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Two Closely Related Human Nuclear Export Factors Utilize Entirely Distinct Export Pathways

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Summary

Nuclear mRNA export mediated by the human protein TAP requires a carboxy-terminal domain that directly interacts with components of the nuclear pore complex. Here we demonstrate that NXF3, a human RNA binding protein related to TAP, lacks this domain yet retains the ability to export tethered RNA transcripts and to shuttle between the nucleus and the cytoplasm. NXF3 contains a novel Crm1-dependent nuclear export signal that compensates in cis for the loss of the nuclear pore targeting domain. NXF3-dependent RNA export is therefore blocked by Crm1-specific inhibitors that do not affect TAP function. Thus, while the related TAP and NXF3 proteins are both capable of mediating nuclear RNA export, they do so via unrelated export pathways.

Introduction

The constitutive transport element (CTE) is an RNA structure found in the retrovirus Mason-Pfizer monkey virus (MPMV) that induces the nuclear export of incompletely spliced MPMV transcripts when present in *cis* (Bray et al., 1994; reviewed by Cullen, 2000). The search for cellular factors able to bind the CTE specifically led to the identification of the TAP protein as a candidate CTE-dependent nuclear mRNA export factor (Grüter et al., 1998). This hypothesis was validated by data showing that expression of human TAP not only enhances CTE function in microinjected *Xenopus* oocytes (Grüter et al., 1998) but also rescues MPMV CTE function in otherwise nonpermissive quail cells (Kang and Cullen, 1999).

Important functional domains within the 619 amino acid (aa) TAP protein, also referred to as NXF1, include a centrally located CTE binding domain and a carboxy-terminal domain that mediates the binding of TAP to components of the nuclear pore complex (NPC), termed nucleoporins (NUPs) (Kang and Cullen, 1999; Katahira et al., 1999; Bachi et al., 2000; Kang et al., 2000). The ability of TAP to directly target the NPC, without the intercession of a member of the importin/exportin family of nuclear transport factors, is highly unusual and distinguishes TAP from Rev, a protein encoded by human immunodeficiency virus type 1 (HIV-1) that induces the nuclear export of incompletely spliced HIV-1 transcripts (Malim et al., 1989). Rev contains a short, leucine-rich nuclear export signal (NES) that recruits the exportin Crm1. Crm1 in turn targets Rev and its associated viral mRNA cargo to the NPC and, hence, to the cytoplasm (Fischer et al., 1995; Fornerod et al., 1997a; Neville et al., 1997; Stade et al., 1997).

Specific inhibition of Crm1 function blocks the Rev-dependent export of HIV-1 transcripts but does not affect CTE function or bulk cellular mRNA export (Fischer et al., 1995; Fornerod et al., 1997a; Bogerd et al., 1998). In contrast, inhibition of TAP function by microinjection of saturating levels of CTE RNA specifically blocks not only CTE-dependent but also cellular mRNA export, although Crm1-dependent RNA export is unaffected (Pasquinelli et al., 1997; Saavedra et al., 1997). Both CTE-dependent and cellular mRNA export can be rescued by coinjection of recombinant TAP, thus implicating TAP as a key factor in the sequence-nonspecific export of cellular mRNAs from the nucleus (Grüter et al., 1998). However, it currently remains unclear how the TAP protein is recruited to cellular mRNAs.

Support for the hypothesis that TAP is critical for cellular mRNA export has also come from efforts to identify factors important for mRNA export in yeast cells. Mutational inactivation of Mex67p, a yeast protein related to human TAP, blocked poly(A)$^+$ RNA export and also led to a loss of viability (Segref et al., 1997). However, these cells could be rescued by expression of TAP together with a proposed human cofactor termed p15-1 or NXT1 (Katahira et al., 1999). Therefore, it appears that the nuclear mRNA export function of TAP has been functionally conserved throughout eukaryotic evolution.

While yeast cells encode a single TAP homolog, higher eukaryotes generally encode several genes that show sequence similarity to human TAP. In humans, four genes related to TAP as well as one pseudogene have been reported, but only two of these (termed NXF2 and NXF3) encode open reading frames (Herold et al., 2000). However, NXF3 seemed unlikely to function as a nuclear export factor because it lacks the carboxy-terminal domain that is critical for both TAP-dependent nuclear export and nucleoporin binding (Kang and Cullen, 1999; Katahira et al., 1999) (Figure 1A). Remarkably, we demonstrate here that NXF3 is a nucleocytoplasmic shuttle protein that is fully capable of inducing the nuclear export of tethered mRNA molecules. Although NXF3 has lost the ability to target the NPC directly, NXF3 remains able to exit the nucleus due to the presence of a novel leucine-rich Crm1 binding site, equivalent to the NES found in HIV-1 Rev (Fischer et al., 1995), that is lacking in TAP.

Results

NXF2 and NXF3 Show Tissue-Specific Expression

A combination of in vitro and in vivo assays has permitted the definition of several functional domains in TAP

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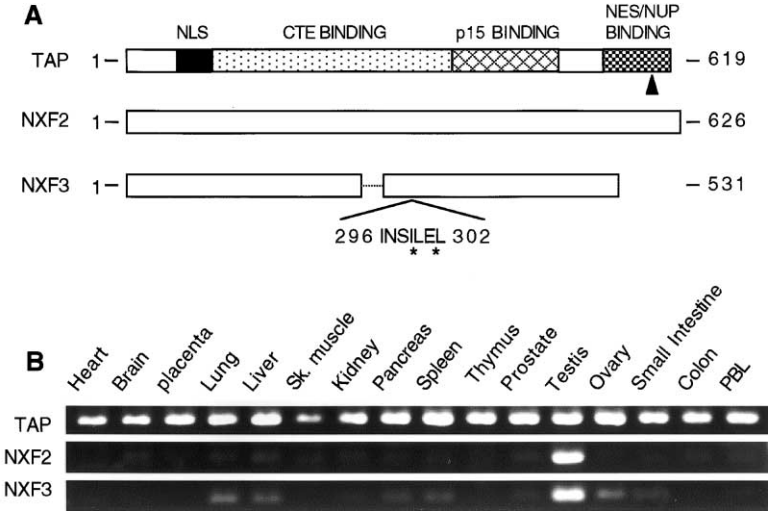

Figure 1. Comparison of TAP, NXF2, and NXF3 Expression Patterns

(A) Schematic representation of the functional domain organization of human TAP. NXF2 is ~58% identical to TAP and lacks significant insertions or deletions. While NXF3 is ~52% identical to TAP, it lacks a 36 aa stretch that maps to the CTE binding domain and has also lost the carboxy-terminal NES/NUP binding domain present in TAP. The NXF3 leucine-rich NES mapped in this paper is indicated, with critical leucine residues marked by asterisks. The closed triangle indicates the position of the TAP A17 mutation.

(B) The tissue-specific expression pattern of TAP, NXF2, and NXF3 mRNAs was determined by quantitative RT-PCR.

(Figure 1A). These include a nuclear localization signal (NLS) (aa 61–102), the MPMV CTE binding domain (aa 96–370), a central domain that recruits the p15-1 cofactor (aa 370–490), and an essential carboxy-terminal domain (aa 550–619) that directly interacts with certain NUPs and that functions as an NES (Kang and Cullen, 1999; Katahira et al., 1999; Bachi et al., 2000). The predicted sequence of the NXF2 protein reveals that TAP and NXF2 are colinear over their entire sequence and are ~58% identical (Figure 1A). In contrast, while NXF3 also displays ~52% sequence identity with TAP, it bears two obvious deletions when compared to both TAP and NXF2: the deletion of 36 aa within the CTE binding domain of TAP (Braun et al., 1999; Kang and Cullen, 1999), and the truncation of the NXF3 carboxyl terminus by 46 aa relative to TAP (Figure 1A). This latter deletion removes residues that in TAP are critical for both NUP binding and NES function (Kang and Cullen, 1999; Katahira et al., 1999; Kang et al., 2000).

As noted above, TAP has been proposed to function as a generic, sequence-nonspecific nuclear export factor for cellular mRNAs (Grüter et al., 1998; Katahira et al., 1999; Cullen, 2000). Analysis of the tissue-specific expression pattern of TAP mRNA (Figure 1B) revealed that the *TAP* gene was expressed at a high level in all tissues examined. In contrast, NXF2 mRNA was found to be expressed almost exclusively in testis. NXF3-specific transcripts were also detected at a high level in testis and at a low level in a small number of other tissues (Figure 1B). These data are consistent with a ubiquitous, essentially housekeeping role for TAP in global nuclear mRNA export, but they argue that NXF2 and NXF3 are unlikely to play such a general role.

NXF3 Can Function as a Nuclear RNA Export Factor

It has previously been demonstrated that TAP can specifically bind to the MPMV CTE and that expression of human TAP can rescue CTE function in nonpermissive quail cells (Grüter et al., 1998; Kang and Cullen, 1999). However, neither NXF2 nor NXF3 proved able to bind the CTE, and it was therefore not surprising that expression of these proteins failed to rescue CTE function in quail cells (data not shown).

Previously, it has been demonstrated that the HIV-1 Rev protein can mediate the nuclear export of an RNA when tethered to that RNA by the MS2 coat protein RNA binding domain (McDonald et al., 1992). This experiment is reproduced in Figure 2A, which utilizes an indicator construct, pDM128/4XMS2, that contains the *cat* indicator gene and four copies of the MS2 operator RNA target flanked by 5′ and 3′ splice sites. The unspliced *cat* mRNA encoded by pDM128 indicator constructs is normally unable to exit the nucleus and is instead exported in a fully spliced form that does not encode CAT (Hope et al., 1990; McDonald et al., 1992; Kang and Cullen, 1999; Yang et al., 2000). However, if a *cis*-acting RNA export signal (such as the MPMV CTE) is inserted into this unspliced *cat* mRNA, then export occurs and CAT enzyme activity is induced.

As shown in Figure 2A, transfection of human 293T cells with pDM128/4XMS2 normally results in very little CAT activity. However, as previously reported (McDonald et al., 1992), expression of an MS2-Rev fusion protein induces CAT expression. This activation is dependent on the integrity of the leucine-rich NES, as shown by the mutational inactivation of this sequence in MS2-RevM10 (Malim et al., 1991). A fusion protein consisting of MS2 fused to the full-length TAP protein activated CAT expression as effectively as did MS2-Rev (Figure 2A). This again required a functional NES, since introduction of the A17 mutation into the carboxy-terminal domain of TAP, which inhibits both NUP binding and NES function (Kang et al., 2000), largely blocked the activation of CAT expression (Figure 2A) without affecting the stability of the MS2-TAP fusion protein (Figure 2B).

We next tested fusion proteins consisting of MS2 linked to full-length NXF2 or NXF3 (Figure 2A). Surprisingly, the MS2-NXF2 fusion protein proved to be essentially inactive even though expression of this protein was readily detectable (Figure 2B). In contrast, the MS2-NXF3 fusion gave rise to a robust activation of the pDM128/4XMS2 indicator construct. To confirm that the observed activation of CAT protein expression indeed reflected the cytoplasmic appearance of the unspliced *cat* mRNA encoded by pDM128/4XMS2, we also per-

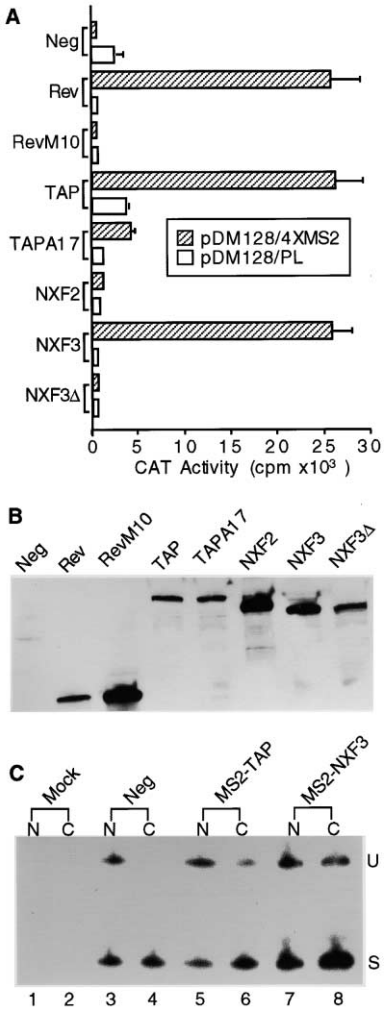

Figure 2. Nuclear Export of an mRNA Induced by Tethering of Nuclear Export Factors

(A) The pDM128/4XMS2 indicator construct contains the *cat* indicator gene and four copies of the MS2 operator RNA target flanked by 5′ and 3′ splice sites. Nuclear export of this RNA, and hence *cat* expression, is dependent on the recruitment of an mRNA export factor to the MS2 sites. 293T cells were transfected with 25 ng of pDM128/4XMS2 or pDM128/PL (as a negative control), 500 ng of the MS2-fusion protein expression plasmid indicated at left, and 50 ng of the pBC12/CMV/β-gal internal control. At 48 hr, cells were harvested and CAT and β-gal activities determined. These data represent the average of three experiments, with standard deviations indicated, after normalization to the internal control.

(B) A Western analysis was performed in parallel using 293T cells transfected with plasmids expressing the indicated MS2-fusion proteins. The antiserum used is a polyclonal rabbit anti-MS2 serum.

(C) RNase protection analysis was performed using nuclear (N) and cytoplasmic (C) RNA fractions derived from 293T cells transfected with pDM128/4XMS2 and either the parental pBC12/CMV plasmid, as a negative (Neg) control, or plasmids expressing the MS2-TAP or MS2-NXF3 fusion protein. The unspliced (U) and spliced (S) mRNAs encoded by pDM128/4XMS2 were detected using a previously described probe (Yang et al., 2000) that traverses the 3′ splice site. Cells transfected with pBC12/CMV only are indicated by "mock."

formed an RNase protection assay using cytoplasmic and nuclear RNA fractions derived from transfected cells. Unspliced *cat* mRNA was essentially undetectable in the cytoplasm of cells transfected with pDM128/4XMS2 alone (Figure 2C, lane 4), as predicted by the very low level of CAT activity measured in these cells (Figure 2A). In contrast, unspliced *cat* mRNA was exported to the cytoplasm of cells transfected with pDM128/4XMS2 plus either pMS2-TAP (Figure 2C, lane 6) or pMS2-NXF3 (Figure 2C, lane 8). Therefore, NXF3, like TAP, is able to activate the nuclear export of an mRNA when tethered to that RNA by fusion to a heterologous RNA binding domain.

NXF3 Interacts with the CRM1 Nuclear Export Factor

Previously, we and others have demonstrated that the TAP carboxy-terminal domain specifically interacts with FG-repeat domains found in certain human NUPs, including NUP153 and CG1, in the yeast two-hybrid assay (Katahira et al., 1999; Kang et al., 2000). This interaction is direct, as it can also be detected in vitro using recombinant proteins (Bachi et al., 2000). The leucine-rich NES of HIV-1 Rev also specifically interacts with the FG-repeat domains found in a distinct set of human nucleoporins in the yeast two-hybrid assay, including residues 1864–2090 of CAN/NUP214 (residues 1864–2090, termed ΔCAN, were chosen because larger CAN fragments also bind to TAP; Kang et al., 2000) and the protein RAB/hRIP (Bogerd et al., 1995; Fritz et al., 1995; Stutz et al., 1996). However, this interaction is indirect and is, in fact, bridged by the endogenous yeast Crm1 homolog (Neville et al., 1997). As a result, this interaction cannot be detected in vitro using recombinant proteins.

Because NXF3 can function as a nuclear RNA export factor (Figure 2A) yet apparently lacks the carboxy-terminal NUP binding domain/NES present in TAP (Figure 1A), we asked if NXF3 would also interact with NUPs in the yeast two-hybrid assay. We were also curious to see if NXF2 and NXF3 would be able to bind to human p15-1, a candidate cofactor for TAP-dependent nuclear mRNA export (Katahira et al., 1999; Guzik et al., 2001). As shown in Figure 3, the HIV-1 Rev protein specifically interacted with the FG-repeat domains of both CAN/NUP214 and RAB/hRIP but failed to interact with the FG-repeat domains of CG1 and NUP153 or with the human p15-1 protein. The interaction of Rev with CAN and RAB/hRIB was blocked by mutational inactivation of the Rev NES (data not shown; Bogerd et al., 1995; Fritz et al., 1995). In contrast, the TAP protein gave a readily detectable interaction with the nucleoporins CG1 and NUP153, and also bound the p15-1 protein, but failed to interact with either the ΔCAN fragment of CAN/NUP214 or with RAB/hRIP. Introduction of the A17 mutation into the TAP carboxy-terminal domain blocked NUP binding but did not affect p15-1 binding (data not shown; Kang et al., 2000).

Analysis of the binding profiles of NXF2 and NXF3 showed that both proteins have retained the ability to specifically bind to the TAP cofactor p15-1 (Figure 3). NXF2 also gave the same NUP binding profile as TAP, although binding to CG1 and NUP153 was attenuated when compared to TAP. Remarkably, the NXF3 protein instead gave the NUP binding profile characteristic of HIV-1 Rev, i.e., NXF3 showed little or no binding to CG1 or NUP153 yet gave rise to a readily detectable interaction with both ΔCAN and RAB/hRIP (Figure 3). Therefore,

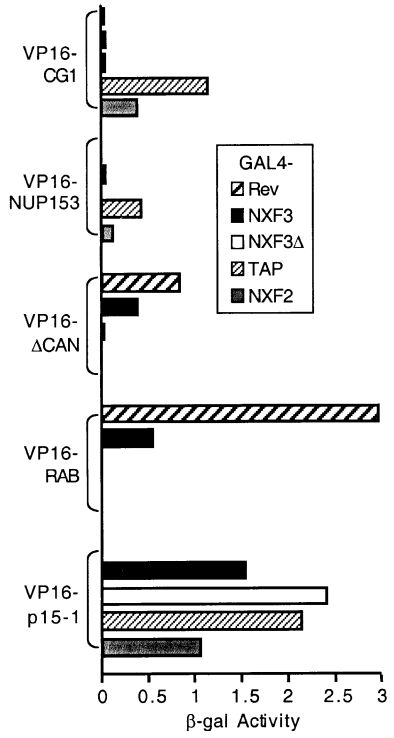

Figure 3. Analysis of the Protein Binding Properties of NXF2 and NXF3

The yeast indicator strain Y190 was transformed with plasmids expressing the indicated GAL4 DNA binding domain and VP16 transcription activation domain fusions. After growth on selective plates, the level of induced β-gal activity was determined as previously described (Bogerd et al., 1995).

these data imply that the carboxy-terminal truncation of NXF3, when compared to TAP, has indeed blocked the ability of NXF3 to interact with NUPs such as CG1 and NUP153 that normally bind to this TAP domain. However, these data also suggest that NXF3 has acquired the ability to instead interact with Crm1. To test this hypothesis, we performed a two-hybrid assay in human cells, as previously described (Bogerd et al., 1998), using a plasmid that expresses a GAL4 DNA binding domain fusion to Crm1 and a second plasmid that expresses the VP16 transcription activation domain fused to Rev, TAP, or NXF3 (for technical reasons, TAP and NXF3 were actually expressed fused to both the MS2 coat protein and the VP16 sequence). As shown in Figure 4A, this assay revealed a readily detectable interaction between Rev and Crm1, while no binding of TAP to Crm1 was detectable. NXF3, like Rev but unlike the far more closely related TAP protein, was also able to bind to Crm1 specifically in this human cell based assay.

Leptomycin B (LMB) is an antibiotic that specifically blocks Crm1 function (Fornerod et al., 1997a; Kudo et al., 1999). If NXF3 is indeed inducing RNA export by recruitment of Crm1, then this export should be inhibited by treatment with LMB. Importantly, LMB does not affect CTE function (Bogerd et al., 1998) and therefore should not affect RNA export mediated by the MS2-TAP fusion protein. As shown in Figure 4C, this indeed proved to be the case. Specifically, addition of LMB to transfected cells entirely blocked activation of the *cat* indicator gene present in pDM128/4XMS2 by either the MS2-Rev or MS2-NXF3 fusion protein, yet it had little or no effect on activation induced by MS2-TAP. Similarly, coexpression of the dominant negative ΔCAN fragment of the nucleoporin CAN/NUP214, which has been shown to selectively inhibit Crm1 function by blocking the ability of Crm1 to bind to the NPC (Fornerod et al., 1997b; Bogerd et al., 1998), also inhibited activation of pDM128/4XMS2 by either MS2-Rev or MS2-NXF3, yet did not affect MS2-TAP function (Figure 4D). We therefore conclude that NXF3 is indeed activating the nuclear export of tethered RNA molecules by recruitment of the Crm1 nuclear export factor.

NXF3 Is a Nucleocytoplasmic Shuttle Protein

Expression of a form of the NXF3 protein bearing a hemaglutinin (HA) epitope Tag, followed by immunofluorescent analysis, revealed that NXF3 is localized to both the nucleus and cytoplasm in transfected cells (Figure 5A), as also reported by Herold et al. (2000). As this tagged protein is ~60 kDa in size and therefore too large to diffuse into the nucleus, its partially nuclear localization implies active nuclear import, i.e., the presence of an NLS. The observation that NXF3 can mediate the nuclear export of tethered RNA molecules (Figure 2) suggests that NXF3 should also contain an NES. We therefore asked whether the partially cytoplasmic localization of NXF3 was the result of ongoing Crm1-dependent nuclear export. To test this hypothesis, we treated cells expressing the HA-tagged NXF3 protein with LMB, which should block all Crm1-dependent nuclear export. As shown in Figure 5B, this resulted in an entirely nuclear localization for the HA-NXF3 protein. We therefore conclude that NXF3 contains not only a Crm1-dependent NES, but also an active NLS, and that the full-length NXF3 protein continuously shuttles between the nucleus and the cytoplasm of expressing cells.

Identification of a Leucine-Rich NES in NXF3

To define the location of the Crm1 binding site in NXF3, we first divided NXF3 into four overlapping fragments of ~150 aa each and determined that only one of these (extending from 260 to 410) was able to interact with RAB/hRIP in the yeast two-hybrid assay (data not shown). This 150 aa segment was then subdivided into three overlapping fragments of 60 amino acids each that, when analyzed by yeast two-hyrid assay, further mapped the Crm1 binding site of NXF3 to between residues 260 and 320. Inspection of this sequence revealed a short leucine-rich sequence (296-INSILEL-302) that displays similarity to the previously defined leucine-rich NES consensus (Bogerd et al., 1996; Kim et al., 1996). Importantly, the equivalent sequences in TAP (338-ISAIRER-344) and in NXF2 (343-VSAIRDC-349) lack the two leucine residues found in NXF3.

To test whether the 296–302 sequence is indeed critical for Crm1 binding by NXF3, we mutated leucine residues 300 and 302 in NXF3 to arginine, as seen in TAP, to create the NXF3Δ mutant. As shown in Figure 2A, this mutation entirely blocked the ability of NXF3 to activate the nuclear export of the *cat* mRNA encoded by

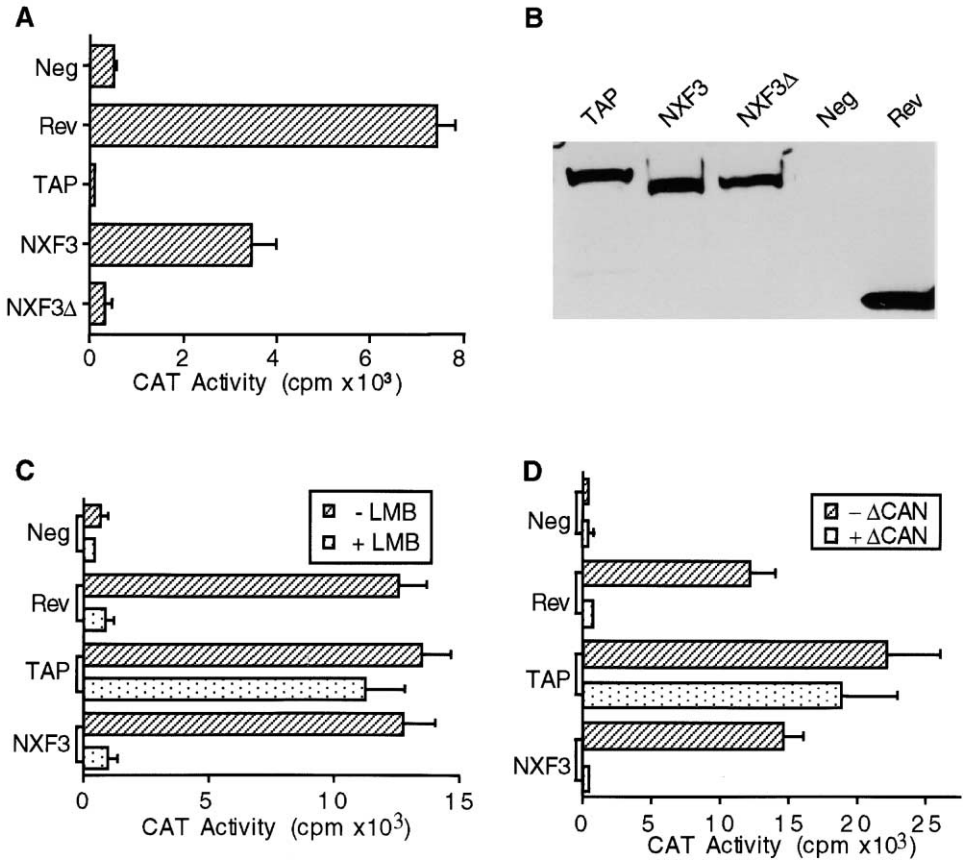

Figure 4. NXF3 Function Is Mediated by Crm1

(A) The NXF3 protein binds Crm1 specifically in a mammalian two-hybrid assay. 293T cells were transfected with 25 ng of the pG6(−31) HIVLTRΔTAR reporter plasmid, 250 ng of the pGAL4-Crm1 expression plasmid, and 1000 ng of a plasmid expressing the VP16/Rev, MS2/VP16/TAP, MS2/VP16/NXF3, or MS2/VP16/NXF3Δ fusion protein. The parental pBC12/CMV served as the negative control. Induced CAT enzyme activity was determined at ~48 hr after transfection.

(B) Western analysis of the level of expression of the VP16 fusion proteins analyzed in (A) was performed using a VP16-specific monoclonal antibody.

(C) Activation of the pDM128/4XMS2 indicator construct by MS2 fusion proteins was assayed as described in Figure 2 except that the indicated cultures were treated with 5 ng of LMB per 35 mm culture starting 16 hr after transfection.

(D) This export assay was performed as described in Figure 2 except that the cultures were also cotransfected with 500 ng of pBC12/CMV/ΔCAN, or 500 ng of pBC12/CMV as a negative control.

pDM128/4XMS2 when expressed as an MS2 coat protein fusion. This lack of activity was not due to instability of the MS2-NXF3Δ mutant, as shown by Western blot analysis (Figure 2B). NXF3Δ had also lost the ability to interact with CAN and RAB/hRIP in the yeast two-hybrid assay, yet it fully retained the ability to bind to the p15-1 cofactor (Figure 3). Consistent with the hypothesis that mutation of leucine residues 300 and 302 in NXF3 selectively inactivates Crm1 binding, the NXF3Δ mutant indeed proved unable to interact with Crm1 in the mammalian two-hybrid assay (Figure 4A), although introduction of this mutation did not affect the level of expression of the VP16-NXF3 fusion protein (Figure 4B). Finally, immunofluorescent localization of an HA-tagged version of the NXF3Δ mutant revealed that this protein, unlike wild-type HA-NXF3, was entirely nuclear (Figure 5C). The HA-NXF3Δ mutant therefore gives the same nuclear localization seen with wild-type HA-NXF3 in the presence of the Crm1 inhibitor LMB (Figure 5B), as would be predicted if this mutation indeed inactivated a Crm1-dependent NES.

NXF3 Is Associated with Poly(A)$^+$ RNA In Vivo

We have shown that the full-length NXF3 protein is a Crm1-dependent nucleocytoplasmic shuttle protein that is able to mediate the nuclear export of mRNA molecules to which it is tethered via the MS2 coat protein (Figure 2). An important additional prediction for a bona fide nuclear mRNA export factor is that it should at least transiently associate with mRNA transcripts in vivo. In fact, a major piece of evidence supporting the hypothesis that TAP mediates nuclear mRNA export in human cells is the demonstration that TAP can be specifically crosslinked to poly(A)$^+$ RNA in human cells by UV irradiation (Katahira et al., 1999)

To examine whether NXF3 is also associated with poly(A)$^+$ RNA in human cells, we transfected human 293T cells with expression plasmids encoding amino-

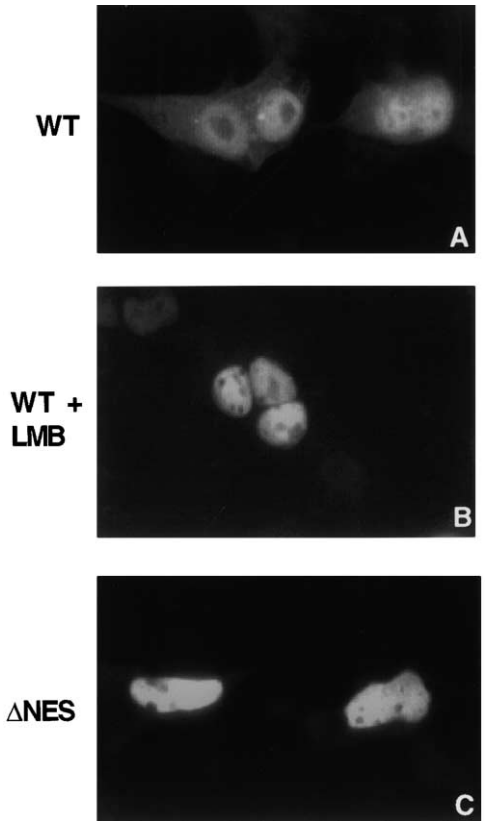

Figure 5. Subcellular Localization of the NXF3 Protein

293T cells were transfected with 1 μg of a plasmid expressing wild-type NXF3 (A and B), or the NXF3Δ mutant (C) bearing an amino-terminal HA tag. The cells in panel B were treated with the CRM1 inhibitor LMB prior to fixation. Fixed cells were stained with a FITC-conjugated anti-HA monoclonal antibody and the subcellular localization of the NXF3 protein visualized by immunofluorescence.

terminally HA-tagged forms of TAP, NXF3, or Importin α (Impα). Impα was chosen as a negative control since this protein nuclear import factor is similar in size to NXF3, also shuttles between the nucleus and the cytoplasm, and (like NXF3) is largely nuclear at steady state (Herold et al., 1998). At 44 hr after transfection, cultures were UV irradiated (Piñol-Roma and Dreyfuss, 1992) and poly(A)$^+$ RNA/protein complexes were isolated from total cell lysates prepared from both UV crosslinked and noncrosslinked cultures using oligo(dT) cellulose. Samples were then treated with RNase A and analyzed by SDS-PAGE followed by Western blotting using an HA-specific mouse monoclonal antibody. Total cell lysates from samples not subjected to oligo(dT) purification were also analyzed to verify that comparable levels of each protein were expressed.

As shown in the middle panel of Figure 6, we were able to detect a specific interaction between endogenous poly(A)$^+$ RNA and both HA-TAP and HA-NXF3. The intensity of this interaction was comparable, thus suggesting that the recruitment of TAP and NXF3 to poly(A)$^+$ RNA is equivalent in vivo. In contrast, an interaction between poly(A)$^+$ RNA and HA-Impα was barely detectable, as was predicted based on the known role of Impα in protein nuclear import rather than in mRNA nuclear export.

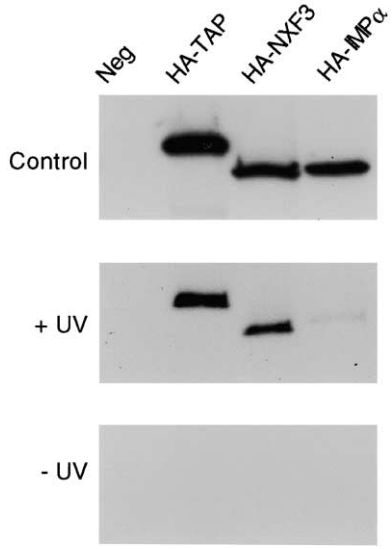

Figure 6. TAP and NXF3, but Not Impα, Associate with Poly(A)$^+$ RNA In Vivo

293T cells were transfected with expression plasmids encoding HA-tagged forms of TAP, NXF3, or Impα, or with the parental pBC12/CMV plasmid (Neg). At 44 hr after transfection, the cultures were UV irradiated and poly(A)$^+$ RNA:protein complexes were isolated with oligo(dT) cellulose. After elution and RNase digestion, cross-linked proteins were detected by Western analysis using an anti-HA monoclonal antibody (middle panel). Equivalent expression levels of the introduced HA-tagged proteins were confirmed by western analysis of samples prior to oligo(dT) cellulose treatment (upper panel). Nonirradiated cells processed in parallel served as an additional negative control (lower panel).

As shown in the upper panel of Figure 6, these differences did not reflect variations in the level of protein expression in transfected cells. Importantly, these RNA:protein interactions were only detectable in UV-irradiated cells.

Discussion

A role for the TAP protein in mRNA nuclear export was first suggested by the identification of TAP as the cellular target for the CTE RNA export sequence encoded by the retrovirus MPMV (Grüter et al., 1998). Because the CTE is not active in certain nonmammalian contexts but can be functionally rescued by expression of human TAP, it has been possible to define functional domains in TAP that are important for mediating CTE-dependent nuclear RNA export (Figure 1A). The most interesting of these is the essential carboxy-terminal NUP binding domain (Kang and Cullen, 1999; Katahira et al., 1999; Bachi et al., 2000). This domain is unique among nuclear export factors in that it permits TAP to directly interact with components of the NPC, and hence mediate nuclear export, without the intercession of a member of the importin/exportin family of nuclear transport factors.

While the importance of TAP for CTE-dependent mRNA export can be readily demonstrated, the precise role of TAP in the sequence-nonspecific nuclear export of human mRNAs remains to be defined (Cullen, 2000). However, a critical role for TAP in this process is strongly

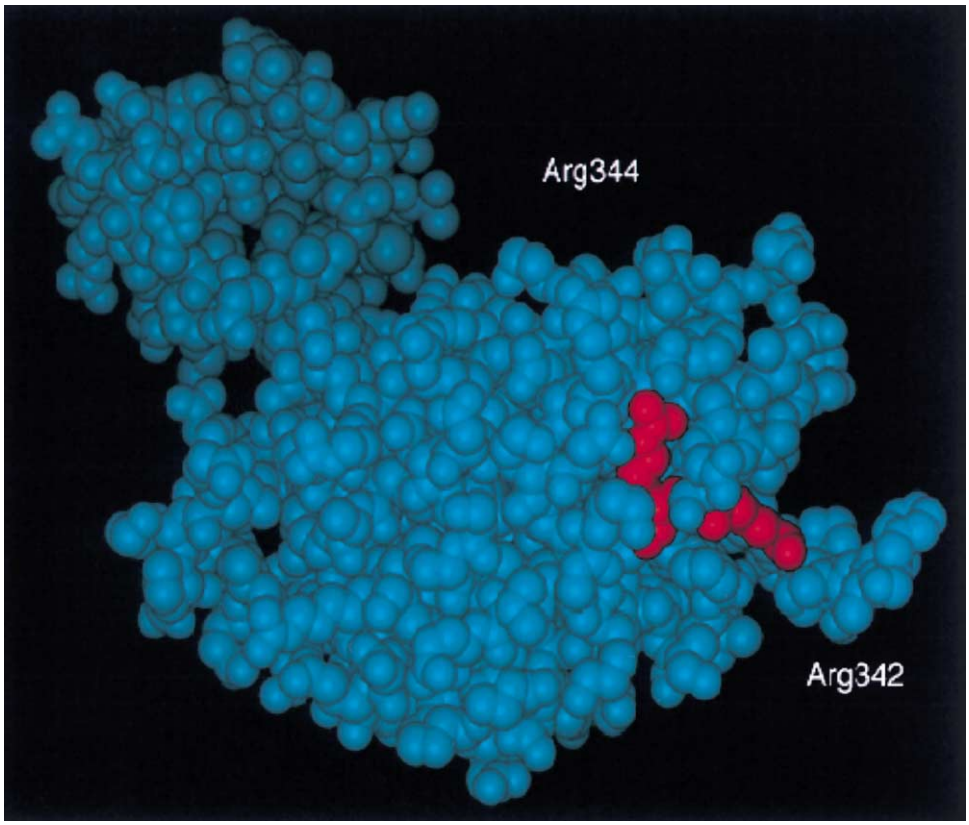

Figure 7. Model of the LRR Domain of Human TAP

The crystallographic structure of part of the CTE binding domain of TAP (residues 102–371) has been reported by Liker et al. (2000). Here, we show the reported structure of the LRR subdomain of TAP (residues 203–362). Arginine residues 342 and 344, which are equivalent to leucine residues 300 and 302 in NXF3, are highlighted to show their location on the surface of TAP.

suggested by the finding that the TAP homologs encoded by *C. elegans* and yeast are required for poly(A)$^+$ RNA export in these eukaryotic organisms (Katahira et al., 1999; Tan et al., 2000). Presumably, the carboxy-terminal NUP binding domain of TAP also plays a critical role in cellular mRNA export from the nucleus. In support of this hypothesis, recent data demonstrate that nuclear mRNA export does not require the GTP bound form of the RAN GTPase, a cofactor that is critical for all exportin-mediated nuclear export but dispensable for nuclear export mediated by the TAP carboxy-terminal domain (Clouse et al., 2000).

Duplication of genes provides a major mechanism for the evolution of proteins with novel mechanisms of action and/or expression patterns (Lynch and Conery, 2000). Because of the evident importance of TAP for global mRNA export, we were interested in the possibility that humans might express proteins related to TAP that would regulate gene expression in specific tissues at the level of nucleocytoplasmic transport. The human genome encodes five genes that are clearly related to TAP but only two of these, *NXF2* and *NXF3*, encode open reading frames (Herold et al., 2000). However, NXF3 appeared unpromising as a candidate nuclear export factor because it lacks sequences equivalent to the critical TAP carboxy-terminal domain (Figure 1A). Remarkably, the data presented in this manuscript demonstrate that NXF3, like TAP, is in fact fully capable of mediating the nuclear export of tethered RNA molecules (Figure 2). This apparent discrepancy is explained by the finding that NXF3, unlike TAP, possesses the ability to effectively interact with the Crm1 nuclear export factor (Figure 4A). Therefore, NXF3, while very similar in sequence to TAP, is (in at least this regard) more closely comparable to the Crm1-dependent Rev nuclear export factor encoded by HIV-1. Consistent with this hypothesis, both the nuclear export of tethered RNA molecules mediated by the MS2-NXF3 fusion protein (Figure 4) and nucleocytoplasmic shuttling by NXF3 (Figure 5) were found to be effectively blocked by specific inhibitors of Crm1 function such as LMB.

These data suggested that NXF3 might contain a Crm1 binding motif comparable to the leucine-rich NES found in HIV-1 Rev and several other viral and cellular proteins (Malim et al., 1991; Fischer et al., 1995; Cullen, 2000). A leucine-rich motif that mapped to around residues 296 to 302 in NXF3 (Figure 1A) was in fact found to be essential for both Crm1 binding and nuclear export mediated by the NXF3 protein (Figures 2A and 4A). Mutation of this motif neither destabilized the NXF3 protein (Figures 2B and 4B) nor interfered with the ability of NXF3 to bind to p15-1, a known cofactor for TAP-dependent nuclear mRNA export (Katahira et al., 1999) (Figure 3). However, mutagenesis of this leucine-rich NES did

result in the same entirely nuclear localization seen with wild-type NXF3 in the presence of LMB (Figure 5).

Comparison of the predicted amino acid sequences of NXF3 and TAP suggests that the mutation of two arginine residues in TAP to two leucine residues in NXF3 was a key step in the acquisition of a leucine-rich NES by NXF3, although the introduction of this mutation into TAP does not suffice to confer Crm1 binding (data not shown). The NXF3 NES falls within a region that has homology to a domain of TAP whose structure was recently solved (Liker et al., 2000). As shown in Figure 7, the two relevant arginines in TAP are located on the surface of the LRR subdomain and would be accessible for binding to Crm1 upon mutation to leucine. We therefore propose that NXF3 evolved from TAP, after gene duplication, by the initial acquisition of a leucine-rich NES due to the introduction of a small number of missense mutations on an exposed surface of the LRR subdomain. At this point, the NXF3 protein would have acquired the ability to use two distinct mechanisms to access the NPC, i.e., either directly via the carboxy-terminal NUP binding domain or indirectly via the newly acquired Crm1-dependent NES. The subsequent introduction of a nonsense mutation that deleted the carboxy-terminal NUP binding domain (Figure 1A) might therefore have little affect on the ability of NXF3 to continue to function as a nuclear export factor. Importantly, other functional domains in TAP that play a key role in TAP-mediated nuclear mRNA export, including the NLS and the p15-1 binding domain, have been conserved during the evolution of the *NXF3* gene.

Recently, Herold et al. (2000) also reported an analysis of NXF2 and NXF3 function and reached somewhat different conclusions. Most importantly, Herold et al. (2000) reported that NXF3 was unable to interact with NUPs in vitro and did not localize to the nuclear rim in expressing cells, two properties that distinguished NXF3 from both TAP and the NXF2 protein. Based on these and other data, these authors proposed that NXF2 could function as a nuclear export factor while NXF3 was likely to be inactive. Clearly, the data presented in this manuscript argue that NXF3 is, in fact, fully capable of mediating the nuclear export of tethered RNA molecules. The reported inability of NXF3 to directly bind to NUPs in vitro is, however, consistent with our observation that the NPC recruitment of NXF3 is actually mediated by Crm1. Although our data also document NUP binding by NXF2 (Figure 3), NXF2 differed from both TAP and NXF3 in being unable to activate nuclear RNA export when expressed as an MS2 fusion protein (Figure 2A). However, we have recently observed that MS2-NXF2 can mediate nuclear mRNA export in cells that overexpress the p15-1 protein (data not shown). While p15-1 overexpression was also recently reported to enhance nuclear mRNA export mediated by a tethered TAP protein (Guzik et al., 2001), we, in fact, observed only a modest positive effect of p15-1 overexpression on MS2-TAP dependent mRNA export (data not shown).

A Possible Role for Crm1 in the Nuclear Export of Specific Human mRNAs

While the autosomal *TAP* gene was found to be expressed in all human tissues examined, the X-linked *NXF2* and *NXF3* genes were predominantly expressed in human testis (Figure 1B). Of interest, it has recently been demonstrated that male-benefit genes, including testis-specific genes involved in the mitotic stages of male germ cell development, have selectively accumulated on the X chromosome during evolution (Wang et al., 2001). These observations, therefore, raised the possibility that NXF2 and/or NXF3 might play a role in male germ cell development, perhaps by acting as tissue-specific nuclear mRNA export factors.

Although we have not yet identified a specific cargo for NXF3-mediated nuclear export, we nevertheless believe that NXF3 is indeed likely to function as a human nuclear mRNA export factor. The evidence in favor of this hypothesis includes the demonstration, using UV crosslinking, that NXF3 is associated with poly(A)$^+$ RNA in human cells (Figure 6). Comparison of NXF3 with TAP, which is likely to be a critical mediator of nuclear mRNA export, suggests that both proteins associate with poly(A)$^+$ RNA in vivo with comparable efficiency. We have therefore provided evidence demonstrating that full-length NXF3 is associated with cellular mRNA transcripts in vivo (Figure 6), that NXF3 is a nucleocytoplasmic shuttle protein (Figure 5), and that NXF3 can export mRNA molecules when tethered to these by fusion to a heterologous RNA binding domain (Figure 2). Altogether, these findings raise the possibility that wild-type NXF3 may function as a tissue-specific nuclear mRNA export factor in vivo. When considered in combination with recent evidence arguing that Crm1 can mediate the selective nuclear export of mRNAs bearing AU-rich elements (Brennan et al., 2000), these data raise the possibility that Crm1 may play an important role in the nuclear export of specific target mRNA molecules in specific human tissues, even while bulk mRNA nuclear export remains dependent on the ubiquitously expressed TAP export factor.

Experimental Procedures

Construction of Molecular Clones

The yeast expression constructs pVP16, pGAL4/TAP, pVP16/ΔCAN, pVP16/p15-1, and pVP16/RAB have been described (Bogerd et al., 1995; Kang et al., 2000). All mammalian expression constructs are based on pBC12/CMV (Malim et al., 1989), which was also used as a negative control. We have previously described the mammalian expression plasmids pDM128/PL, pVP16/Rev, pGAL4/Crm1, pBC12/CMV/ΔCAN, and pBC12/CMV/βGal (Malim et al., 1991; Bogerd et al., 1998). Sequences encoding full-length NXF2 and NXF3 were cloned by PCR amplification from cDNAs reverse transcribed from human testis mRNA (Clontech) by means of primers that introduced flanking restriction sites; they were then cloned into appropriate vectors. In order to construct plasmids expressing MS2 fusion proteins, sequences encoding the MS2 coat protein were PCR amplified from genomic DNA isolated from the yeast strain L40-coat (SenGupta et al., 1996), digested with BspHI and NcoI, and then inserted into the NcoI site of pBC12/CMV to generate pMS2. PCR-amplified *Rev*, *TAP*, *NXF2*, and *NXF3* cDNAs were then ligated into NcoI/XhoI-digested pMS2.

Mammalian expression plasmids encoding fusion proteins consisting of the VP16 transcription activation domain linked to TAP, NXF3, or NXF3Δ were derived by insertion of the VP16 activation domain between the MS2 and the export factor coding sequences in pMS2-TAP or pMS2-NXF3. These plasmids therefore encode MS2-VP16-TAP and MS2-VP16-NXF3 fusion proteins. The pDM128/4XMS2 indicator construct was generated by PCR amplification of the MS2 operator RNA dimer from pIII/MS2-2 (SenGupta et al., 1996)

followed by ligation of two copies of the dimer into the BglII and ClaI sites of the pDM128/PL polylinker.

An expression plasmid encoding HA-tagged TAP has been previously described (Kang and Cullen, 1999). The pHA-NXF3 expression plasmid was constructed by inserting a BspHI-XhoI fragment encoding full-length NXF3 into pBC12/CMV/HA (Kang and Cullen, 1999) digested with NcoI and XhoI. The pHA-Impα2 plasmid was constructed similarly by introducing a full-length *Impα2* cDNA (Herold et al., 1998) into pBC12/CMV/HA. Yeast constructs expressing NXF fusion proteins were made using pGBT9 (Clontech) digested at EcoRI and SalI sites. Sequences encoding full-length NXF2 or NXF3 were cloned as MfeI and XhoI fragments into pGBT9 in order to generate GAL4 DNA binding domain fusions. The leucine → arginine double point mutant of NXF3 (NXF3Δ) was generated by QuickChange mutagenesis (Stratagene) of the appropriate wild-type templates. Plasmids expressing the FG repeats of CG1 and NUP153 fused to the VP16 transcription activation domain were obtained from a yeast two-hybrid cDNA library using pGAL4/TAP(61–619) as bait.

Cell Culture and Transfection

Human 293T cells were maintained as previously described (Bogerd et al., 1998). Cells were transfected with either Fugene-6 (Roche Molecular Biochem.) or calcium phosphate, which generated similar results. Cells were cultured in 35 mm 6-well plates and harvested ~48 hr after transfections. Induced CAT enzyme expression levels were normalized to the activity of the β-gal internal control, as previously described (Yang et al., 2000). For LMB treatment, 293T cells were transfected with Fugene-6 and then incubated at 37°C for 16 hr, at which time fresh medium containing LMB (5 ng/2 ml medium) was substituted. At 48 hr after transfection, the cells were harvested and CAT enzyme activity assayed. The amount of plasmid used in transfections is given in each figure legend. Western analyses were carried out using a rabbit polyclonal anti-MS2 protein antiserum (a gift of Dr. P. Stockley) or a monoclonal anti-VP16 activation domain antibody (Santa Cruz) following standard protocols.

In Vivo Expression Profile

The tissue expression profiles of *TAP*, *NXF2*, and *NXF3* were determined by PCR on normalized, first-strand cDNA preparations from 16 human tissues/cells (Clontech) as previously described (Wang et al., 2001). Briefly, 2.5 μl of cDNA was used in each 25 μl PCR reaction. Thirty-five cycles were performed under the following conditions: 94°C, 30 s; 56°C, 30 s; 72°C, 90 s. PCR primers for each gene are as follows: TAP— forward primer GTTCCCTGAGCATTCCTTTC, reverse primer CCGCACAAATAGCTCATCATT; NXF2—forward primer AGGCCATCCAGCTGGTACAG, reverse primer AAGTATGACAAGG CAGGCTAA; NXF3—forward primer CTCTTTGTGCGGGATACCAG, reverse primer CATGCAAGAACATGAGGAGC.

Yeast and Mammalian Two-Hybrid Assays

Yeast two-hybrid assays were performed as previously described (Fields and Song, 1989; Kang and Cullen, 1999). Plasmids expressing GAL4 DNA binding and VP16 transcription activation domain fusions were cotransformed into the yeast indicator strain Y190 (Harper et al., 1993) by standard procedures followed by incubation at 30°C on selective culture plates for approximately 4 days. Yeast cells were then harvested and analyzed for induced β-gal activity as previously described (Kang and Cullen, 2000).

Two-hybrid assays in human 293T cells were performed as described (Bogerd et al., 1998). Briefly, 293T cells were transfected with the indicator construct pG6(−31)HIVLTRΔTAR (Southgate and Green, 1991) together with plasmids expressing the GAL4 DNA binding domain fused to full-length human Crm1 (Bogerd et al., 1998) and the VP16 activation domain fused to full-length Rev, TAP, NXF3, or the NXF3ΔNES mutant. Induced CAT activities were determined at 48 hr after transfection.

Immunofluorescent Analysis of the Cellular Localization of NXF3

Human 293T cells were seeded onto Biocoat collagen-treated coverslips (Becton Dickinson Labware) and then transfected with either wild-type pCMV/HA-NXF3 or the NXF3Δ mutant. At 48 hr after transfection, the cells were fixed using 3% paraformaldehyde in PBS and stained with a mouse anti-HA monoclonal antibody conjugated to FITC (Santa Cruz) (Kang and Cullen, 1999). The coverslips were then mounted on a microscope slide, and NXF3-expressing cells were visualized with a Leica DMRB fluorescent microscope. LMB treatment of cells expressing wild-type HA-NXF3 was performed as described above.

In Vivo UV Crosslinking

UV crosslinking was performed essentially as previously described (Piñol-Roma and Dreyfuss, 1992; Katahira et al., 1999). Briefly, 293T cells were transfected with an HA-tagged expression plasmid or with the parental pBC12/CMV plasmid as a negative control, using the calcium phosphate method. Approximately 44 hr posttransfection, cells were washed with PBS and UV crosslinked in a Stratalinker (Stratagene) at 100 mJ. Poly(A)$^+$ mRNA/protein complexes from total cell lysates (crosslinked and noncrosslinked) were purified on oligo(dT) cellulose [MicroPoly(A)Pure, Ambion]. Samples were RNase A treated and then analyzed by SDS-PAGE and Western blotting with an anti-HA monoclonal antibody (Covance). In addition, total cell lysates from samples that were not subjected to oligo(dT) purification were analyzed by Western blot to verify levels of protein expression.

Molecular Modeling of the Structure of TAP

The crystal structure of the RNA binding domain of human TAP was solved by Liker et al. (2000). The three-dimensional data were downloaded from the web site http://www.umass.edu/microbio/chime/explorer/. In order to obtain a better view of the two arginine residues, the RNP domain was removed and only the LRR domain was shown. Two arginine residues (342 and 344), which correspond to the two critical leucine residues (300 and 302) in NXF3, were highlighted in red using RasMol software and are located on the surface of the TAP LRR domain.

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Accession Numbers

The GenBank accession numbers for the cDNA sequences for NXF2 and NXF3 are AF285596 and AF346619, respectively.