that apparently utilizes an export receptor other than Crm1 or CAS.

MATERIALS AND METHODS

Construction of Eukaryotic PTB Expression Plasmids—The human PTB cDNA plasmid pG15 was kindly provided by P. Sharp and P. McCaw (Massachusetts Institute of Technology, Cambridge, MA) (19). The Xenopus laevis nucleoplasmin cDNA plasmid was kindly provided by I. Mattaj and L. Englemier (European Molecular Biology Laboratory, Heidelberg, Germany). PTB codons 1–60 were made by PCR amplification of pG15 to generate a 208-bp DNA fragment, which had a NcoI site at the start codon and a XhoI site right after the 60th codon, using primers PTB001 and PTB002 (see Table I for primer sequences). The nucleoplasmin core region (NPC) was made by PCR amplification of the nucleoplasmin cDNA with a XhoI site just before the 2nd codon and a NolI site right after the 150th codon (primers NPC001 and NPC002, respectively). The PTB1–60 fragment digested with Ncol and XhoI and the NPC fragment digested with XhoI and NolI were simultaneously subcloned between the Ncol and NolI sites of pcMV/myc/cyto (Invitrogen) to make pmCV/PTB1–60/NPC/myc. The DNA encoding the SV40 large T antigen NLS (amino acid sequence PKKKRKVEDP) was made by annealing with codon-optimized oligonucleotides with a XhoI site at an end and a NotI site at the other end (oligonucleotides NLS001 and NLS002). This DNA fragment was digested with XhoI and NolI and subcloned between the Ncol and NolI sites of pcMV/pcMV/myc/cyto to make pcMV/NLNS/myc. A NPC DNA fragment with a SalI site just before the 2nd codon and a XhoI site right after the 150th codon was amplified by PCR from the cDNA clone using primers NPC003 and NPC004, digested with SalI and XhoI, and subcloned between the SalI and XhoI sites of pcMV/NPC/myc to make pcMV/NPC/myc/myc. To fuse portions of PTB with the NPC coding region, PTB cDNA fragments were generated by PCR from pcG15, digested with Ncol and SalI, and subcloned between the Ncol and SalI sites of pcMV/NPC/myc/myc. Primers used were as follows: for PTB codons 1–25, PTB001 and PTB009; for PTB codons 18–42, PTB007 and PTB008; for PTB codons 35–60, PTB005 and PTB006, for codons PTB1–20, CMV001 and PTB015; for PTB codons 1–15, CMV001 and PTB016; for PTB codons 5–25, PTB013 and NPC004; for PTB codons 10–25, PTB014 and NPC004.

Site-directed mutagenesis of pmCV/PTB1–25/NPC/myc was done with the PCR-based QuikChange kit from Stratagene following the protocol in the instruction manual. Primers used were as follows: for M1, PTB025 and PTB026; for M2, PTB027 and PTB028; for M3, PTB029 and PTB030; for M4, PTB031 and PTB032; for M5, PTB033 and PTB034; for M6, PTB035 and PTB036; for M7, PTB037 and PTB038; for M8, PTB039 and PTB040; for T12A, PTB041 and PTB042; for K13A, PTB043 and PTB044; for R14A, PTB045 and PTB046; for S16A, PTB047 and PTB048; for G11A, PTB050 and PTB051; for G15A, PTB052 and PTB053; for T12D, PTB054 and PTB055; for S16D, PTB056 and PTB057.

The plasmid pmCV-PTB/myc-cyto expresses human PTB with a Myc epitope tag at the C terminus and was constructed using the PCR method to generate the entire human PTB cDNA and inserting this fragment between the XhoI and NolI sites of the plasmid pmCV/myc-cyto (Invitrogen). The plasmid pmCV-PTB/NE5/myc-cyto expresses PTB with codons 2–25 deleted. The partial PTB cDNA was obtained by PCR amplification of pG15 with primers PTB058 and PTB061. The PCR product was digested with XhoI and cloned into the XhoI site of pcMV/NLNS/myc. All of the PCR-generated regions of the above plasmids were sequenced at the University of California San Francisco Biomolecular Resource Center, to confirm the lack of unintended mutations.

Construction of Yeast Two-hybrid Plasmids—The bait and prey plasmids (BTM116 and pVP16, respectively) (20) were kindly provided by S. Hollenberg (Oregon Health Sciences University) via E. Huang (University of California, San Francisco, CA). PTB codons 1–30 were made by PCR amplification of pG15 with primers PTB023 and PTB024. This fragment was digested with BamHI and BgII and subcloned into the EcoRI and HindIII sites of BTM116 to make pBTM116–30. Construction of the pBTM116–30 plasmid was confirmed by restriction enzyme digestion. Full-length PTB was amplified by PCR with pG15 as a template and primers PTB062 and PTB063. The PCR product was digested with EcoRI and SalI and cloned into pBTM116 to make plexPTB–FL. Two complementary oligonucleotides PKI01 and PKI02, which encode the protein kinase inhibitor NES (21), were annealed to each other and cloned between the EcoRI and SalI sites of pBTM116 to make plexNES. The human importin α cDNA plasmid pRS7T-hSRP1α was kindly provided by K. Weis (University of California, Berkeley, CA) (22). The whole coding region of hSRP1α was amplified from this plasmid by PCR using primers SRP01 and SRP02. This DNA fragment was digested with BglII and BgII and subcloned into the BamHI site in BTM116 to make plexSRP; orientation was confirmed by restriction enzyme digestion.

All of the PC-generated regions of the above plasmids were sequenced at the University of California San Francisco Biomolecular Resource Center, to confirm the lack of unintended mutations.

HBV RNA Export Assay—The chloramphenicol acetyltransferase (CAT) expression plasmid pDM138 was kindly provided by T. Parslow (University of California, San Francisco, CA) and contains the SV40 enhancer early promoter driving the expression of a chimeric CAT/HBV-1 mRNA that is not highly expressed unless an export element is present within the transcribed region (24). The plasmid pDM138-PRE contains the entire HBV export element (PRE) inserted downstream of the CAT gene in the transcribed region of pDM138 (25). HuH-7 human hepatoma cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection of HuH-7 cells was done with the calcium phosphate method in 60-mm dishes (5 Prime M1002, Boulder, CO) (26). Each dish was transfected with 2.5 μg of the CAT plasmid and 1.5 μg of the PTB-expressing plasmid or the empty vector. Two days after transfection, the cells were harvested for CAT assay, as described previously (25).

Nuclear Export Assays—To look for nuclear export of PTB derivatives, the human-mouse heterokaryon assay was used (17, 27). HuH-7 human hepatoma cells and mouse NIH 3T3 cells were separately cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection of HuH-7 cells was done with the calcium phosphate method in 60-mm dishes (5 Prime → 3 Prime, Inc.) (26). At 30 h after transfection, the transfected HuH-7 cells were mixed with an equal number of NIH 3T3 cells and seeded onto a two-well chamber slide. After overnight incubation, 50 μg/ml cycloheximide was added to the culture medium to inhibit protein synthesis. After 45 min, the cocultured cells were fused with 50% polyethylene glycol 8000 in serum-free medium for 1 min (27), washed once with medium containing 50 μg/ml cycloheximide, and incubated in medium containing 50 μg/ml cycloheximide for another 1 h before fixation and immunostaining. The effect of a mutant Ran-binding protein 1 (RanBP1) with a mutated NES on export mediated by the PTB NES was determined by co-transfection, as described previously (18, 48). Because a small but significant portion of Rev can be present in the cytoplasm at steady state (28), the heterokaryon assay is not suitable for examining nuclear export of Rev. Instead, we examined the subcellular localization of Rev following actinomycin D treatment, which decreases the relative rate of Rev import versus export and hence causes Rev to localize to the cytoplasm at steady state (29, 30). Briefly, HuH-7 cells were transfected with pMDM121–30 (courtesy of T. Parslow), which expresses HIV-1 Rev (24). After 2 days, the cells were treated with 50 μg/ml cycloheximide for 45 min, followed by 1 μg/ml actinomycin D for 1 h and processed for immunostaining. In some experiments, 2.5 ng/ml leptomycin B was added 1 h before addition of cycloheximide.

 Immunofluorescence Microscopy—The cells were fixed and permeabilized with 100% methanol for 15 min. Immunostaining with primary
antibody was done by incubating the cells with a mouse anti-Myc antibody (Invitrogen) at 1:100 dilution and a rabbit anti-/H9252-α-galactosidase antibody (5 Prime 3 Prime, Inc.) at 1:250 dilution in Dulbecco's phosphate-buffered saline (PBS) for 1 h. After washing with PBS, the secondary antibody staining was done by incubating the cells with PBS containing FITC-labeled goat anti-mouse antibody (Sigma) at 1:25 di-

| Name       | Sequence (5' to 3')                     |
|------------|----------------------------------------|
| CAS01      | CGCGGCGGCCGCTGGAACTCAGCGATGCAAATC     |
| CAS02      | CGCGGCGGCCGAACGATCTGCACTGTGGCGC       |
| CMV001     | ATAGGGTTCGCGCACATTTCCCCC              |
| NLS001     | CGCGCTCGAGCCAAAGAAAGAGAAGAGACCCCG     |
| NLS002     | CGCGGCGCCGCGGCTTCACTTTTCTCTCTTTTGCTGAGCGCG |
| NSF01      | CGCGGCGGCGGAGTAAGCACGCTGGAGTGCGTTACAAAGCGGGTATTTTTCACAGCAGGATGAGCG |
| NSF02      | TGCAGGGATGCTCCTACAGTATGTTATTTTTAAAGCAGGATGAGCG |
| NSF03      | GCTTATCGTCACGTCACTGTGCTATAGGTGAGCGCAAC |
| NSF04      | GCTTAAGGATACACTGATGACCGACTCCGCGCGC |
| NPC001     | CGCGCTCGAGCCGCTTACAGTGAACGCGCGGACGC |
| NPC002     | CGCGGCGGCGCTGGAGATTCTTGATCTTCGCGCGG |
| NPC003     | CGCGGCGGCGCTGGAAGGAATTTTGGATTTTTCT |
| NPC004     | CGCGGCGGCGCTGGAAGGAATTTTGGATTTTTCT |
| PKI01      | AAATTCAATGAATTGCTTGAATTAGCACGCTTTGATATGCTAATTCAAGAGACGG |
| PKI02      | TCGAGCATTGCTCCTGATAACAGCTACCGCTACTTACG |

| Name       | Sequence (5' to 3')                     |
|------------|----------------------------------------|
| PTB001     | CGGTGTCGCTGTGCGCAGCT                          |
| PTB002     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB005     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB006     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB007     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB008     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB009     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB010     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB011     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB012     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB013     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB014     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB015     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB016     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB017     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB018     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB019     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB020     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB021     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB022     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB023     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB024     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB025     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB026     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB027     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB028     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB029     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB030     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB031     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB032     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB033     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB034     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB035     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB036     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB037     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB038     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB039     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB040     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB041     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB042     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB043     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB044     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB045     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB046     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB047     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB048     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB049     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB050     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB051     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB052     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB053     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB054     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB055     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB056     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB057     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB058     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB059     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB060     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB061     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB062     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| PTB063     | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| SRN01      | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| SRN02      | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| XP001      | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |
| XP002      | CGCGGCTCGAGCCGCTGCTGCGGAGGAGG               |

antibody was done by incubating the cells with a mouse anti-Myc antibody (Invitrogen) at 1:100 dilution and a rabbit anti-β-galactosidase antibody (5 Prime → 3 Prime, Inc.) at 1:250 dilution in Dulbecco's phosphate-buffered saline (PBS) for 1 h. After washing with PBS, the secondary antibody staining was done by incubating the cells with PBS containing FITC-labeled goat anti-mouse antibody (Sigma) at 1:25 di-
lution, Cy3-labeled sheep anti-rabbit antibody (Sigma) at 1:50 dilution, and PBS-saturated 4,6-diamidino-2-phenylindole (DAPI) at 1:100 dilution for 1 h. The stained cells were washed, coverslipped, and visualized with a Zeiss epifluorescence microscope. Staining for Rev was performed in the identical manner with a mouse anti-Rev antibody (Intracel Corp.).

**Two-hybrid Assay—** Yeast transformation of strain L40 was done with the CLONTECH Yeast Transformation System following the protocol provided. Filter-lift β-galactosidase assay and liquid β-galactosidase assay using o-nitrophenylβ-galactoside were performed using standard protocols (30).

**RESULTS**

**Autonomous NES in PTB Distinct from Its NLS—** It has been previously reported that, in a heterokaryon assay, PTB is rapidly exported from the nucleus to the cytoplasm (17), and we have confirmed this observation (18). However, because PTB binds RNA, it remained possible that PTB was leaving the nucleus by “piggy-backing” on RNA molecules being exported by other factors. To confirm that PTB has an autonomous NES, we utilized this assay to determine whether PTB without its RNA binding domains is a shuttle protein.

The RNA binding domains of PTB have been mapped to be downstream of amino acid residue 60 (31, 32). We therefore fused the first 60 codons of the human PTB cDNA to the cDNA coding for NPc, which forms a large pentamer in the nucleus and hence cannot leave the nucleus without an exogenous NES (27). The fusion gene also codes for the Myc epitope tag at the C terminus, to facilitate visualization of the expressed chimeric protein with fluorescence microscopy; it should be noted that all other chimeric proteins used in this study also contain this epitope. The fusion gene was placed under the control of the CMV promoter in a mammalian expression plasmid, which was transfected into HuH-7 human hepatoma cells. After 2 days, the cells were fixed and stained for the chimeric protein by immunofluorescence and for DNA with DAPI. Most of the human cells showed no immunostaining; because only a minority of cells are transfected with our protocol. Scattered cells showed staining that was restricted to the nucleus (see example in Fig. 1A; compare with nuclear staining in Fig. 1B), confirming previous data showing that the N-terminal 60 residues of PTB comprise its NLS (32, 33). The experiment was then repeated, but, before being fixed, the cells were pre-treated with cycloheximide, fused to murine 3T3 cells, and then incubated for 1 h in the presence of cycloheximide. Immunofluorescence microscopy revealed staining not only of human nuclei but also of murine nuclei, identified by the presence of distinctive nuclear granules (Fig. 1, C and D, arrow identifies one murine nucleus)/(27, 34, 35). Heterokaryon formation was confirmed by staining for β-galactosidase (Fig. 1E), which was expressed from a plasmid co-transfected into the human cells. The human nuclei from the cell(s) that had been transfected could be identified by the presence of more intense staining for the chimeric protein (Fig. 1C, chevrons; note the lack of nuclear granules in Fig. 1D). Because the murine cells were not transfected with any plasmids, the only manner by which their nuclei could contain the chimeric protein is by the protein being rapidly exported out of the transfected human nuclei and then imported into the murine nuclei of heterokaryons. As negative control, we used NPc fused to the SV40 T antigen NLS. In this case, scattered human nuclei showed staining for the chimeric protein (chevrons, Fig. 1, F and G), whereas no staining was seen in any murine nuclei. The formation of heterokaryons was confirmed by the presence of cytoplasmic β-galactosidase expressed from a co-transfected plasmid (Fig. 1F). The lack of export was not caused by interference from the T antigen NLS, because we could demonstrate export of another chimera comprising the N-terminal 60 residues of PTB, NPc, and the T

![Fig. 1. Nuclear import and export mediated by PTB residues 1–60.](image-url)
antigen NLS (Fig. 1, I–K). Therefore, the N-terminal 60 residues of PTB can function as both NLS and NES.

The M9 NES of hnrNPA1 shows both NLS and NES activity, which cannot be separated from each other by extensive mutagenesis or deletion analysis (27, 36). To determine whether such is the case with the PTB NES, we constructed plasmids that expressed smaller fragments of the PTB N-terminal region fused to Npc and T antigen NLS. As seen in Fig. 2 (C–E) and Table II, just the N-terminal 25 residues of PTB retained full function as NES. It should be noted that this chimeric protein, because of the presence of the T antigen NLS, is localized to the nucleus in transfected cells (Fig. 2A, compare with nuclear staining in Fig. 2B), thus ruling out import of cytosolic protein into the murine nuclei without export from the human nucleus.

A previous paper showed that the entire N-terminal 60 residues of PTB are needed for NLS activity (33). This conclusion is confirmed by our observation that a chimeric protein comprising just the N-terminal 25 residues of PTB and Npc, without the T antigen NLS, is localized to the cytoplasm (Fig. 2F, contrast with nuclear staining in Fig. 2G), unlike the comparable chimera comprising all 60 residues at the N terminus (Fig. 1A). Therefore, the NES of PTB is separable from the NLS, although it is entirely contained within the NLS.

Mapping of the PTB NES—The result shown in Fig. 2C indicated that the N-terminal 25 residues function as NES, but further experiments showed that residues 18–42 and 35–60 (together with the initiator methionine residue) cannot (Fig. 3, A–F and Table II). Therefore, the NES activity of PTB appears to reside solely within the N-terminal 25 residues. To determine whether even smaller fragments have export activity, we looked for export mediated by residues 1–20 or 1–15 of PTB. These experiments revealed that neither fragment has NES activity (Table II). Therefore, the C-terminal boundary of the NES is between residues 21 and 25. Smaller fragments of PTB missing the extreme N-terminal region (other than the initiating methionine) were also tested for export activity. As seen in Table II, residues 5–25 and 10–25 both showed moderate export activity. In contrast, residues 18–42 showed no detectable activity (Table II). Therefore, residues between positions 10 and 17 are critical for NES function, whereas residues 2–9 play an accessory role. It should be noted that all of these chimeric proteins are localized to the nucleus in transfected cells (data not shown), thus ruling out import of cytosolic protein into the murine nuclei without export from the human nucleus.

To map the PTB NES in more detail, we substituted clusters of 3 residues from positions 2–25 with alanine (Table III) in the context of PTB residues 1–25 fused to Npc and T antigen NLS, to generate mutants M1–M8. As seen in Table III, most of the mutations had no significant impact on export activity. However, M4 and M5, which affected residues 11–13 and 14–16, respectively, showed no export activity. Therefore, the central 6 residues of the PTB N terminus are critical for NES function. These results correlate well with the deletional analysis in Table II, although the latter also indicated a function for residues 17–25.

To dissect further the core PTB NES (residues 11–16), individual residues in this region were mutated to alanine, and the mutants tested for export activity. As shown in Table III, the lysine 13 residue and the arginine 14 residue are both important for NES function, whereas the threonine 12 residue and serine 16 residue are unimportant. The two glycine residues at positions 11 and 15 are of intermediate importance. These results point to a critical role for the 2 basic residues, as well as some function of the glycine residues, in the export of PTB.

No Interaction of PTB NES with Known Export Receptors—We have previously shown that the export of full-length

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**TABLE II**

Deletional analysis of PTB NES using heterokaryon assay

| Chimeric protein tested | Export activity |
|------------------------|----------------|
| PTB1–60/Npc/myc        | ++             |
| PTB1–25/Npc/NLS/myc    | ++             |
| PTB18–42/Npc/NLS/myc   | –              |
| PTB35–60/Npc-NLS/myc   | –              |
| PTB1–20/Npc-NLS/myc    | –              |
| PTB1–15/Npc-NLS/myc    | –              |
| PTB5–25/Npc-NLS/myc    | +              |
| PTB10–25/Npc-NLS/myc   | +              |

* The numbers indicate the residues of PTB present in the chimera.

*+*, >50% of heterokaryons show export; *, 20–40% of heterokaryons show export.
PTB is blocked by a mutant RanBP1 that is missing its NES and hence is present in both the cytoplasm and nucleus (18). This mutant RanBP1 also blocks the export of Rev (44, 48).

Because RanBP1 is an accessory factor for the hydrolysis of RanGTP to RanGDP, this result is evidence that the PTB NES, like the leucine-rich NES of Rev, depends on high levels of RanGTP in the nucleus for export function and hence probably utilizes a transportin family member as export receptor (48). However, these data are subject to alternative interpretations, because full-length PTB is presumably bound to RNA and/or other cellular factors that may affect its export. Therefore, we repeated this experiment using PTB1–25/NPc/NLS/myc. In a human-mouse heterokaryon that expresses the mutant RanBP1 (Fig. 4A), this protein could not be exported out of the human nucleus, as revealed by the failure of the murine nucleus to show any staining with anti-Myc antibodies (Fig. 4B).

In other words, the export of this chimeric protein was blocked by the mutant RanBP1. Therefore, the PTB NES appears to utilize a transportin family member as export receptor.

Two members of the transportin family, Crm1 and CAS, are known to ferry cargo proteins out of the nucleus. Crm1 mediates the export of proteins containing a leucine-rich NES, the prototype of which is the HIV-1 Rev protein (8–11). The fungal metabolite leptomycin B specifically inhibits the formation of the ternary complex between Crm1, Ran-GTP, and the cargo protein and thus blocks Crm1-mediated export (37, 38). To determine whether Crm1 is the export receptor for PTB, we looked for export in the presence of leptomycin B (kind gift of M. Yoshida, Kyoto University, Kyoto, Japan) (39). We first confirmed that leptomycin B was functional in our cells, by looking at the subcellular distribution of HIV-1 Rev. As seen in Fig. 5A, Rev is mostly cytoplasmic in cells treated with actinomycin D, because this drug favors Rev export over import (28, 29). When the cells were also treated with leptomycin B, Rev was strictly nuclear, indicating that export of HIV-1 Rev was totally blocked (Fig. 5C, compare with nuclear staining in Fig. 5D). In contrast, leptomycin B did not block the export of the chimeric protein containing PTB residues 1–60 fused to NPc and T antigen NLS (Fig. 5, E and F). Therefore, Crm1 does not appear to interact with the PTB NES and mediate PTB export. As further confirmation of this inference, we used the yeast two-hybrid system with a β-galactosidase reporter gene to look for binding of PTB NES to Crm1. Using either a filter-lift assay or liquid β-galactosidase assay, no detectable β-galactosidase activity was observed with either the PTB NES alone or full-length PTB as bait and Crm1 as prey (Fig. 6, B and C, respectively), despite the fact that the fusion proteins between PTB and lexA can be demonstrated to be expressed well in the yeast cells by Western blotting (data not shown). In contrast, β-galactosidase activity was easily detected when the protein kinase inhibitor NES, which is a Rev-like NES that uses RanGTP to RanGDP, this result is evidence that the PTB NES, like the leucine-rich NES of Rev, depends on high levels of RanGTP in the nucleus for export function and hence probably utilizes a transportin family member as export receptor (48). However, these data are subject to alternative interpretations, because full-length PTB is presumably bound to RNA and/or other cellular factors that may affect its export. Therefore, we repeated this experiment using PTB1–25/NPc/NLS/myc. In a human-mouse heterokaryon that expresses the mutant RanBP1 (Fig. 4A), this protein could not be exported out of the human nucleus, as revealed by the failure of the murine nucleus to show any staining with anti-Myc antibodies (Fig. 4B).

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These data render it highly unlikely that Crm1 is the export factor for the PTB NES.

CAS is another transportin that is known to export the importin/H9251 family of proteins (13, 14). No specific inhibitors of this export pathway are available. However, CAS can be shown to bind to importin/H9251 in the yeast two-hybrid assay (40, 41). We therefore again used this assay to determine whether the PTB NES might bind to CAS. The amount of β-galactosidase activity in yeast cells was undetectable when CAS as prey was paired with either the PTB NES or full-length PTB as bait (Fig. 6, E and F, respectively), but, as expected, weak blue staining was seen when CAS was paired with importin α1 (Fig. 6D). Therefore, CAS also probably does not mediate PTB NES export.

Functional Importance of PTB NES—Our data thus far pin-point a domain of PTB that can function as NES in a heterologous context, but do not necessarily assign any functional importance to this domain in the intact PTB protein. The large fragment of PTB downstream of the NES is also exported from the nucleus to the cytoplasm (data not shown), presumably because of passive export via heteromultimerization with endogenous full-length PTB and/or “piggy-backing” on exported RNA. Thus, to determine more directly the functional role of the NES, we compared the ability of wild type and NES-deleted PTB to activate export of HBV mRNA. The HBV genome encodes a cis-acting RNA element called the posttranscriptional regulatory element (PRE) that is needed for efficient export of viral mRNA (25, 42). We have previously shown that PTB binds to the PRE and acts as a positive effector of PRE function (18). A facile method to measure this export is to use the reporter plasmid pDM138-PRE, which contains the CAT gene within a poorly utilized intron from the human immunodeficiency virus I (43, 44). Export of the unspliced CAT mRNA transcribed from this plasmid is inefficient, unless an export element such as the PRE is present within the 3′-untranslated region of the message (25, 43). We co-transfected HuH-7 cells with pDM138 or pDM138-PRE and a plasmid expressing either full-length PTB or PTB deleted of the NES (residues 2–25). As shown in Fig. 7A, co-transfection of full-length PTB resulted in a 3-fold increase in PRE activity, similar to our previous results using stably transfected cells (18), whereas co-transfection of NES-deleted...
PTB actually caused a 2-fold decrease in PRE activity. Failure to express the deleted PTB cannot explain this difference, because we could easily detect this protein in transfected cells (Fig. 7B). Indeed, the decrease in PRE activity caused by it suggested that the NES-deleted PTB can partially interfere with PTB function, probably by competing for binding to the PRE.

**DISCUSSION**

Our results demonstrate that PTB contains an autonomous NES that is localized at the N terminus of this protein and that overlaps with but is distinct from its NLS. The presence of this NES, together with the fact that PTB is found associated with exposed regions of hnRNA (31), is consistent with the suggestion that PTB may be involved in the export of cellular mRNA. This hypothesis is further strengthened by our finding that full-length PTB, but not PTB deleted of its NES, activates the export of HBV mRNA that contains PTB binding sites. In addition, Xenopus PTB has been implicated in targeting Vg1 mRNA to the vegetal hemisphere of the Xenopus oocyte (45). Therefore, PTB may also have a role in directing mRNA to specific regions of the cytoplasm.

The identity of the cellular receptor that mediates the export of the PTB NES is unknown. Crm1 is highly unlikely to be the receptor, because export mediated by the PTB NES is not sensitive to leptomycin B and because this NES does not bind to Crm1 in the yeast two-hybrid assay. This conclusion is further bolstered by the fact that the PTB NES shows no sequence similarity to the leucine-rich NES known to bind to Crm1 (46). CAS, the other transportin family protein known to export proteins, also does not appear to be the factor, because we could detect no binding between CAS and the PTB NES in the yeast two-hybrid assay. However, because this is a negative result, other interpretations are not entirely excluded. For example, it is possible that the NES in the fusion protein with LexA is not accessible for binding to CAS. We believe this is unlikely, however, because the NES in the fusion protein binds strongly to a translation elongation factor.2 Other NES whose export is mediated by unknown factors include those for hnRNP K and hnRNP A1 (27, 47). The PTB NES does not resemble in sequence either of these NES (27, 36, 47). Furthermore, the hnRNP A1 NES, known as M9, cannot be separated from its NLS, despite extensive mutagenesis studies (27, 36). Therefore, the PTB NES may represent a different class of NES from other known NES, although further experiments are needed to confirm this inference.

The PTB NES is small, with an active core sequence that is only 6 residues long, although the adjacent 9 residues on either side contribute to full activity. The exact role played by these adjacent residues is as yet unknown. Possibly, they provide the proper structural context for optimal function of the core sequence. In the core sequence, the most critical residues appear to be 2 basic residues, with 2 glycine residues also contributing to the export activity. Not surprisingly, these residues are entirely conserved among all PTB sequences in the data bases (Table III).

In summary, we have localized and begun to characterize the NES of PTB, which does not appear to be exported by known export receptors. Therefore, future studies on the factors responsible for export of PTB may shed new light on nuclear export pathways and mRNA export in mammalian cells.

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**REFERENCES**

1. Gorlich, D., and Kutay, U. (1999) *Annu. Rev. Cell Dev. Biol.* 15, 607–660
2. Nakanishi, S., and Dreyfuss, G. (1999) *Cell* 99, 677–690
3. Weis, K. (1998) *Trends Biochem. Sci.* 23, 185–189
4. Mattaj, I. W., and Englmeier, L. (1998) *Annu. Rev. Biochem.* 67, 265–306
5. Hille, K., Lipowsky, G., Kent, H. M., Stewart, M., and Gorlich, D. (1998) *EMBO J.* 17, 6587–6598
6. Smith, A., Brownwell, A., and Macara, I. G. (1999) *Curr. Biol.* 9, 1433–1436
7. Bachi, A., Braun, I. C., Rodrigues, J. P., Pante, N., Hille, K., von Kobbe, C., Kutay, U., Wilm, M., Gorlich, D., Carmo-Fonseca, M., and Izaurralde, E. (1999) *RNA* 5, 136–158
8. Neville, M., Stutz, F., Lee, D. L., and Rosbash, M. (1997) *Curr. Biol.* 7, 767–775
9. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997) *Cell* 90, 1041–1050
10. Pfeifer, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) *Cell* 90, 1051–1060
11. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) *Nature* 389, 308–311
12. Paraskeva, E., Izaurralde, E., Bischoff, F. R., Huber, J., Kutay, U., Hartmann, E., Luhrmann, R., and Gorlich, D. (1999) *J. Cell Biol.* 145, 255–264
13. Kutay, U., Bischoff, F. R., Kotzka, S., Kraft, R., and Gorlich, D. (1997) *Cell* 90, 1061–1071
14. Hood, J. K., and Silver, P. A. (1998) *J. Biol. Chem.* 273, 35142–35146
15. Valcarcel, J., and Gebauer, F. (1997) *Curr. Biol.* 7, R705–R708
16. Griesbeck, O., Chang, K. H., Rijnbrand, R., Yi, M., Sangar, D. V., and Lemon, S. M. (2000) *Cell Biol. Rep.* 18, 1538–1555
17. Michael, W. M., Siomi, H., Choi, M., Pinol-Roma, S., Nakanishi, S., Liu, Q., and Dreyfuss, G. (1998) *Cold Spring Harbor Symp. Quant. Biol.* 63, 663–668
18. Zhang, W. Q., Li, R., Hou, P. Y., Lai, M. M. C., and Yen, T. S. B. (2001) *J. Virol.* 75, 10779–10786
19. Gili, A., Sharp, P. A., Jamison, S. F., and Garcia-Blanco, M. A. (1991) *Genes Dev.* 5, 1224–1236
20. Hollenberg, S. M., Strunk, L., Cheng, P. F., and Weintraub, H. (1995) *Mol. Biol. Cell.* 15, 3813–3822
21. Wilt, W., Knuith, J. L., Tsien, R. Y., and Taylor, S. H. (1995) *Cell* 82, 463–473
22. Weis, K., Mattaj, I. W., and Lamond, A. I. (1995) *Science* 268, 1049–1053
23. Brinkmann, U., Brinkmann, E., Gallo, M., and Pastan, I. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 10447–10451
24. Hope, T. J., McDonald, D., Huang, X. J., Low, J., and Parslow, T. G. (1990) *J. Virol.* 64, 5360–5366
25. Huang, Z. M., and Yin, T. S. (1995) *Mol. Cell. Biol.* 15, 3864–3869
26. Gorman, C. (1985) in DNA Cloning (Glover, D. M., ed) Vol. 2, pp. 143–190, IRL, Oxford
27. Michael, W. M., Choi, M., and Dreyfuss, G. (1995) *Cell* 83, 415–422
28. Soros, V., and Ciechaczek, A. (2001) *Virology* 290, 199–210
29. Staub, R., Gaitanaris, G. A., and Pavlikas, G. N. (1995) *Virology* 213, 439–447
30. Golemis, E. A., Gyuri, J., and Brent, R. (1996) in Current Protocols in Molecular Biology (Ausubel, F. M., ed) pp. 13.6.2–13.6.6, John Wiley & Sons, Inc., New York
31. Ghiotti, A., Pinol-Roma, S., Michael, W. M., Morandi, C., and Dreyfuss, G. (1992) *Nucleic Acids Res.* 20, 3671–3678
32. Perez, I., McCaffee, J. G., and Patton, J. G. (1997) *Biochemistry* 36, 11881–11890
33. Romanelli, M. G., Weighardt, P., Biamonti, G., Riva, S., and Morandi, C. (1997) *Exp. Cell Res.* 235, 300–304
34. Fan, X. C., and Steitz, J. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 15295–15298
35. Donnelly, M., and Elliott, G. (2001) *J. Virol.* 75, 2566–2574

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2 B. Li and T. S. B. Yen, unpublished observations.
36. Bogerd, H. P., Benson, R. E., Truant, R., Herold, A., Phingbodhipakkiya, M., and Cullen, B. R. (1999) *J. Biol. Chem.* **274**, 9771–9777
37. Kudo, N., Matsumori, N., Takata, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9112–9117
38. Wolff, B., Sanglier, J. J., and Wang, Y. (1997) *Chem. Biol.* **4**, 139–147
39. Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S., and Beppu, T. (1994) *J. Biol. Chem.* **269**, 6320–6324
40. Herold, A., Truant, R., Wiegand, H., and Cullen, B. R. (1998) *J. Cell Biol.* **143**, 309–318
41. Kunaler, M., and Hurt, E. C. (1998) *FEBS Lett.* **433**, 185–190
42. Huang, Z. M., and Yen, T. S. (1994) *J. Virol.* **68**, 3193–3199
43. Huang, Z. M., Zang, W. Q., and Yen, T. S. (1996) *Virology* **217**, 573–581
44. Zang, W. Q., and Yen, T. S. B. (1999) *Virology* **259**, 299–304
45. Cote, C. A., Gautreau, D., Denegre, J. M., Kress, T. L., Terry, N. A., and Mowry, K. L. (1999) *Mol. Cell.* **4**, 431–437
46. Bogerd, H. P., Fridell, R. A., Benson, R. E., Hua, J., and Cullen, B. R. (1996) *Mol. Cell. Biol.* **16**, 4297–4324
47. Michael, W. M., Eder, P. S., and Dreyfuss, G. (1997) *EMBO J.* **16**, 3587–3598
48. Zolotukhin, A. S., and Pelber, B. K. (1997) *J. Biol. Chem.* **272**, 11556–11560