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The mRNA export in *Caenorhabditis elegans* is mediated by Ce-NXF-1, an ortholog of human TAP/NXF and *Saccharomyces cerevisiae* Mex67p

WEI TAN,* ANDREI S. ZOLOTUKHIN,* JENIFER BEAR, DANIEL J. PATENAUDE,
and BARBARA K. FELBER

Basic Research Laboratory, Human Retrovirus Pathogenesis Section, National Cancer Institute—
Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201, USA

ABSTRACT

Human TAP and *Saccharomyces cerevisiae* Mex67p belong to a family of proteins that mediate mRNA export. Computer searches identified previously two *Caenorhabditis elegans* genes, C15H11.3 and C15H11.6, that encode putative homologs of hTAP and Mex67p (Segref et al., *EMBO J*, 1997, 16:3256–3271). Using RNA interference experiments in *C. elegans*, we found that functional knockout of C15H11.3 resulted in nuclear accumulation of poly(A)-containing RNAs and was lethal for both embryos and adult nematodes. No embryonic or progeny abnormality was observed in functional knockout of C15H11.6. Taken together, these data established that the C15H11.3 gene product is an ortholog of hTAP and Mex67p; thus, it was named Ce-NXF-1. Ce-NXF-1 binds RNA directly and is a nucleocytoplasmic shuttle protein accumulating in the nucleoplasm and at the nuclear rim. The rim association is mediated via unique signals present in the C-terminal portion of all TAP/NXF and Mex67p proteins. This region was shown to interact with the FG-repeat domains of nucleoporins Nup98, Nup153, and Nup214, indicating that the rim association occurs through components of the nuclear pore complex. In summary, Ce-NXF-1 belongs together with hTAP and Mex67p to a family of proteins that participate in mRNA export and can provide a direct molecular link between mRNAs and components of the nuclear pore complex. Therefore, despite differences in mRNA metabolism between these species, they utilize a conserved mRNA transport mechanism.

Keywords: import; nuclear rim; nucleocytoplasmic transport; nucleoporin; RNA binding; RNA interference; shuttling protein

INTRODUCTION

After synthesis, processing, and assembly into ribonucleoprotein (RNP) complexes, transcripts are usually subjected to export into the cytoplasm. This transport involves a complex interaction of the RNP complex with components of the nuclear pore (for recent reviews, see Nakielnny & Dreyfuss, 1997; Mattaj & Englmeier, 1998; Gorlich & Kutay, 1999). All retroviruses depend on transport of their unspliced, full-length mRNA from the nucleus to the cytoplasm. The full-length viral RNA serves not only as mRNA for the production of the Gag/Gag-Pol polyproteins, but also as genomic RNA for the progeny virions. Therefore retroviruses provide

a unique tool to study the regulation of nuclear export. The study of the posttranscriptional regulatory mechanisms employed by retroviruses has led to the discovery of major nucleocytoplasmic export pathways (Felber, 1998; Izaurralde & Adam, 1998; Stutz & Rosbash, 1998).

The export of the unspliced simian type D retroviruses (SRV/D) RNA was shown to be mediated via the *cis*-acting constitutive transport element (CTE) (Bray et al., 1994; Tabernero et al., 1997). The CTE forms an extended secondary RNA structure with two conserved loop regions (Tabernero et al., 1996, 1997), which are the binding sites for the human TAP (hTAP) (Grüter et al., 1998). hTAP is a nucleocytoplasmic shuttle protein (Bear et al., 1999; Kang & Cullen, 1999; Katahira et al., 1999; Bachi et al., 2000) and was shown to promote CTE-dependent RNA export (Grüter et al., 1998; Bear et al., 1999; Kang & Cullen, 1999). Another nu-

Reprint requests to: Barbara K. Felber, Human Retrovirus Pathogenesis Section, NCI-FCRDC, Building 535, Room 110, Frederick, Maryland 21702-1201, USA; e-mail: felber@mail.ncifcrf.gov.

*These authors contributed equally to this work.

cleocytoplasmic shuttle protein, the cellular RNA helicase A, also binds to CTE (Tang et al., 1997a, 1997b, 1999); however, its exact role in CTE RNA export remains under investigation.

Human TAP belongs to a family of proteins that were also found to be involved in mRNA export (Segref et al., 1997; Grüter et al., 1998). The first recognized member, the *Saccharomyces cerevisiae* Mex67p, was identified by the Hurt lab (Segref et al., 1997). Mex67p is a nuclear rim-associated protein that binds RNA and is essential for mRNA export in yeast (Segref et al., 1997; Hurt et al., 2000). Similarly, hTAP was also shown to bind, albeit with lower affinity, to cellular mRNAs (Grüter et al., 1998; Braun et al., 1999; Katahira et al., 1999) and to participate in their nucleocytoplasmic export (Grüter et al., 1998). It is thought that hTAP uses two distinct mechanisms to participate in the export of CTE and mRNA. While hTAP has to mediate the export of the retroviral RNA prior to splicing, it should only promote export of the terminally spliced cellular mRNAs. Importantly, hTAP was shown to replace Mex67p in yeast (Katahira et al., 1999). This finding clearly established that hTAP and Mex67p share not only structural but also functional features.

Like Mex67p, hTAP is a nuclear protein (Bear et al., 1999; Kang & Cullen, 1999; Katahira et al., 1999; Bachi et al., 2000) and was shown to be imported into the nucleus via transportin (Truant et al., 1999; Bachi et al., 2000). Both Mex67p (Segref et al., 1997) and hTAP (Bear et al., 1999; Katahira et al., 1999; Bachi et al., 2000) were found to accumulate in the nuclear rim and were shown to directly associate with components of the nuclear pore complex (NPC) (Katahira et al., 1999; Bachi et al., 2000). Because both Mex67p and hTAP can be crosslinked to mRNA in vivo and bind RNA in vitro, they likely provide a direct link between the mRNAs and the NPC. Several additional proteins were shown to directly interact with hTAP and/or Mex67p (Santos-Rosa et al., 1998; Katahira et al., 1999; Bachi et al., 2000; Strasser & Hurt, 2000; Stutz et al., 2000). Their roles in TAP/Mex67p function remain unclear.

Using computer searches, Segref et al. (1997) found that the predicted *Caenorhabditis elegans* C15H11.3 and C15H11.6 genes could encode proteins that are homologous to TAP and Mex67p. In this work, we investigate the role of these putative proteins in *C. elegans*. We demonstrate that the C15H11.3 gene product, termed Ce-NXF-1, is essential for mRNA export in *C. elegans*. This finding established that the Ce-NXF-1 is an ortholog of human TAP and *S. cerevisiae* Mex67p. We found that Ce-NXF-1 is a nucleocytoplasmic shuttle protein that localizes to the nuclear rim. Taken together, our data reveal that the family of TAP/Mex67p proteins shares molecular determinants for RNA binding, nuclear localization, and association with the NPC as well as the ability to shuttle between the nucleus and the cytoplasm.

RESULTS

C15H11.3 is an ortholog of human TAP and *S. cerevisiae* Mex67p

The RNA interference (RNAi) method had been shown to be an effective way to specifically knock out expression of target genes in *C. elegans* (Harbinder et al., 1997; Fire et al., 1998; Montgomery et al., 1998). We designed RNAi experiments to inhibit expression of the putative gene products of C15H11.3 and C15H11.6 as identified on cosmid C15H11 (accession Z81035). C15H11.3 and C15H11.6 were previously referred to as C15H11.e and C15H11.d, respectively (Segref et al., 1997). First, total RNA isolated from *C. elegans* was subjected to reverse transcription and the cDNAs spanning the complete or partial predicted coding region of C15H11.3 and C15H11.6 were PCR amplified and cloned. RNAi was performed by either direct microinjection into the gonads or by soaking of the nematodes with double-stranded RNA (dsRNA) overnight. Upon RNAi with dsRNA from C15H11.3 (spanning amino acids 62–411), we observed that all the laid embryos were arrested before morphogenesis (see below; Fig. 1), and, consequently, none of them were able to hatch. Therefore, we found that RNAi was reproducibly 100% embryonic lethal. We observed the same phenotype using both methods of RNAi. Interestingly, we found that the soaking method was also lethal for the adult nematodes, 100% of which lost their mobility and died within 3 days. This finding demonstrated that C15H11.3 is an essential factor. On the other hand, RNAi targeted to C15H11.6 had no effect on the embryonic or larval development or on fertility of progeny nematodes. We also did not observe any effect on the mother nematodes. We used both methods of RNAi and different dsRNA fragments (spanning the amino acids 98–264 and 2–421) to support this finding. Similarly, incubation of the nematodes in buffer did not have any effect and was indistinguishable from untreated nematodes.

Because C15H11.3 had been identified as a homolog of mRNA export factors TAP/Mex67p, we examined the effect of its functional knockout on *C. elegans* mRNA localization. After RNAi with dsRNA to C15H11.3, the embryos and adult nematodes were fixed, and subjected to in situ hybridization with a biotinylated oligo(dT) probe (Fig. 1). We found that RNAi of C15H11.3 resulted in nuclear accumulation of poly(A)-containing RNAs both in the embryos (Fig. 1G–J) and adults (Fig. 1K–L). Figure 1G/H and I/J show embryos at different developmental stages before morphogenesis. In contrast, untreated embryos (Fig. 1A–D) and adult nematodes (Fig. 1E, F) showed the expected more uniform or cytoplasmic localization of poly(A)-containing RNAs. Similar data were obtained after incubation of the nematodes in buffer only.

FIGURE 1. RNA interference with C15H11.3 results in nuclear accumulation of poly(A) mRNA. *C. elegans* nematodes were subjected to RNAi with *Ce-nxf-1*. Untreated nematodes were indistinguishable from mock-treated (not shown) nematodes. The embryos and adult nematodes were fixed and subjected to in situ hybridization. Poly(A)-containing RNAs (pseudocolored in red) are shown in **B, D, F, H, J, and L**. The same samples were stained for nuclei (pseudocolored in green) in **A, C, E, G, I, and K**. Untreated and RNAi embryos are shown in **A–D** and **G–J**, respectively. Intestines of adult nematodes are shown without (**E, F**) and upon RNAi treatment (**K, L**).

Taken together, these data established that the C15H11.3 gene product is an ortholog of TAP/Mex67p, and it was named Ce-NXF-1. We adopted the new name NXF (nuclear export factor) designated for TAP and TAP-like proteins based on a recent nomenclature agreement with the Human Genome Nomenclature Committee (HUGO) (Herold et al., 2000). Although RNAi of C15H11.6 did not have the effect we observed with C15H11.3, we propose to name it Ce-NXF-2 due to its structural homology to Ce-NXF-1. In the subsequent experiments, we focused entirely on Ce-NXF-1.

Ce-NXF-1 is an RNA-binding protein

To test whether Ce-NXF-1 can bind RNA, we performed in vitro binding experiments. We used human histone H4 mRNA as a representative probe because this RNA has been shown to depend on hTAP for export (Bachi et al., 2000). The bacterially produced complete Ce-NXF-1 fused in frame to glutathione S-transferase (GST) was immobilized on glutathione-

Sepharose beads and bound to the radiolabeled probe in the presence of varying amounts of unlabeled histone H4 RNA. In parallel, the reactions were performed with GST moiety alone and with recombinant human TAP spanning amino acids 61–372 fused to GST (GST hTAP61–372). As shown in Figure 2A, GST Ce-NXF-1 bound efficiently to histone H4 RNA, whereas binding to GST moiety was barely detectable. This finding indicated that Ce-NXF-1 can bind RNA in vitro. We then compared Ce-NXF-1 and human TAP61–372 for their ability to bind to the histone H4 mRNA. We found that both proteins bound histone H4 mRNA similarly and have an apparent K_B of less than 50 nM (Fig. 2B). Thus, Ce-NXF-1, like its human ortholog, is an RNA-binding protein.

Ce-NXF-1 is a nuclear protein

We transiently expressed the predicted complete Ce-NXF-1 protein to study its subcellular localization. We used human HeLa cells because no *C. elegans* cell

FIGURE 2. Ce-NXF-1 is an RNA-binding protein. In vitro binding was performed using ^{32}P -labeled histone H4 RNA as a probe, in the presence of cold H4 RNA at different concentrations (plotted on x axis in nM). **A:** The amount of radiolabeled histone H4 RNA bound to GST Ce-NXF-1 (diamonds) or GST (squares) is plotted on the y axis. **B:** The percentage of the radiolabeled histone H4 RNA bound to GST Ce-NXF-1 (diamonds) or GST hTAP61-372 (squares) is plotted on the Y axis. One hundred percent corresponds to the binding in the absence of cold RNA.

line is available. Ce-NXF-1 was tagged with different protein moieties. As neutral moieties, we used the green fluorescent protein (GFP) or the hemagglutinin (HA) epitope tag. We also used two moieties that are found exclusively in the cytoplasm and cannot enter the nucleus by diffusion, such as GST and GFP- β -galactosidase (GFP- β Gal) hybrid protein (Bear et al., 1999). Although nuclear localization of GFP alone can occur via diffusion, only the presence of an active import signal (NLS) mediates nuclear translocation of GST or GFP- β Gal.

Figure 3 shows that Ce-NXF-1 containing the GFP at the C-terminus or the HA-tag at the N-terminus was found predominantly in the nucleus, where it accumulated in the nucleoplasm but not in the nucleoli. In addition, we observed that Ce-NXF-1 also localized at the nuclear rim (see also below). This association was resistant to in situ digitonin treatment (+ digitonin), pointing to strong association of Ce-NXF-1 with the nuclear membrane.

To study whether Ce-NXF-1 enters the nucleus by active import or by diffusion, we tagged NXF-1 with GST or GFP- β Gal at the N- or C-terminus, respectively. The presence of these tags had a profound effect on localization of Ce-NXF-1, resulting in a predominant cytoplasmic accumulation of these hybrid proteins (Fig. 3). These findings suggested that the presence of a large protein moiety either due to its size (GFP- β Gal, ~250 kDa; Bear et al., 1999) and/or its ability to mul-

timerize (GST; Heger et al., 1999) drastically reduced the nuclear accumulation of Ce-NXF-1. Therefore, although Ce-NXF-1 (HA- or GFP-tagged) is found primarily in the nucleoplasm, its nuclear accumulation is mediated by passive diffusion rather than by active import. Because these studies were performed in HeLa cells, we cannot exclude that Ce-NXF-1 has signal(s) for active nuclear import whose function is restricted to *C. elegans*. In summary, Ce-NXF-1 is a nuclear protein that accumulates at the nuclear rim, similarly to hTAP (Bear et al., 1999; Kang & Cullen, 1999; Katahira et al., 1999; Bachi et al., 2000) and *S. cerevisiae* Mex67p (Segref et al., 1997).

Interestingly, although the GFP- β Gal- and GST-tagged Ce-NXF-1 remained in the cytoplasm, these proteins also accumulated at the nuclear rim (Fig. 3). We have previously shown that a mutant hTAP lacking its N-terminal NLS and remaining in the cytoplasm was still able to accumulate at the nuclear rim (Bear et al., 1999). Taken together, these data suggest that these proteins are able to associate with components of the NPC (see also below), independent of the presence of the molecular determinants that mediate nuclear accumulation.

Ce-NXF-1 has two independent signals that mediate nuclear accumulation

We examined the subcellular localization of different N- and C-terminal deletion mutants of Ce-NXF-1 upon tag-

FIGURE 3. Ce-NXF-1 is a nuclear protein. Ce-NXF-1 was tagged with GFP, HA, GST, and GFP- β Gal, respectively. HLtat cells were transfected with the different expression vectors. GFP- and GFP- β Gal-tagged proteins were detected in live cells. GST- and HA-tagged proteins were fixed in 3.7% formaldehyde and subjected to indirect immunofluorescence using anti-GST and anti-HA antibodies as described (Bear et al., 1999). The cells in the indicated panel were subjected to digitonin treatment (+ digitonin) before fixation (see Fig. 5). The images were obtained by fluorescent microscopy and by use of the CCD camera.

ging with GFP (Table 1, Fig. 4). We found that the N-terminal portion spanning amino acids 1–396 and 1–244 localized preferentially to the nucleus. Mutant 1–164 was excluded from the nucleus and therefore was not further investigated, while a fragment spanning amino acids 84–351 was distributed equally in both compartments, like GFP alone. Taken together, these data showed that the region spanning amino acids 1–244 is sufficient for nuclear accumulation.

We next examined whether the C-terminal portion participated in the nuclear localization. We noticed that all N-terminal deletion mutants (removing amino acids 1–499) accumulated in the nucleus (Table 1, Fig. 4). The nuclear accumulation was only impaired by further deletion to amino acids 513 or 570. These data identified the presence of a second region within Ce-NXF-1, spanning amino acids 499–628, that mediated nuclear accumulation.

The experiments shown in Figure 4 also revealed that removal of amino acids 1–498 did not affect the rim association. However, further N-terminal deletions

TABLE 1. Ce-NXF-1 has two nuclear localization determinants.

Ce-TAP-1	Localization	Rim association
N-terminal deletions		
1–628	N	Yes
62–628	N	Yes
244–628	N	Yes
396–628	N	Yes
454–628	N	Yes
499–628	N	Yes
513–628	N + C	No
570–628	N + C	No
C-terminal deletions		
1–396	N	No
1–244	N	No
1–164	C	No
84–351	N + C	No

N: nucleus; C: cytoplasm.

(mutants 513–628 and 570–628) abolished rim accumulation. Therefore, Ce-NXF-1 and hTAP shared the ability to associate with the nuclear rim via their C-terminal portions (see also below) (Bear et al., 1999; Katahira et al., 1999; Bachi et al., 2000). In conclusion, the region spanning amino acids 499–628 contains both signals that mediate nuclear accumulation as well as nuclear rim localization.

FIGURE 4. N- and C-terminal signals in Ce-NXF-1 contribute independently to nuclear accumulation. HLtat cells were transfected with the indicated N- and C-terminal deletion mutants of the GFP-tagged Ce-NXF-1 and with GFP alone, and visualized as described for Figure 3.

Identification of novel conserved motifs in Ce-NXF-1 that mediate interaction with the nuclear rim

We compared the C-terminal sequences of Ce-NXF-1 to those of hTAP and Mex67p as well as to the TAP homologs from *Drosophila melanogaster* (dmTAP) (ac-

cession #CAB64382) and *Schizosaccharomyces pombe* (spMex) (accession #AAD43831). Interestingly, this alignment identified several conserved motifs located within the N- and C-terminal subregions (Fig. 5A). Similarly, we also found that the predicted TAP/Mex67p homologs in rat (GenBank accession AF093140) and mouse (GenBank accession AF093139), which share

FIGURE 5. Ce-NXF-1 associates with the nuclear rim. **A:** Comparison of the amino acid sequences of the C-terminal regions of Ce-NXF-1, hTAP, and Mex67p (Segref et al., 1997) with those of *S. pombe* Mex (spMex) and *D. melanogaster* TAP (dmTAP) revealed conservation of several motifs. Identical (black) and similar (gray) amino acids are indicated. Alanine substitutions were introduced into regions M1–M4 as shown generating the corresponding mutants. The vectors expressing the GFP-hybrid proteins of Ce-NXF-1 499–628 (**B**) and of hTAP 509–619 (**C**) were transfected into HLtat cells. All cells were subjected to treatment with 0.004% digitonin and fixed in 3.7% formaldehyde.

overall an extensive homology to hTAP (Bear et al., 1999), also share these motifs (data not shown).

Next, we examined the role of these regions for the subnuclear localization of Ce-NXF-1. Focusing on two conserved clusters located within the N- and C-terminal portions of 499–628, we introduced multiple alanine substitutions (M1, M2, M4), and examined the localization of the GFP-tagged proteins after digitonin treatment. As shown in Figure 5B, amino acid changes in M1 or in M2 abolished accumulation at the rim. Therefore, both changes (M1, M2) as well as removal (mutant 513–628, lacking region including M1; 570–628, lacking region spanning both M1 and M2; see Fig. 4) of these conserved motifs abolished rim association. In addition, alanine substitutions in M4 also abolished rim association (Fig. 5B). These findings indicate that there are several motifs within the C-terminal region of Ce-NXF-1 that independently contribute to its association with the nuclear rim. Only the presence of all these elements results in accumulation of Ce-NXF-1 at the nuclear rim.

This conclusion was further supported by our analysis of the C-terminal region of the hTAP. The GFP-tagged 509–619 associates with the nuclear rim (Fig. 5C), as previously shown (Bachi et al., 2000). We introduced the alanine changes described in Figure 5A, generating M1, M2, M3, and M4. We also found that changes in any of these four regions abolished rim association (Fig. 5C). We had previously shown that removing the region spanning M1 and M2 (in mutant 540–619) or the region spanning M3 and M4 (deletion 560–619) resulted in loss of rim association (Bear et al., 1999). Similarly, Bachi et al. (2000) reported recently that internal deletions within this region resulted in loss of rim localization.

Taken together, these data demonstrate that Ce-NXF-1 shares with the hTAP/Mex67p family the ability to accumulate at the nuclear rim. Here, we showed that for both Ce-NXF-1 and hTAP, this association is mediated through a complex interaction of conserved motifs located at the C-terminus of these proteins.

Ce-NXF-1 associates with nucleoporins

hTAP and Mex67p were previously shown to interact directly with components of the NPC (Segref et al., 1997; Bachi et al., 2000). We then examined whether Ce-NXF-1 is able to interact with selected nucleoporins. We used the yeast two-hybrid interaction-mating assay and examined interactions by growth (not shown) and β Gal production (Fig. 6). The complete Ce-NXF-1, the N-terminal (amino acids 1–244), and the C-terminal (amino acids 499–628) regions were inserted into the bait plasmid pEG202 in frame with LexA. The FG-repeat domains of different human nucleoporins were cloned into the prey plasmid pJG4-5 in frame with the activation domain. Expression of the Lex-A fusion pro-

FIGURE 6. Interaction of Ce-NXF-1 with FG-repeat domains of nucleoporins. Yeast strains were cotransformed with the indicated plasmids and were subjected to the interaction-mating assay under selective growth conditions. After replica-plating onto nitrocellulose, the strains were assayed for β Gal production.

teins was verified by western immunoblot analysis (data not shown). We found that only the complete Ce-NXF-1 as well as its C-terminal portion, but not the N-terminal portion, interacted with the NP domains of the nucleoporins tested (Fig. 6). These effects were specific, as no interactions were observed for the empty bait plasmid with any of the prey plasmids. We next assayed these interactions using the liquid culture assay. Table 2 shows mean values of the normalized β Gal activities from five to six experiments. We found that the C-terminal fragment of Ce-NXF-1 interacted to a similar extent with the different NP domains tested (Nup98, Nup153, and Nup214). As a comparison, sim-

TABLE 2. Two-hybrid interaction determined by the liquid growth assay.

Bait plasmid containing	Prey plasmids containing			
	Empty	NP Nup98	NP Nup153	NP Nup214
Ce-NXF-1				
1–244	1	1	1	1
499–628	1	8	6	3
499–628 M1	1	3	9	7
hTAP				
412–619	1	2	6	3
509–619 M1	1	3	13	3

Values are given as normalized β Gal activities (fold induction).

ilar interactions were also obtained using the C-terminal portion of hTAP. We noted that the interaction of NXF/TAP with these nucleoporins was overall weaker than that of the NES of HIV-1 Rev using both interaction mating assays (incubation time required for Rev- and NXF/TAP-containing colonies to turn blue is ~ 4 h versus ~ 16 h) and the liquid β Gal assay. A typical experiment using the liquid β Gal assay showed activation of the interaction of Rev with NP98 of $>79\times$, with NP153 of $14\times$, and with NP214 of $>78\times$. In the same experiment, the activation of the complete and the C terminus of TAP with these nucleoporins yielded values of $2\text{--}6\times$ and $5\text{--}6\times$, respectively. These assays also showed the expected preferential interactions of Rev with NP-Nup98 and NP-Nup214 as previously published (Fritz & Green, 1996; Stutz et al., 1996).

Interestingly, we also observed that the M1 mutations introduced into Ce-NXF-1 and hTAP did not abolish interaction with any of the FG-repeat domains tested, although these mutants lack the ability to accumulate at the nuclear rim (Fig. 5). Although the two assays address the association of NXF/TAP with nucleoporins, they are of different natures, which may explain this discrepancy. In the yeast two-hybrid assay, interactions with individual nucleoporins are analyzed, whereas the rim accumulation measures the association with NXF/TAP with the complete set of possible interaction partners at the NPC. Alternatively, our yeast two-hybrid data may indicate that Ce-NXF-1 is able to interact with generic FG-repeats, whereas nucleoporins distinct from those tested provide the key link to the NPC.

Ce-NXF-1 is a nucleocytoplasmic shuttle protein

We have shown that Ce-NXF-1 is essential for mRNA export (Fig. 1), that it binds RNA directly (Fig. 2), and is a nuclear protein (Fig. 3). To further understand the mechanism of Ce-NXF-1-mediated RNA export, we investigated whether it is a nucleocytoplasmic shuttle protein. We studied the trafficking of Ce-NXF-1 by using a polyethylene glycol (PEG)-mediated fusion assay in HeLa cells. We generated an expression vector that provides the SV40 NLS as a strong import signal to obtain exclusive nuclear localization of the GFP- β Gal-tagged Ce-NXF-1. Due to the size of the hybrid protein, this allowed us to distinguish active export from diffusion. The transfected HeLa cells were mixed with an excess of untransfected cells, and the next day, the cells were subjected to fusion using PEG in the presence of cycloheximide. As shown in Figure 7, the intact Ce-NXF-1 translocated efficiently to the acceptor nuclei. No translocation was observed of the SV40-GFP- β Gal protein in the absence of an export signal (Fig. 7). These data demonstrated that the Ce-NXF-1 is a *bona fide* shuttle protein. Therefore, nucleocytoplasmic shuttling is another common feature among the *C. elegans* and the human TAP/NXF proteins (Bear et al., 1999; Kang & Cullen, 1999; Bachi et al., 2000).

DISCUSSION

In this study, we report that the gene product encoded by the C15H11.3 (termed Ce-NXF-1), is an ortholog of

FIGURE 7. Ce-NXF-1 is a nucleocytoplasmic shuttle protein. Ce-NXF-1 was cloned into the SV40NLS-GFP- β Gal vector. HLtat cells were transfected, mixed with an excess of untransfected cells and subjected to fusion using polyethylene glycol in the presence of cycloheximide as described (Bear et al., 1999). After 1 h, the cells were fixed with 3.7% formaldehyde. The strong fluorescent nucleus represents the donor.

the hTAP and Mex67p mRNA export factors. We demonstrated that Ce-NXF-1 is an RNA-binding protein and is essential for the nuclear export of poly(A)-containing mRNAs in *C. elegans*. Ce-NXF-1 is a nucleocytoplasmic shuttle protein that accumulates in the nucleoplasm and at the nuclear rim and interacts with FG-repeat domains of nucleoporins. Therefore, like hTAP and Mex67p, the Ce-NXF-1 has the features necessary for an export receptor that directly binds RNA and tethers it to the NPC for export.

Although Ce-NXF-1 shares only limited homology of 42% with hTAP and of 33% with Mex67p, they share several functional features. Both Ce-NXF-1 and hTAP (Bear et al., 1999; Kang & Cullen, 1999) contain two independent determinants located at the N- and C-terminus that contribute to nucleoplasmic accumulation. Examining the import of Ce-NXF-1, we found that both signals promote nuclear accumulation but were unable to act as active NLS. Analyzing the import of hTAP, we had reported that its N-terminal NLS could act as an active import signal, whereas the C-terminal signal promoted nuclear accumulation rather than being an active NLS (Bear et al., 1999). The hTAP NLS is located between amino acids 67–100 and contains several crucial arginine residues (Bear et al., 1999; Kang & Cullen, 1999). Recently, transportin was shown to mediate hTAP's import by binding to this NLS (Truant et al., 1999; Bachi et al., 2000). Sequence alignments and motif searches did not reveal any resemblance to this NLS within Ce-NXF-1. This observation may explain the inability of Ce-NXF-1 to promote active nuclear import. Although other aspects of Ce-NXF-1 trafficking were similar to that of hTAP, we cannot rule out the possibility that Ce-NXF-1 contains an NLS that mediates active import in *C. elegans* but not in human HeLa cells. In any case, whether the nuclear import is mediated via an active process or via diffusion, we found that the Ce-NXF-1 is able to actively export from the nucleus. In addition, we found that the N- and the C-terminus of Ce-NXF-1 contain transferable signals that confer nucleoplasmic accumulation to a neutral protein moiety like GFP.

We also found that hTAP and Ce-NXF-1 utilize shared signals at their C-termini for the rim association. These signals are unique to the TAP/Mex67p family and are not found in other known proteins. Further dissection of these signals revealed that they include four clusters of conserved residues that contribute independently to the accumulation of the TAP/NXF proteins on the nuclear membrane. Analyzing the M1 mutations in Ce-NXF-1 and hTAP in more detail, we found that they still interacted with the FG domains of Nup98, Nup153, and Nup214. In agreement with this finding, Bachi et al. (2000) also observed that mutants of the C-terminal portion of hTAP that abolished nuclear rim accumulation still interacted with nucleoporins. Alternatively, this data led us to speculate that these regions may provide

binding sites for NPC components other than those tested. Comparison of the nucleoporin interactions with the TAP proteins to those with Rev/CRM1 in the yeast two-hybrid assay, we noted two major differences. First, both TAP proteins interacted similarly with all three NP-domains tested, whereas Rev/CRM1 preferentially interacted with Nup98 and Nup214 (Fritz & Green, 1996; Stutz et al., 1996; our own observation). From other studies, we know that the Rev export proceeds via Nup98 and Nup214 (Zolotukhin & Felber, 1999) and not via any generic FG-repeat-containing nucleoporins. In this case, the efficiency of the yeast two-hybrid interactions of Rev/CRM1 with nucleoporins (Fritz & Green, 1996) was predictive of their involvement in export. In addition, the TAP proteins interacted overall weaker with these nucleoporins than Rev/CRM1. Therefore, although hTAP (Katahira et al., 1999; Bachi et al., 2000) and Ce-NXF-1 can interact with Nup98, Nup153, and Nup214, nucleoporins other than those tested in this work may link the TAP proteins to the NPC. Hence, the dedicated nucleoporin partners may still remain to be elucidated.

Although the basic mRNA utilization mechanisms are conserved between *C. elegans* and *Homo sapiens*, these two species differ substantially in the cistronic organization of their genes and subsequently in pre-mRNA organization and splicing (for recent reviews, see Blumenthal, 1998). Despite the differences in post-transcriptional processes leading to the generation of mature mRNAs in these species, important aspects of the eukaryotic mRNA export are conserved. The orthologous TAP proteins of *C. elegans* and *H. sapiens* not only share the basic mechanism of mRNA export, but also have major functional determinants conserved that are essential for nucleocytoplasmic trafficking.

MATERIALS AND METHODS

Recombinant DNA

Total RNA of the *C. elegans* N2 strain was isolated using the RNazol (Tel-test, Inc.) followed by DNase I treatment. cDNAs encoding the predicted complete and partial proteins were generated by reverse transcription, followed by PCR amplification and cloning into the Bluescript KS+ vector. The cloned regions span the amino acids 62–411 for C15H11.3 (Ce-NXF-1), and amino acids 98–264 and amino acids 2–421 for C15H11.6 (Ce-NXF-2), respectively. The cDNA encoding the complete Ce-NXF-1 protein was PCR amplified and cloned into TOPO TA cloning vector pCR®2.1-TOPO® (Invitrogen). This cDNA clone contains two nucleotide changes at T967C changing S323P and A1713G, which is a silent change as compared to the published sequence in cosmid C15H11. pCMV-GFPsg25 contains the GFP gene driven by the human cytomegalovirus (CMV) promoter (Stauber et al., 1998). The TAP coding sequences were PCR amplified and cloned into the *SacII* and *NheI* sites located at the 5' end of GFP. pCMVGFP-βGal (Bear et al., 1999) encodes the GFP-βGal

hybrid protein. TAP1-628 was inserted into the *SacII* and *NheI* sites. p40NLS-GFP- β Gal contains the simian virus 40 nuclear localization signal (MAPKKRKRVAAA) inserted into *SacII*-*NheI* sites located at the 5' end of GFP in pCMVGFP- β Gal and encodes a SV40 NLS-GFP- β Gal hybrid protein. TAP fragments were inserted in frame into the *EcoRI* and *BlnI* sites located at the C-terminus of β Gal. To construct pCMV-HA-Ce-NXF-1, the complete coding sequence was PCR amplified using a sense primer that provides the HA epitope and cloned into *BssHII* and *XhoI* sites of pCMV37M1-10D (Schneider et al., 1997). pCMV-GST-Ce-NXF-1 contains the GST-NXF cDNA inserted into *BssHII* and *XhoI* sites of pCMV37M1-10D. For bacterial expression, TAP was inserted into the *BamHI* and *EcoRI* sites of pGEX-2T. For yeast expression, the indicated TAP coding sequences or the HIV-1 Rev NES (amino acids 72–84) were cloned into the *EcoRI* and *BamHI* sites of pEG202. The nucleoporin repeat domains of Nup98 (amino acids 2–494), Nup214 (amino acids 1864–2090), and Nup153 (amino acids 894–1475) were cloned into the *EcoRI* and *XhoI* sites and the *XhoI* site, respectively, of pJG4-5. Mutations were introduced by using oligonucleotide-directed mutagenesis or by QuickChange site-directed mutagenesis protocol (Stratagene). All the mutations and PCR products were confirmed by double-stranded sequencing by the PCR-assisted fluorescent terminator method and analyzed by using Sequencher (Genes Code Corp.).

RNAi

The plasmids containing the C15H11.3 and C15H11.6 sequences were linearized with *XbaI* and *XhoI*, respectively. Sense and antisense RNAs were synthesized *in vitro* by using T3 and T7 polymerases (Ambion), respectively, followed by RNase-free DNase treatment at 37°C for 15 min and heat inactivation of the enzyme at 70°C for 15 min. Five microliters of sense and of antisense RNAs were mixed, heated to 80°C for 1 min with a 30-min ramp to 37°C in the Perkin Elmer 9600 PCR machine, and frozen in 10- μ L aliquots. Two microliters of the annealed dsRNA were diluted 1:10 in sterile distilled water prior to microinjection or soaking of the nematodes. Mock-treated nematodes were incubated in the same buffer without RNA.

Standard methods were used to culture the wild-type strain N2 of the nematode *C. elegans*. For microinjection, the dsRNA was injected into one arm of the gonad of L4 stage *C. elegans*. For the soaking experiments, L4 nematodes were transferred into 20 μ L of the diluted dsRNA solution or 1:10 diluted RNA synthesis buffer in a 0.1-mL sterile Eppendorf tube. After incubation overnight at 20°C, the nematodes were transferred onto a plate containing fresh bacteria strain OP 50 and they were transferred to a new plate every 24 h. The injected or soaked animals were monitored for movement and survival; the embryos laid were monitored for hatching. For RNA localization, several nematodes or embryos were collected, freeze-cracked, and fixed as previously described (Seydoux & Fire, 1995). *In situ* hybridization was performed using a biotinylated T₃₀ oligodeoxyribonucleotide hybridization probe and streptavidin conjugated to Alexa 594 red fluorophore (Molecular Probes). The cell nuclei were visualized by staining with Hoechst 33342 blue fluorescent dye. Fluorescence was detected using an inverted microscope (Zeiss Axiovert 135TV)

and appropriate filter sets. The images were captured by using a digital charge-coupled device (CCD) camera (Photometrics) and processed by using IPLab Spectrum software. Digital images were pseudocolored and merged using Adobe Photoshop software.

In vitro RNA binding

The DNA template for human histone H4 mRNA (Jarmolowski et al., 1994; Saavedra et al., 1997) was prepared by PCR using a sense primer containing the T7 promoter. *In vitro* transcription, radioactive labeling and RNA purification were performed using standard techniques. The RNAs were mixed in binding buffer (15 mM HEPES, pH 7.7, 50 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 5% glycerol, 0.5 mM DTT, 0.2% Triton X-100) with recombinant GST-fusion proteins immobilized on glutathione-Sepharose beads. After an incubation for 15 min at room temperature followed by three washes with binding buffer, the radioactivity retained on the beads was quantitated by Cerenkov counting.

Indirect immunofluorescence, microscopy, and fusion assay

HLtat is a HeLa-derived cell line expressing human immunodeficiency virus type 1 (HIV-1) Tat. Cells were transfected according to SuperFect (Qiagen) or FuGENE™ 6 protocol (Roche Molecular Biochemicals). Indirect immunofluorescence, microscopic analysis of transfected cells and fusion assays were performed as described previously (Bear et al., 1999). For digitonin treatment, the cells were washed and incubated for 1.5 min in 0.004% digitonin in PBS. The cells were fixed immediately in 3.7% formaldehyde.

Yeast strains and two-hybrid interaction analysis

The pEG202- and pJG4-5-derived plasmids were transformed into EGY48 and RFY206 yeast strains, respectively, and were subjected to the interaction-mating assay under selected growth conditions according to the standard protocol (Finley & Brent, 1994, 1995). Replica-plated filters were assayed for β Gal production. The detection of blue colonies required an overnight incubation. For the liquid β Gal assay, the indicated plasmids were cotransformed into EGY48, and 5-mL cultures were grown overnight in the presence of glucose. The next day, the yeast cells were washed, refed with galactose-containing medium, and grown overnight. The β Gal activity was determined using similar amounts of cells (optical density of 0.5 at 600 nm) for 30 min.

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