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A Role for Nucleoporin FG Repeat Domains in Export of Human Immunodeficiency Virus Type 1 Rev Protein and RNA from the Nucleus

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The human immunodeficiency virus type 1 Rev protein contains a nuclear export signal (NES) that is required for Rev-mediated RNA export in mammals as well as in the yeast *Saccharomyces cerevisiae*. The Rev NES has been shown to specifically interact with a human (hRIP/RAB1) and a yeast (yRip1p) protein in the two-hybrid assay. Both of these interacting proteins are related to FG nucleoporins on the basis of the presence of typical repeat motifs. This paper shows that Rev is able to interact with multiple FG repeat-containing nucleoporins from both *S. cerevisiae* and mammals; moreover, the ability of Rev NES mutants to interact with these FG nucleoporins parallels the ability of the mutants to promote RNA export in yeast and mammalian cells. The data also show that, after *Xenopus* oocyte nuclear injection, several FG nucleoporin repeat domains inhibit the export of both Rev protein and U small nuclear RNAs, suggesting that these nucleoporins participate in Rev-mediated and cellular RNA export. Interestingly, not all FG nucleoporin repeat domains produced the same pattern of RNA export inhibition. The results suggest that Rev and cellular mediators of RNA export can interact with multiple components of the nuclear pore complex during transport, analogous to the proposed mode of action of the nuclear protein import receptor.

The human immunodeficiency virus type 1 Rev protein directs the export of unspliced or partially spliced viral transcripts from the nucleus to the cytoplasm in mammalian cells. Rev contains an RNA binding domain, which binds the Rev response element present on target transcripts. Export activity is mediated by a genetically defined effector domain, which has also been identified as a nuclear export signal (NES) (3, 8). Rev has also been shown to promote the export of Rev response element-containing transcripts in *Xenopus* oocytes (9), insect cells (21) and *Saccharomyces cerevisiae* cells (38), suggesting that the cellular component(s) targeted by the Rev NES is evolutionarily conserved. Nonviral nuclear export signals related to the Rev NES have also been identified in TFIIIA (8, 11) and PKI, the cyclic AMP-dependent protein kinase inhibitor (10, 39).

Recently, a yeast two-hybrid approach led to the identification of a yeast protein (yRip1p) (37) and a human protein (hRIP/RAB1) (2, 13), both of which interact with the Rev NES. yRip1p and hRIP exhibit a series of degenerate tetrapeptide repeats containing the amino acids phenylalanine and glycine (FG repeats); these repeats are a trademark of a class of yeast and vertebrate nuclear pore proteins called FG nucleoporins (34). We have previously shown that Rev also interacts with the FG repeats of two other yeast nucleoporins, yNup159p/RAT7 and yNUP100p (37).

There is evidence that the interaction between yRip1p or hRIP and the Rev NES is specific and relevant for RNA export in both yeast and mammalian systems, respectively (2, 4, 37). The PKI NES also interacts with FG nucleoporin repeats in the two-hybrid system and was proposed to mediate the export of protein substrates by accessing the same export pathway(s) as Rev (10).

The *Xenopus* oocyte system provides an entirely different approach to study Rev-mediated RNA export (9). In this system, export is saturable by a peptide conjugate comprising the Rev NES coupled to bovine serum albumin (BSA) (Rev NES-BSA); this conjugate competes with the nuclear export of 5S rRNA and U1 small nuclear RNA (snRNA), but not mRNA, tRNA, and rRNA; this indicates that in oocytes the Rev NES mediates export by usurping a pathway dedicated to a specific class of RNAs (8). The oocyte nuclear proteins interacting with the Rev NES are unknown.

In this report, we present studies that link features of the yeast, mammalian tissue culture, and *Xenopus* oocyte systems. We found that the Rev NES interacts with an expanded panel of FG nucleoporins and shows a preference for a subset of FG repeats. A series of Rev NES mutants that affect Rev function in vivo show parallel effects on the two-hybrid interactions with all the FG nucleoporin repeat domains (2, 13, 30). This indicates a generic structural interaction between the Rev NES and FG repeats, and it suggests that Rev can target multiple FG nucleoporins in vivo. A role for these repeats in Rev-mediated export is supported by their ability to prevent cytoplasmic accumulation of Rev after nuclear injection into *Xenopus* oocytes. Finally, the injection of different FG repeats results in distinct patterns of RNA export inhibition, suggesting that individual FG repeats have different affinities or specificities for endogenous NESs.

MATERIALS AND METHODS

Two-hybrid cloning and mating assay. The two-hybrid bait constructs were obtained by cloning wild-type or mutant Rev sequences (30) as *Eco*RI-*Xho*I PCR fragments into the pEG202+PL vector (HIS3 2 μm) cut with *Eco*RI and *Xho*I, thereby creating in-frame fusions with the LexA DNA binding domain (7, 17). The two-hybrid prey constructs were obtained by cloning the different FG repeat domains as *Eco*RI-*Xho*I PCR fragments into the pJG4-5 vector (TRP1 2μm) cut with *Eco*RI and *Xho*I, thereby creating in-frame fusions with an *Escherichia coli* activation domain (7, 17). The PCR fragments encoded the indicated amino acids of the following proteins: yRip1p (37), 151 to 275; hRIP/RAB1 (2, 13), 388 to 562; yNup159p/RAT7 (15), 497 to 701; hCAN/Nup214 (26), 1691 to 1894; yNup49p (41, 42), 7 to 239; yNup57p (16), 1 to 247; yNup100p (41), 278 to 539;

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Nup145p (6, 40), 24 to 216; rNup98 (32), 41 to 515; yNup1p (5), 438 to 737; yNsp1p (18), 296 to 606; and yNup2p, 182 to 537. The interaction between Rev and yNup116p was examined by cloning an *Eco*RI-*Xho*I PCR fragment encoding amino acids 459 to 672 of yNup116 (41) into the pEG202+PL bait vector and by cloning wild-type Rev as an *Eco*RI-*Xho*I fragment into the prey vector pJG4-5; no interaction between Rev and yNup116p FG repeats was detected when yNup116p was expressed from the prey construct (37). The y*RIP1* prey truncations were obtained by cloning *Eco*RI-*Xho*I PCR products into the pJG4-5 vector.

A mating assay was used to examine two-hybrid interactions. The bait constructs were transformed (20) into RFY206 (*MAT***a** *his3Δ200 leu2-3 lys2Δ201 ura3-52 trp1*Δ::*hisG*) with the *lacZ* reporter plasmid pSH18-34 (*URA3* 2μm) (7). The prey constructs were transformed into EGY48 (*MAT*α *his3 leu2*::*3lexAop-LEU2 ura3 trp1 LYS*) with pSH18-34. The bait- and prey-containing yeast strains were mated, and diploids were selected on His^- Trp^- Ura^-–2% glucose plates (7). Two-hybrid interactions between the bait and prey fusion proteins were determined by replica plating the diploids to His^- Trp^- Ura^-–3% galactose–1% raffinose–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates as described previously (37). The β-galactosidase activities in the diploid strains (see Table 2) were determined by using a liquid assay as previously described (28).

***E. coli* GST fusion proteins.** The glutathione *S*-transferase (GST) fusion constructs were obtained by cloning the different FG repeat regions as *Eco*RI-*Xho*I PCR fragments into pGEX4T-1 (Pharmacia) cut with *Eco*RI and *Xho*I. The GST-yRip1p fusion constructs encoded amino acids 153 to 219 (five FG repeats) or 121 to 230 (nine FG repeats) of yRip1p. The GST-yNup159p/RAT7, GST-yNsp1p, and GST-yNup116p fusion constructs encoded the same FG repeat regions as the two-hybrid prey and bait constructs described above. The GST fusion constructs were introduced into *E. coli* BL21 (DE3) cells (Novagen) and purified on glutathione agarose beads (Sigma) as described previously (36). The elution of recombinant proteins was performed in phosphate-buffered saline (PBS) buffer containing 20 mM glutathione. Recombinant proteins were concentrated and desalted with microconcentrator devices (Pall Filtron).

***Xenopus* oocyte injections.** Oocyte injections and analyses of microinjected RNA by denaturing gel electrophoresis and autoradiography were performed as described previously (24); the bands were quantified with a PhosphorImager. The injected samples contained 5 mg of recombinant protein per ml. In vitro translation and microinjection of in vitro-translated protein into oocytes, isolation of protein from oocytes, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis were all carried out as described previously (25). For in vitro translations, the human immunodeficiency virus Rev cDNA was cloned into the *Nco*I and *Hin*dIII sites of the pRSETB vector (Invitrogen Corporation). The DNA was linearized with *Hin*dIII and transcribed with T7 RNA polymerase (Promega).

RESULTS

Rev interacts with the FG repeats of multiple yeast and mammalian nucleoporins. To examine the ability of Rev to interact with a larger set of FG repeat-containing proteins, we used the two-hybrid assay to test several other yeast and mammalian proteins belonging to the three previously described subclasses of FG nucleoporins, XXFG, GLFG, and FXFG (4, 34). As shown in Table 1, Rev was found to interact with the repeat regions of yNup159p (15) and hCAN/Nup214 (26), which are the two closest relatives of hRIP (2, 13) and yRip1p (37) and the two other known members of the XXFG family. Rev also interacts with the repeats of yNup49p (41, 42), yNup57p (16), yNup100p, yNup116p (41), yNup145p (6, 40), and rNup98 (32), which are the six GLFG proteins currently identified in *S. cerevisiae* and mammals. The level of interaction between Rev and all these repeat domains varies, but there is no evidence so far that these differences are relevant to the function of Rev in vivo. As a common theme in all these Rev-interacting proteins, FG repeats are almost certainly important for the interaction. However, we found no interaction between Rev and the FXFG repeat domains of yNsp1p (18), yNup1p (5), and yNup2p (29) (Table 1). A two-hybrid interaction between Rev and the repeat region of the human Nup153 FG nucleoporin was recently reported (12). The repeat region of Nup153 tested in that study contains both FXFG repeats and more degenerate XXFG repeats at the carboxy terminus (4); it is not known whether the XXFG repeats and/or the FXFG repeats are responsible for the interaction with Rev.

TABLE 1. Two-hybrid interactions between wild-type Rev and FG nucleoporins[a]

Repeat	Nucleoporin	Rev interaction[b]
XXFG	yRip1p	4
	hRIP/RAB1	3
	yNup159p/RAT7	1
	hCAN/Nup214	5
GLFG	yNup49p	2
	yNup57p	3
	yNup100p	1
	yNup116p	1
	yNup145p	2
	rNup98	6
FXFG	yNsp1p	0
	yNup1p	0
	yNup2p	0

[a] The FG repeat regions of the indicated nucleoporins were tested for interaction with wild-type Rev by a two-hybrid mating assay (7); the FG repeats were expressed as prey fusions, and wild-type Rev was expressed as a bait fusion, except in yNup116p, in which the prey and bait were reversed.

[b] Rev interaction is expressed as the relative intensity of blue color (0 to 6, least to most intense) reached by the diploid strains on X-Gal indicator plates after 2 days at 30°C.

Both the FG repeats and the linker sequences of yRip1p are important for interaction with Rev. To assess the relative importance of the yRip1p FG repeats and spacer sequences, different regions of the yRip1p protein were tested for interaction with Rev in the two-hybrid assay (Fig. 1). In general, the apparent strength of the interaction with Rev decreased with the size of the yRip1p piece analyzed: no interaction with yRip1p portions smaller than 80 amino acids and containing fewer than seven repeats was observed. Similarly, the very strong signals observed with the rNup98 and hCAN/Nup214 fusion constructs (Table 1), which encode 30 and 15 FG repeats, respectively, were also weakened by progressive deletions (data not shown). However, the size of the interacting region and the number of repeats were not the only determinants, since regions of yRip1p containing from 8 to 13 FG repeats interacted less well than the region between amino acids 151 and 275, which contains only 7 repeats and was initially isolated in the two-hybrid screen (37). Moreover, the Rev-interacting XXFG and GLFG repeat domains are rich in asparagine, serine, and threonine and devoid of acidic residues. In contrast, the spacer sequences of the noninteracting FXFG repeat domains are highly charged (4). As all of these fusion proteins were expressed at comparable levels in yeast cells (data not shown), the repeat spacing and/or the sequences between the repeats contribute directly or indirectly to the interaction with the Rev NES.

The Rev-FG nucleoporin interactions are affected similarly by Rev mutations. As described above, there is an excellent correlation between the relative biological activity of a set of Rev NES mutants in mammalian cells (30) and their ability to interact with hRIP/RAB1 in the two-hybrid system (2). Although Rev's activity in *S. cerevisiae* is more modest (38), all the mutants tested have similar effects in yeast and mammalian cells: strong mutants are strong and weak mutants are weak in both systems (Table 2; also reference 37). Moreover, there is the same excellent correlation between biological activity and the ability of the mutants to interact with yRip1p in the two-hybrid system, consistent with yRip1p being a functional target for Rev in *S. cerevisiae* (Table 2).

The same mutant series was tested for interaction with all

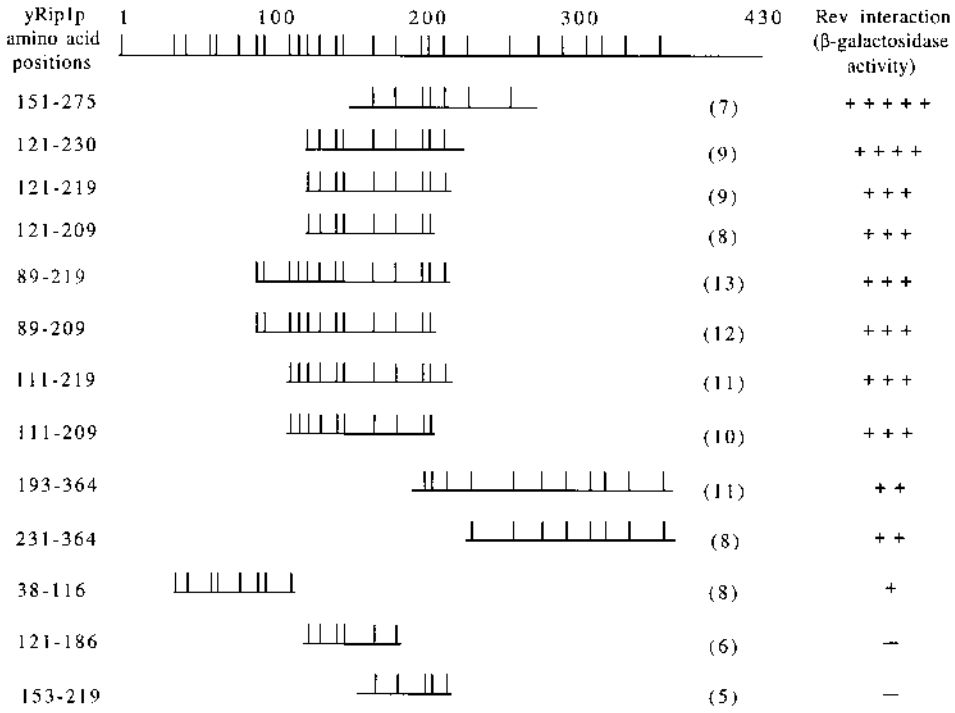

FIG. 1. Two-hybrid interactions between Rev and yRip1p truncations. The ability of a Rev bait fusion to interact with a series of prey fusions encoding various portions of yRip1p was examined by the two-hybrid mating assay (7). The yRip1p amino acids encoded by each prey fusion are indicated on the left. Each vertical bar corresponds to one FG repeat; the number of FG repeats present in each fusion is indicated in parentheses. The pluses reflect the relative intensities of blue color produced by the diploid strains on X-Gal indicator plates after 2 days at 30°C, with + + + + + being dark blue and + being light blue (−, no color). The complete yRip1p prey fusion (amino acids 1 to 430, shown at the top of the figure) was not suitable for comparison with the other fusions, as it was less stable and induced lower levels of β-galactosidase activity.

TABLE 2. Correlation of abilities of Rev-NES mutants to function in yeast or mammalian cells with their abilities to interact with yRip1p or hRIP/RAB1 in the two-hybrid assay[a]

Rev variant	Yeast cells		Human cells	
	In vivo Rev activity (resistance [mM $CuSO_4$])	yRip1p interaction (% β-Gal activity)	hRIP/RAB1 interaction (% β-Gal activity)	In vivo Rev activity[b]
wt	1.1	100	100	++
M15	1.1	86	57	++
M16	1.1	92	106	++
M17	1.1	52	25	++
M19	1.1	108	179	++
M25	1.1	72	187	++
M20	1.0	8.4	16	+
M24	0.9	7.0	12	+
M18	0.9	4.3	10	+
M10	0.8	<1	1	–

[a] The Rev effector domain mutations listed in the stub (M15 through M10) and their effects on export activity in mammalian cells and in yeast versus wild-type (wt) Rev have been reported previously (30, 37); in yeast cells, Rev activity is reflected by changes in the copper resistance of a relevant reporter strain (38). The levels of two-hybrid interaction between the Rev variants and yRip1p or hRIP/RAB1 are expressed as percentages of the β-galactosidase (β-Gal) activity measured in the presence of wild-type Rev. The data for hRIP/RAB1 are from Bogerd et al. (2).

[b] ++, high-level activity; +, low-level activity; −, no activity detected.

the FG repeat domains listed in Table 1 except yNup49p, yNup116p, and yNup145p. Interestingly, the interaction pattern was identical; i.e., every tested FG nucleoporin showed a comparable decrease in the strength of interaction with the different Rev mutants (Fig. 2). The data indicate that the interaction between Rev and FG repeats is generic.

Injection of FG repeats inhibits nuclear export of U snRNAs and Rev in *Xenopus* oocytes. Rev-mediated RNA export in *Xenopus* oocytes is inhibited efficiently by the injection of saturating amounts of the Rev NES-BSA peptide conjugate (8). This competition is also seen when the export substrate is radiolabelled NES-BSA conjugate itself, Rev protein, or U1 or U5 snRNAs (reference 8 and our unpublished data). On the basis of the two-hybrid data, it is likely that this inhibition is due to the titration of NES binding sites on FG nucleoporins required for nuclear export. Injection of isolated FG nucleoporin repeats should similarly block nuclear export by competing with endogenous FG nucleoporins for NES binding. To test this prediction, we expressed a number of FG repeats as GST fusion proteins in *E. coli* and, after purification, assayed their effects on oocyte nuclear export.

We first assayed the effect on RNA export of GST and two GST-yRip1p fusion proteins, namely, GST fused to five-FG repeats and GST fused to nine-FG repeats. The five-repeat fusion failed to interact with Rev NES in the two-hybrid assay, but the nine-repeat fusion showed a strong NES interaction (Fig. 1). We microinjected the following as substrates for the export assay: a mixture containing U1 and U5 snRNAs, whose export is inhibited by the Rev NES-BSA conjugate; an mRNA and tRNA, whose export is not Rev NES sensitive; and U6 snRNA, which is not exported from the nucleus and is a con-

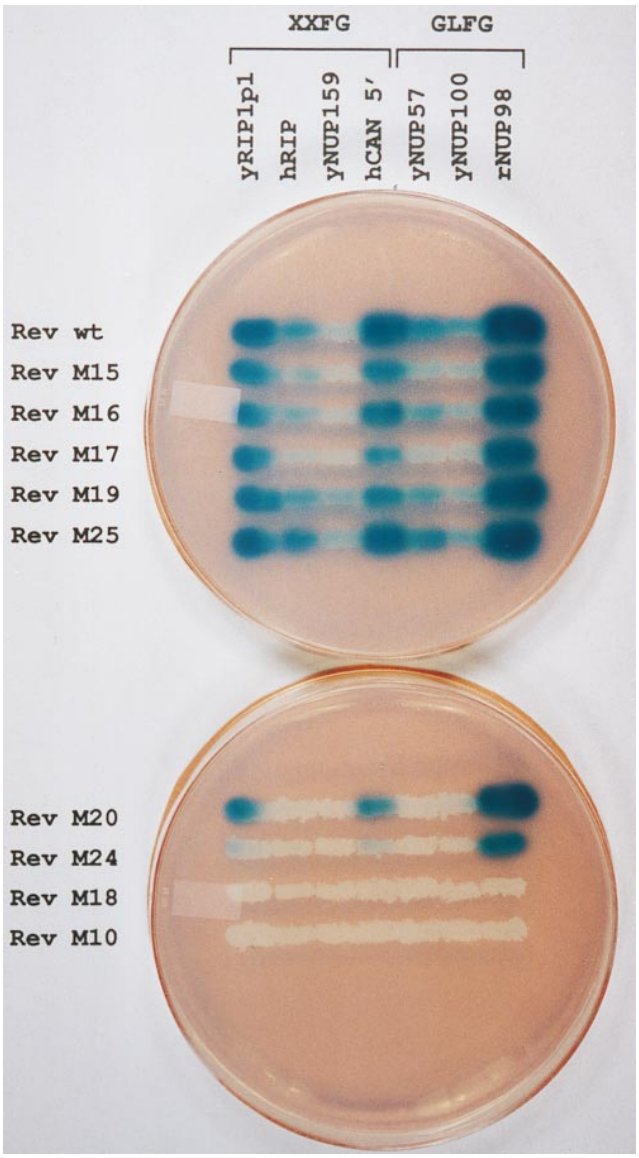

FIG. 2. Matrix of two-hybrid interactions between wild-type (wt) or mutant Rev and FG nucleoporins. The two-hybrid interactions were examined in diploid strains expressing the indicated Rev bait and FG repeat prey fusions. The bait constructs, shown on the left, encode the wild type or the Rev NES mutants M15 through M10 (30). The prey constructs, indicated at the top of the figure, encode amino acids 151 to 275 of yRip1p (seven FG repeats), amino acids 388 to 562 of hRIP (six FG repeats), amino acids 497 to 701 of yNup159p (17 FG repeats), amino acids 1691 to 1894 of hCAN/Nup214 (13 FG repeats), amino acids 1 to 247 of yNup57p (14 FG repeats), amino acids 278 to 539 of yNup100p (15 FG repeats), and amino acids 41 to 515 of rNup98 (32 FG repeats). The diploid strains were replica plated to Ura^- His^- Trp^-–3% galactose–1% raffinose–X-Gal indicator plates and incubated for 2 days at 30°C.

trol for nuclear integrity (reference 8 and references therein). While neither GST nor the five-repeat fusion affected RNA export (Fig. 3, lanes 4 to 9), the nine-repeat fusion specifically inhibited U snRNA export to a level of roughly 50% (Fig. 3, lanes 10 to 12; Table 3). This result not only provides strong evidence that FG nucleoporin repeats are a target of factors involved in nuclear export but also is in excellent agreement with the two-hybrid assay results, suggesting that the effect of the FG nucleoporin repeats is due to their association with endogenous and functionally relevant NES-containing proteins.

We next wished to examine the effect on export of the other FG repeats tested in the two-hybrid assay. This effort was hampered by our inability to express most of the repeat fusion proteins in a stable, soluble form in *E. coli*. However, it was possible to obtain fusion proteins containing repeats from yNup159p/RAT7, yNsp1p, and yNup116p. Injection of all three fusion proteins into *Xenopus* oocyte nuclei also resulted in the efficient inhibition of U1 and U5 snRNA export (Fig. 4, lanes 7 to 15; Table 3).

To obtain additional evidence that the FG repeat inhibition was due to an interaction with endogenous Rev NES-like signals, the effect of the fusion proteins on the export of in vitro-translated Rev protein was assayed. This experiment was possible since the rate of Rev export from the nucleus is greater than the rate of its reimport (Fig. 5, lanes 1 to 6). None of the FG nucleoporin repeats completely inhibited Rev export, but the partial inhibitory effect of the yNup159p/RAT7 and yNsp1p repeat fusions was readily detectable (Fig. 5, lanes 13 to 18; Table 3). yNup116p repeats behaved similarly to yNsp1p and yNup159p/RAT7 repeats in other experiments (data not shown). As in the U snRNA export assay (Fig. 3), the yRip1p fusion was less inhibitory; the effect of this nine-repeat fusion was reproducibly very low but detectable (Fig. 5, lanes 10 to 12; Table 3).

In addition to the quantitative differences in the ability of the fusion proteins to inhibit U snRNA export, a second difference was observed. Uniquely among the repeats tested, the yNsp1p fusion caused a significant inhibition of the export of two different mRNAs, which encode dihydrofolate reductase (DHFR) and histone H4 (Fig. 4, lanes 10 to 12). Of the other repeat fusions tested, neither the yNup159p/RAT7 nor the nine-repeat fusion from yRip1p affected mRNA export (Fig. 4, lanes 7 to 9; Fig. 3, lanes 10 to 12). The yNup116p fusion also appeared to have a small effect on mRNA export, although this result is difficult to interpret as injection of this protein resulted in some mRNA instability (Fig. 4, lanes 13 to 15). None of the fusions detectably affected tRNA export.

DISCUSSION

The data presented in this work indicate that FG repeat-containing nucleoporins interact with Rev and are part of the Rev export pathway through the nuclear pore complex, consistent with a number of genetic studies implicating FG nucleoporins in the process of yeast nuclear export (23). A yRip1p portion containing nine FG repeats interacts with the Rev NES in two-hybrid assays, while one containing five repeats does not (Fig. 1). Similarly, only injection of the nine-repeat fusion interferes specifically with the export of U1 and U5 snRNAs from *Xenopus* oocyte nuclei (Fig. 3). This inhibition recapitulates the effect of nuclear injection of the Rev NES-BSA peptide (8). The data support the relevance of the two-hybrid interactions and suggest that U snRNA export involves an endogenous Rev-like NES. They further suggest that this NES interacts with the same subset of FG repeats as Rev, in a manner that resembles the Rev NES-FG repeat interaction. A cap binding complex has been proposed to mediate U snRNA export (22). The CBP80 subunit contains a degenerate NES-like sequence (14), but we have not been able to detect a two-hybrid interaction between CBP80 and FG nucleoporins, even in the presence of overexpressed CBP20, the other subunit of the cap-binding complex (36a). One explanation for this negative result could be that CBP80 is not able to interact with FG repeats as a two-hybrid fusion protein. Another possibility is that the interaction involves an additional, as yet unidentified component (14).

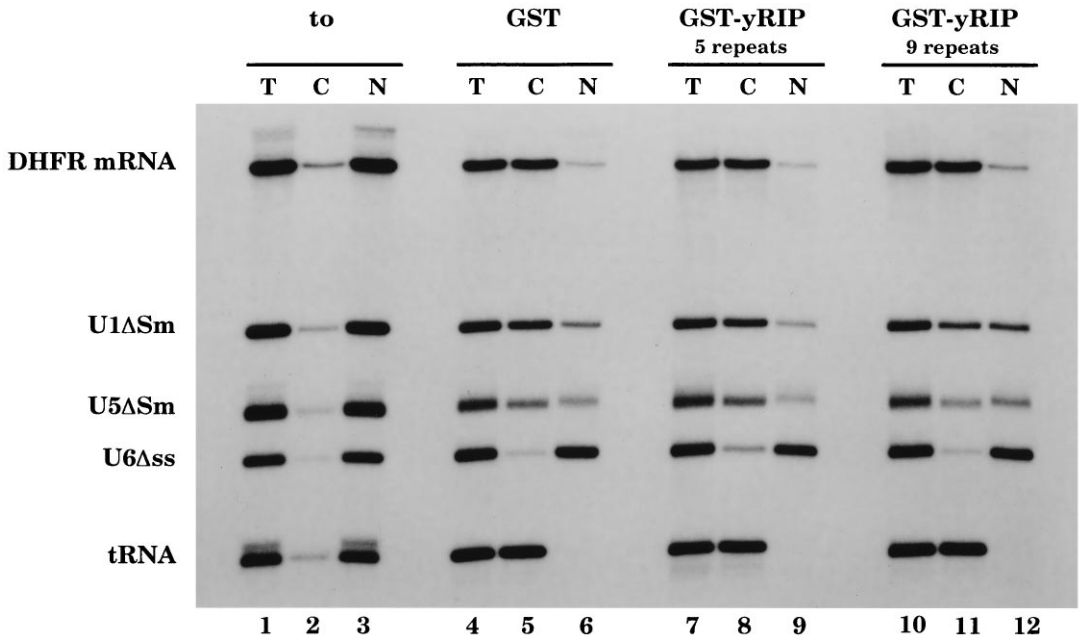

FIG. 3. Injection of GST-Rip1p inhibits U snRNA export. *Xenopus laevis* oocyte nuclei were injected with a mixture containing the following radioactively labelled RNAs: DHFR mRNA, U1ΔSm, U5ΔSm, U6Δss and human initiator methionyl tRNA in PBS. In lanes 4 to 12, the RNA mixture was coinjected with recombinant GST (lanes 4 to 6) or with GST-yRip1p fusions encoding amino acids 153 to 219 with 5 FG repeats (lanes 7 to 9) or amino acids 121 to 230 with 9 FG repeats (lanes 10 to 12). RNAs extracted either from total oocytes (T) or from cytoplasmic (C) or nuclear (N) fractions were visualized by autoradiography after separation on denaturing polyacrylamide gels and quantified with a PhosphorImager. In lanes 4 to 12, RNA was extracted 150 min after injection; in lanes 1 to 3, RNA was extracted immediately after injection ($t = 0$ [to]). Synthesis of DHFR, U1ΔSm, and U5ΔSm RNAs was primed with the m^7GpppG cap dinucleotide, whereas synthesis of U6Δss RNA was primed with γ-methyl-GTP. U6Δss is a control for nuclear integrity. See Table 3 for quantification of the data.

There is strong evidence to support a role of multiple FG repeat domains in protein import (14, 32, 33). The interactions of Rev with multiple FG nucleoporins suggest that Rev might also promote RNA export by interacting with several FG nucleoporins spanning the nuclear pore complex. This possibility is consistent with microscopy studies that have localized hRIP (2, 13), rNup98 (32), and hCAN/Nup214 (26) to the nucleoplasm and the nuclear side and the cytoplasmic side of the nuclear pore complex, respectively. Alternatively, Rev's interaction with multiple FG nucleoporins could reflect targeting to several parallel export pathways. The genetic analysis of Rev mutants and the finding that the yRIP1 gene disruption does not eliminate Rev activity in yeast cells (37) are consistent with this second possibility. In any case, one can only speculate on the relationship of the Rev-nucleoporin interaction to the mechanism of translocation across the nuclear membrane, in large part because of a lack of any detailed understanding of the position and accessibility of the FG repeats within the large and complex nuclear pore.

TABLE 3. Quantification of *Xenopus* oocyte nuclear injections

Fusion	% Inhibition of export[a] of:		
	U1ΔSm RNA	DHFR mRNA	HIV-1 Rev protein
GST-yRIP1 (five repeats)	—[b]	—	—
GST-yRIP1 (nine repeats)	49 ± 5 ($n = 3$)	—	16 ± 4 ($n = 2$)
GST-NSP1	74.5 ± 0.5 ($n = 2$)	37 ± 4 ($n = 2$)	37 ± 3 ($n = 2$)
GST-RAT7	82 ± 5 ($n = 2$)	—	58 ± 2 ($n = 2$)
GST-NUP116	79.5 ± 5.5 ($n = 2$)	ND[c]	ND[d]

[a] The inhibition of export was quantitated either by PhosphorImager analysis or by scanning of autoradiographs with a HeroLab Easy Image plus system. The values are averages of two or three experiments as indicated. The full range of variation between the experiments is indicated by the ± values. Quantitations were performed at 150 min after injection (for U1ΔSm RNA and DHFR mRNA) or at 120 min after injection (for human immunodeficiency virus type 1 [HIV-1] Rev protein).
[b] —, no measurable inhibition compared with control oocytes.
[c] Not determined because of partial mRNA degradation.
[d] Not determined.

Despite the apparently general nature of the Rev NES-FG repeat interaction, the repeats did not behave identically. For example, the nine-repeat yRip1p fusion interacts most strongly in the yeast two-hybrid assay but had less of an inhibitory effect on U snRNA export and on Rev protein export, when expressed in *E. coli* and injected into oocytes, than did the repeats from yNup159p/RAT7 or yNup116p (Fig. 3 to 5; Table 3). The many differences in the two experiments make it hard to evaluate the significance of these quantitative effects. A more notable discrepancy is that the Nsp1p FXFG repeats strongly inhibit mRNA as well as Rev and U snRNA export; however, neither the FXFG repeats of Nsp1p nor those of Nup1p or Nup2 detectably interact with Rev in the yeast two-hybrid system.

The absence of a yeast interaction between Rev and FXFG repeats could be trivial and due to mislocalization or some other unusual feature of the FXFG repeat fusion proteins in yeast cells. A more interesting explanation is that an interaction between an NES and FXFG nucleoporins requires one or more metazoan export cofactors, which are absent from yeast cells or unable to target the two-hybrid fusion proteins. Cofactors might greatly enhance the affinity of Rev for some of the nucleoporin repeats, perhaps by cooperative binding to these FG repeats. Indeed, we have failed to detect an interaction between recombinant Rev and FG repeat fusions produced in

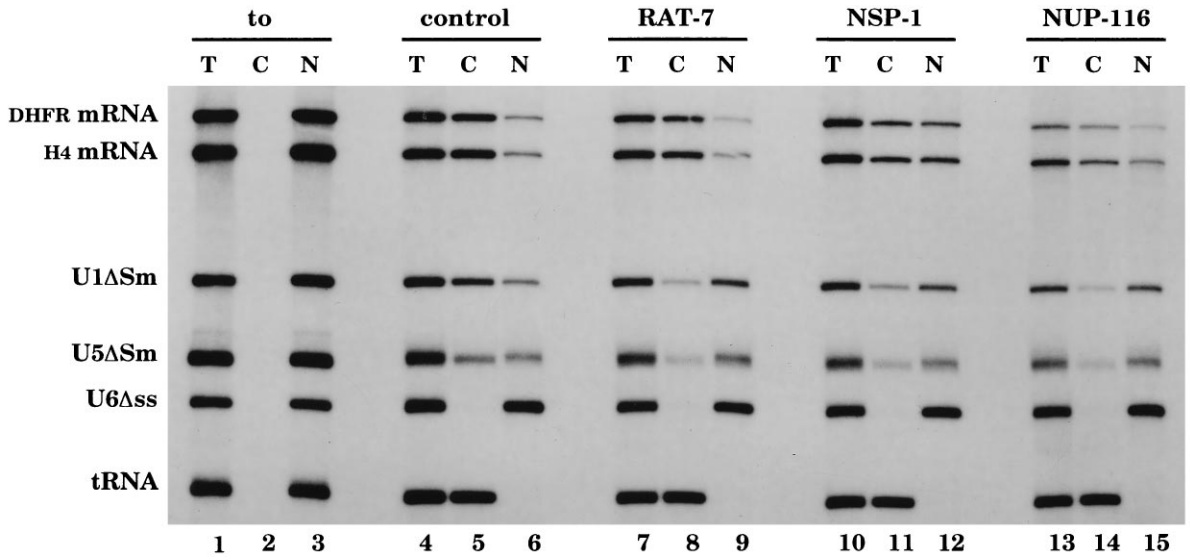

FIG. 4. Inhibition of different RNA export pathways with different FG repeat GST fusion proteins. *Xenopus laevis* oocyte nuclei were injected with the RNA mixture described in the legend to Fig. 3 but containing in addition histone H4 mRNA carrying an m^7GpppG cap structure. In lanes 7 to 15, the RNA mixture was coinjected with GST fusion proteins containing repeats from yNup159p/RAT7 (lanes 7 to 9), yNsp1p (lanes 10 to 12), and yNup116p (lanes 13 to 15). In lanes 4 to 6, the RNA mixture was injected with PBS alone. RNA extraction and analysis were performed as described in the legend to Fig. 3. See Fig. 3 legend for abbreviations and Table 3 for quantification of the data.

E. coli (36a), whereas Rev produced in a reticulocyte lysate has been reported to bind to hRIP/RAB1 (2). This could indicate that the Rev NES-FG repeat interaction is weak and aided by additional factors present in the reticulocyte lysate and *Xenopus* oocyte. The translation initiation factor eIF5A has also been shown to specifically bind the effector domain of Rev (1, 35).

Some of these putative cofactors could be different for different export pathways. Therefore, the ability of the yNsp1p fusion protein to inhibit mRNA export could indicate that the FXFG subclass of FG repeats might interact with an mRNA-specific export cofactor. However, the yNup116p fusion also weakly inhibits mRNA export, and the yNsp1p repeat fusion also inhibits both Rev and U snRNA export. Therefore, even dramatic differences in the inhibition pattern between fusion proteins could reflect differences in affinity rather than specificity for different endogenous export signals and factors. Thus, the interactions of nucleoporin repeats with endogenous export factors might be generic in nature. Finally, a mixed scenario could be that the oocyte export inhibitions are due to an interaction between nuclear export signals and FG repeats in the case of Rip1p, Nup116p, and Nup159/RAT7p but that they

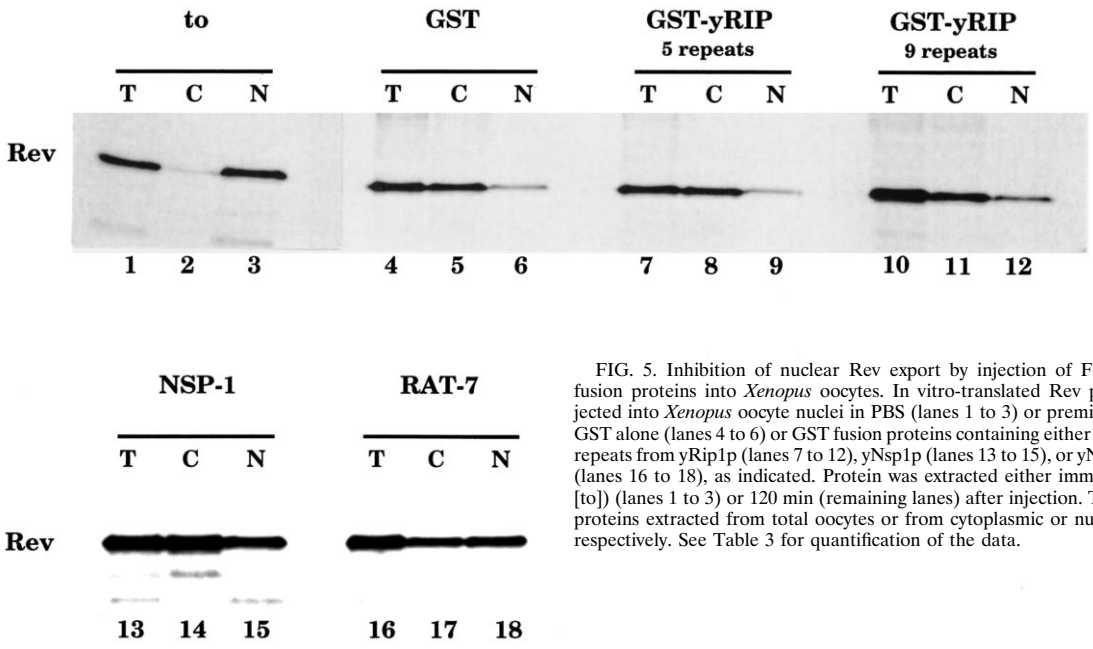

FIG. 5. Inhibition of nuclear Rev export by injection of FG repeat GST fusion proteins into *Xenopus* oocytes. In vitro-translated Rev protein was injected into *Xenopus* oocyte nuclei in PBS (lanes 1 to 3) or premixed with either GST alone (lanes 4 to 6) or GST fusion proteins containing either five or nine FG repeats from yRip1p (lanes 7 to 12), yNsp1p (lanes 13 to 15), or yNup159p/RAT7 (lanes 16 to 18), as indicated. Protein was extracted either immediately ($t = 0$ [to]) (lanes 1 to 3) or 120 min (remaining lanes) after injection. T, C, and N are proteins extracted from total oocytes or from cytoplasmic or nuclear fractions, respectively. See Table 3 for quantification of the data.

are due to the titration of a common soluble factor required for U snRNA, mRNA, and Rev export in the case of the Nsp1 FXFG repeats.

Nuclear protein import is mediated by a heterodimeric receptor, variously called importin, karyopherin, PTAC, or NLS receptor (reviewed by Gorlich and Mattaj [14]). The smaller, alpha subunit interacts directly with NLS signals, and the larger, beta subunit interacts directly with nucleoporins through their FG repeats (19, 27, 31, 33). It is possible that an analogous export factor exists and that this nuclear karyopherin beta-like protein targets a subset of substrates to the nuclear pore complex by interacting with FG repeats.

In any case, the combination of the yeast two-hybrid results, *Xenopus* oocyte injections, and Rev NES genetic analysis suggests that a U snRNA export pathway factor interacts with FG nucleoporins. The predicted cellular NES and the involvement of distinct FG nucleoporins in other export pathways are currently under investigation.

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