Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2936–2940, April 1996
Biochemistry

Amphibian transcription factor IIIA proteins contain a sequence element functionally equivalent to the nuclear export signal of human immunodeficiency virus type 1 Rev

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Communicated by W. K. Joklik, Duke University Medical Center, Durham, NC, December 8, 1995

ABSTRACT **The human immunodeficiency virus type 1 (HIV-1) Rev protein is required for nuclear export of late HIV-1 mRNAs. This function is dependent on the mutationally defined Rev activation domain, which also forms a potent nuclear export signal. Transcription factor IIIA (TFIIIA) binds to 5S rRNA transcripts and this interaction has been proposed to play a role in the efficient nuclear export of 5S rRNA in amphibian oocytes. Here it is reported that amphibian TFIIIA proteins contain a sequence element with homology to the Rev activation domain that effectively substitutes for this domain in inducing the nuclear export of late HIV-1 mRNAs. It is further demonstrated that this TFIIIA sequence element functions as a protein nuclear export signal in both human cells and frog oocytes. Thus, this shared protein motif may play an analogous role in mediating the nuclear export of both late HIV-1 RNAs and 5S rRNA transcripts.**

Human immunodeficiency virus type 1 (HIV-1) encodes a nuclear regulatory protein, termed Rev, that is essential for the expression of viral structural proteins (1–3). These structural proteins are encoded by incompletely spliced viral mRNAs that, in the absence of Rev, are sequestered in the infected cell nucleus (3–8). Rev induces the nuclear export, and hence translation, of these late viral mRNAs by binding to, and multimerizing on, a *cis*-acting RNA target site termed the Rev response element or RRE (3–14). Subsequently, this ribonucleoprotein (RNP) complex recruits a cellular cofactor that induces the entry of these viral mRNA species into a preexisting nuclear export pathway (15–18). The Rev protein domain that mediates this protein–protein interaction, termed the Rev activation domain, has been mapped to a short, ≈10-aa motif that contains three critical leucine residues (19–21) (Fig. 1). Recently, it has been demonstrated that the Rev activation domain is a nuclear export signal (NES) that induces the efficient and rapid export of proteins from the nucleus (15, 24, 25).

Transcription factor IIIA (TFIIIA) is an RNA polymerase III transcription factor that binds to the internal control region of 5S rRNA genes and is critical for the assembly of a functional transcription initiation complex (26). TFIIIA also binds 5S rRNA transcripts (27, 28) and is found in large amounts in the cytoplasm of amphibian oocytes in the form of a 7S RNP storage particle that consists of one molecule of TFIIIA bound to one molecule of 5S rRNA (28, 29). Evidence has been presented indicating that this direct interaction between TFIIIA and 5S rRNA is also important for the efficient nuclear export of 5S rRNA in amphibian oocytes (30), thus suggesting that TFIIIA, like Rev, might function as a sequence-specific nuclear RNA export factor.

The possibility that TFIIIA might mediate 5S rRNA export through a cellular pathway similar to the one used by HIV-1 Rev was recently raised by the finding that the microinjection of peptides encoding the Rev activation domain/NES into frog oocytes results in specific inhibition of not only Rev-dependent RNA export but also of 5S rRNA export (15). The observation (15) that TFIIIA proteins contain a short sequence element located toward their C termini that displays homology to the leucine-rich activation domain of HIV-1 Rev (Fig. 1) was therefore of considerable interest. Here we demonstrate that this TFIIIA sequence can (*i*) functionally replace the Rev activation domain's role in mediating the nuclear export of late HIV-1 mRNA and (*ii*) function as an effective NES in both human cells and frog oocytes.

MATERIALS AND METHODS

Construction of Molecular Clones. The parental pBC12/CMV mammalian expression plasmid and derivatives encoding wild-type (pcRev) and M10 mutant (pcRevM10) forms of HIV-1 Rev have been described (19, 21). M10 contains aspartic acid/leucine in place of leucine/glutamic acid at positions 78 and 79 of Rev (19) (Fig. 1). The pM9XL, pM9BA, and pM9ΔBA expression plasmids were constructed using the Rev mutant expression plasmid pcRevM9, which contains a unique, inserted *Bgl* II site underlying Rev residues 67 and 68 (19). Synthetic oligonucleotides encoding wild-type or mutant forms of the C-terminal 19 aa of *Xenopus laevis* or *Bufo americanus* TFIIIA (22, 23) were inserted between this *Bgl* II site and a unique 3′ *Xho* I site, thereby also deleting the C-terminal 50 aa of Rev. The derivation of the Rev indicator plasmids pDM128/CMV, pHIV-1ΔRev, and pgTat has been described (4, 19, 21, 31).

Glutathione *S*-transferase (GST) fusion protein expression plasmids were constructed based on pGEX-2T (Pharmacia). Plasmid pGST:REV encodes GST linked to residues 67–116 of wild-type HIV-1 Rev, while pGST:REVM10 is identical except that it contains the M10 Rev mutation. Similarly, pGST:BA and pGST:ΔBA express fusion proteins consisting of GST linked to wild-type or mutant forms of a 19-aa *B. americanus* TFIIIA sequence (Fig. 1). In each case, GST was linked to the fused protein sequence via a hinge consisting of two glycine residues.

Mammalian Cell Culture and Transfection. COS cells and HeLa cells were maintained as described (21). Transfection assays for Rev function in COS cells, using the indicator constructs pDM128/CMV, pHIV-1ΔRev, and pgTat, have

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Abbreviations: NES, nuclear export signal; TFIIIA, transcription factor IIIA; HIV-1, human immunodeficiency virus type 1; GST, glutathione *S*-transferase; CAT, chloramphenicol acetyltransferase.

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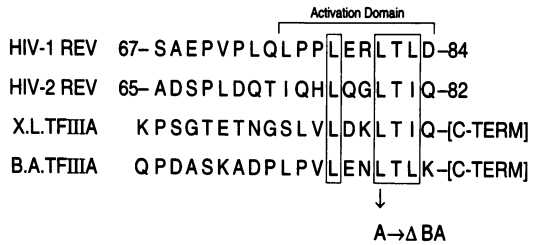

FIG. 1. Protein sequence alignment of selected Rev and TFIIIA proteins. The sequence of an 18-aa stretch of the 116-aa HIV-1 Rev protein, including the mutationally defined ≈10-aa Rev activation domain (21), is shown. Leucine residues critical to activation domain function are boxed. These leucine residues are generally well conserved among lentiviral Rev proteins, although they are sometimes substituted by other bulky hydrophobic amino acids such as isoleucine (21). These Rev sequences are aligned with the C-terminal 19 aa of TFIIIA proteins expressed by the African clawed frog *X. laevis* (X.L.) and the American toad *B. americanus* (B.A.) (22, 23). The ΔBA mutant of *B. americanus* TFIIIA contains an alanine substituted for the indicated leucine residue, while the M10 mutant of HIV-1 Rev substitutes aspartic acid/leucine at positions 78 and 79.

been described in detail (4, 19, 21, 31). Western blot analysis of protein expression levels in transfected COS cells was performed with a rabbit polyclonal anti-Rev antiserum (19).

Mammalian Cell Microinjection. All GST-containing proteins were purified by standard procedures in the absence of any detergents (24, 25). Two days prior to microinjection, HeLa cells were seeded onto glass coverslips at a density of 5 × 10^5 cells per 60-mm dish. GST fusion proteins were then injected at final concentrations of 6–8 mg/ml and the cells were returned to 37°C. Each protein preparation was supplemented with 5–6 mg of rabbit immunoglobulin G (IgG) per ml (Jackson ImmunoResearch) so that the site of injection could be determined. At 30 min after injection, the cells were fixed using 3% paraformaldehyde, and the subcellular localization of the injected proteins was determined by double-label indirect immunofluorescence (23). GST-containing proteins were detected by incubation with an anti-GST monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a goat anti-mouse antibody conjugated to Texas Red; IgG was visualized using a donkey anti-rabbit antibody conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch). Samples were observed by epifluorescence using a Nikon Microphot-FXA microscope at a magnification of ×400.

***Xenopus* Oocyte Microinjection.** Bovine serum albumin (BSA)–peptide conjugates were prepared as described (15). The BSA–R conjugate consisted of BSA linked to the wild-type Rev activation domain sequence NH_2-CLPPLERLTL-COOH, while BSA–M included a similar peptide bearing the M10 mutation. BSA–TF consisted of BSA conjugated to the 19-aa *B. americanus* TFIIIA sequence shown in Fig. 1, extended by the N-terminal cysteine required for cross-linking.

BSA conjugates and unconjugated BSA were radioiodinated using $Na^{125}I$, mixed, and then injected into oocytes as a solution containing ≈0.25 μg of iodinated protein per ml, as described (15). After incubation at 20°C for 1–2 h, oocytes were manually dissected in TNE medium (100 mM NaCl/1 mM EDTA/10 mM Tris·HCl, pH 7.4). Nuclei were fixed and pelleted in 95% ethanol. The cytoplasms were homogenized in TNE, the insoluble fraction was removed by centrifugation, and proteins in the supernatant were precipitated with acetone. Precipitated proteins were dissolved in protein sample buffer and fractionated by SDS/polyacrylamide gel electrophoresis. The resultant gel was then fixed, dried, and subjected to autoradiography.

RESULTS

To test whether the observed homology between Rev and TFIIIA (Fig. 1) had any functional significance, we deleted sequences encoding the C-terminal 50 aa of the 116-aa HIV-1 Rev protein in the Rev expression plasmid pcRev (4, 21) and replaced these with sequences encoding the C-terminal 19 aa of *X. laevis* or *B. americanus* TFIIIA (22, 23). These chimeric gene products, which we term M9XL and M9BA, respectively, would therefore retain the HIV-1 Rev RNA binding and multimerization domains, which are known to be fully contained within the N-terminal 66 aa (19–21, 32, 33), but would completely lack the essential Rev activation domain/NES. Instead, these chimeras would contain the TFIIIA sequences shown in Fig. 1. In addition, we constructed a point mutant in the context of the M9BA chimeric protein, termed M9ΔBA, in which a single leucine residue was changed to alanine (Fig. 1). In HIV-1 Rev, the equivalent mutation blocks activation domain function (21).

To test the functionality of these chimeras, we initially used a transient transfection assay for Rev function in primate cells based on the previously described Rev indicator construct pDM128/CMV (21, 31). This expression plasmid encodes an mRNA that contains the chloramphenicol acetyltransferase (CAT) coding sequence and the HIV-1 Rev response element in an intron defined by two HIV-1 splice sites. Cytoplasmic expression of the unspliced form of this RNA, and hence of the CAT protein, is therefore dependent on Rev (21, 31).

As previously shown (21), the pDM128/CMV plasmid induced only a low level of CAT activity when transfected into the monkey cell line COS, and this activity was enhanced ≈15-fold by cotransfection of a plasmid encoding HIV-1 Rev (Fig. 2*A*). As expected, expression of a form of Rev, termed RevM10, that is mutated at a critical activation domain leucine residue (19), failed to enhance CAT expression. However, the chimeric M9XL and M9BA proteins, in which the entire Rev activation domain was substituted by TFIIIA sequences, were both able to significantly enhance CAT expression. In M9BA, this activity was dependent on the integrity of the TFIIIA-derived leucine motif in that the M9ΔBA point mutant lacked function (Fig. 2*A*). These distinct activities did not reflect differential stabilities of these proteins because Western blot analysis showed all five proteins to be expressed at equivalent levels *in vivo* (Fig. 2*B*).

We next tested whether these chimeric Rev/TFIIIA proteins could rescue virion production from a full-length HIV-1 provirus that lacks a functional Rev gene. Transfection into COS cells of plasmid pHIV-1ΔRev (19, 21), containing such a defective HIV-1 provirus, did not result in the release of significant levels of HIV-1 virions, as determined by measurement of supernatant levels of the HIV-1 capsid protein p24 (Fig. 2*C*). However, high levels of HIV-1 virions were detected when the Rev expression plasmid pcRev was cotransfected. This activity was again dependent on the Rev activation domain, as shown by the inability of the RevM10 mutant to rescue p24 release from the pHIV-1ΔRev construct. However, the M9BA and, to a slightly lesser extent, the M9XL Rev/TFIIIA chimera could both rescue HIV-1 structural protein expression (Fig. 2*C*). This activity was, however, blocked by mutation of the leucine motif present in the M9BA protein.

To further confirm the finding that the essential Rev activation domain can be replaced by a sequence present in the C terminus of amphibian TFIIIA proteins, we analyzed the effect of the M9BA chimera on cytoplasmic expression of an unspliced HIV-1 mRNA with the use of previously described S1 nuclease protection assay (4, 8, 19) (Fig. 3). Upon transfection into mammalian cells, the pgTat plasmid expresses the genomic HIV-1 Tat gene, which contains a large intron derived primarily from the viral envelope gene. In the absence of Rev, this RNA is expressed in the cytoplasm exclusively in a fully spliced (S) form (lane 2) (8, 15). However, in the presence of Rev, unspliced (U) Tat mRNA becomes readily detectable in the transfected cell cytoplasm (lane 3). This induction was fully dependent on the integrity of the Rev activation domain (lane

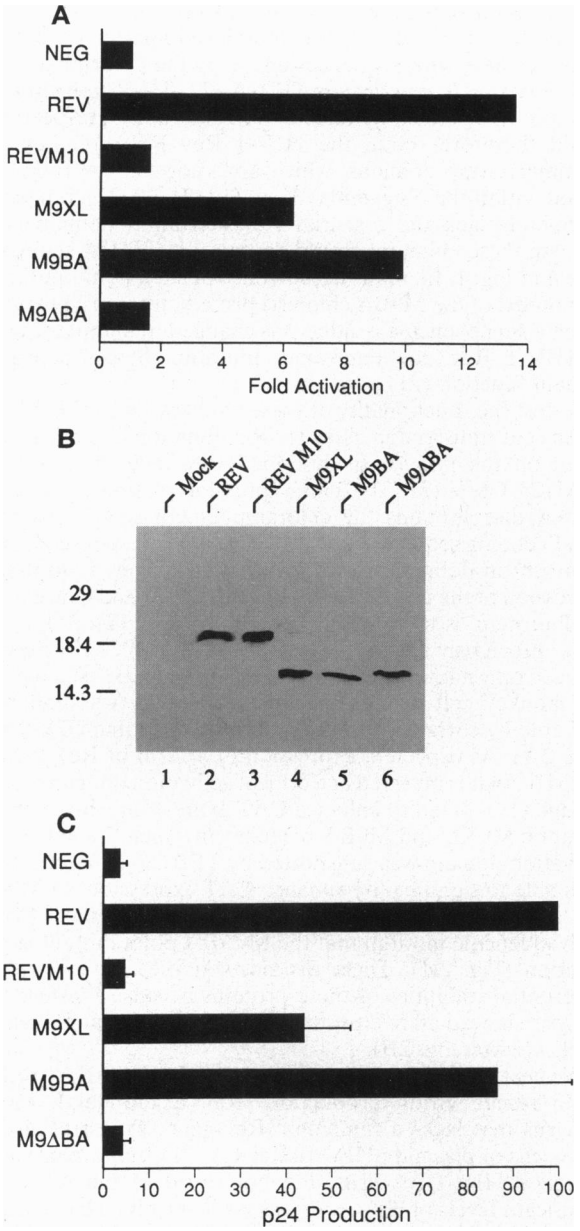

FIG. 2. Sequences derived from amphibian TFIIIA can functionally substitute for the HIV-1 Rev activation domain. (*A*) Relative activation of the *CAT* gene present in the Rev indicator construct pDM128/CMV (21, 31), induced by Rev or by the indicated Rev/TFIIIA fusion proteins, was measured 48 h after transfection into COS cells. (*B*) Western blot analysis of expression levels of the indicated Rev protein variants in transfected COS cells. Mock, mock-transfected culture. The relative mobility of protein molecular mass markers is shown at right in kilodaltons. (*C*) Ability of the indicated Rev derivatives to rescue p24 capsid protein expression from the Rev-deficient HIV-1ΔRev provirus was determined ≈72 h after transfection into COS cell cultures as described (19, 21). Data are given relative to the activity of wild-type HIV-1 Rev, which was arbitrarily set at 100. Results are averages of four independent experiments with SD indicated by error bars. Parental pBC12/CMV expression plasmid (21) served as a negative control in *A* and *C*.

4). Transfection of the pgTat indicator construct together with a plasmid expressing the M9BA fusion protein also induced expression of readily detectable levels of cytoplasmic unspliced HIV-1 mRNA (lane 5) but only if the TFIIIA-derived leucine motif in the chimeric M9BA protein was intact (lane 6).

TFIIIA Contains a NES. The activation domain of HIV-1 Rev has recently been shown to form a NES (15, 24, 25). We

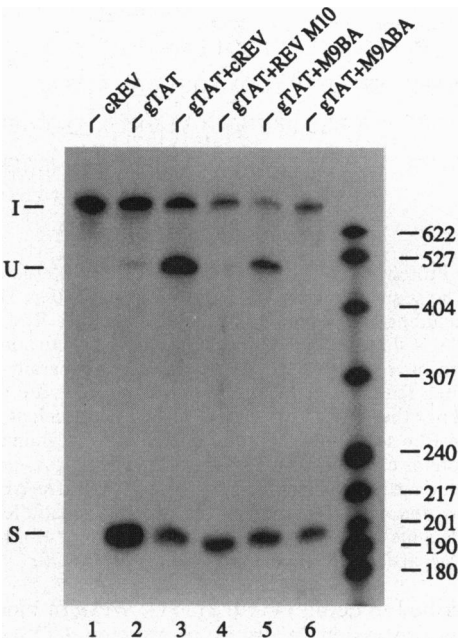

FIG. 3. Amphibian TFIIIA sequences can activate cytoplasmic expression of an unspliced HIV-1 mRNA. Quantitative S1 nuclease protection analysis of cytoplasmic RNA derived from COS cells transfected with the indicated expression plasmids was performed as described (4, 19). The end-labeled input probe (I) is designed to permit visualization of both unspliced (U) and spliced (S) cytoplasmic RNAs derived from the genomic *tat* gene expression plasmid pgTat. End-labeled DNA markers were run in parallel and are shown at right, with size in base pairs indicated.

used a microinjection assay in HeLa cells (23) to determine whether the C-terminal region of *B. americanus* TFIIIA also formed a NES. Fusion proteins consisting of GST linked to wild-type (GST:REV) or mutant (GST:REVM10) forms of aa 67–116 of HIV-1 Rev or wild-type (GST:BA) or mutant (GST:ΔBA) forms of the 19-aa *B. americanus* TFIIIA sequence shown in Fig. 1 were expressed in bacteria and purified. These proteins were then microinjected into HeLa cell nuclei along with IgG, which remains in nuclei after injection (24). At 30 min after injection, the cells were fixed and subjected to double label indirect immunofluorescence using anti-GST and anti-IgG antisera (25). As shown in Fig. 4*A*, microinjected GST:REV (*a*) was efficiently exported to the cytoplasm, while the GST:REVM10 mutant remained entirely nuclear (*c*). Similarly, the GST:BA fusion protein was also rapidly exported from the nucleus (*e*), while the GST:ΔBA mutant remained exclusively nuclear (*g*). In each case, the coinjected IgG internal control remained nuclear (Fig. 4*A Lower*).

We next asked whether the leucine motif found in *B. americanus* TFIIIA could also function as a NES in amphibian oocytes. Fischer *et al.* (15) have previously described an assay for NES function in *Xenopus* oocytes in which a peptide containing the putative NES sequence is first conjugated to BSA. This protein conjugate is then ^{125}I-labeled and microinjected into the oocyte nucleus. At 1–2 h after injection, the oocytes were harvested and nuclear and cytoplasmic protein fractions were analyzed by gel electrophoresis (15).

As shown in Fig. 4*B*, and as previously reported (15), BSA conjugated to a minimal Rev activation domain peptide (BSA–R) is localized almost entirely to the cytoplasm within 1 h of injection (lanes 1 and 2). In contrast, BSA conjugated to a Rev activation domain peptide bearing the M10 mutation (BSA–M) remains almost exclusively nuclear at the end of the same period (Fig. 4*B*, lanes 3 and 4). When a labeled BSA conjugate (BSA–TF) bearing the 19-aa *B. americanus* TFIIIA sequence shown in Fig. 1 was injected into the oocyte nucleus,

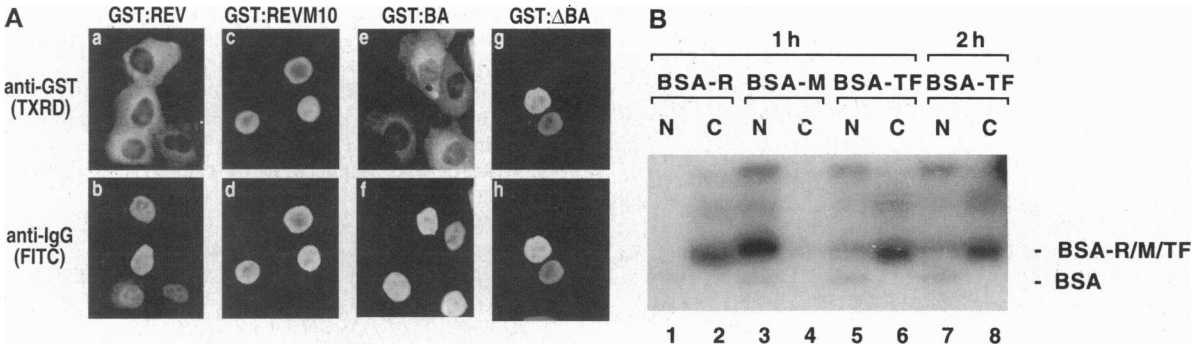

FIG. 4. *B. americanus* TFIIIA contains a NES. (*A*) Purified recombinant fusion proteins consisting of GST linked to C-terminal sequences from wild-type (GST:REV) or mutant (GST:REVM10) Rev or wild-type (GST:BA) or mutant (GST:ΔBA) *B. americanus* TFIIIA (see Fig. 1) were microinjected into HeLa cell nuclei along with rabbit IgG (24, 25). After incubation at 37°C for 30 min, cells were fixed and subjected to double-label indirect immunofluorescence (25) to visualize the intracellular locations of the injected GST fusion proteins and the IgG internal control (TXRD, Texas red; FITC, fluorescein isothiocyanate). (*B*) ^{125}I-labeled BSA conjugated to wild-type (BSA–R) or mutant (BSA–M) forms of the HIV-1 Rev NES or conjugated to the C-terminal 19 aa of *B. americanus* TFIIIA (BSA–TF) was microinjected (15) into the nuclei of frog oocytes along with radiolabeled unconjugated BSA. Export was examined 1 or 2 h later by protein extraction from nuclear (N) and cytoplasmic (C) fractions followed by analysis on a SDS/polyacrylamide gel (15).

≈80% of the labeled protein was detected in the cytoplasm 1 h after injection (Fig. 4*B*, lanes 5 and 6). Surprisingly, little additional protein entered the cytoplasm upon further incubation at 20°C (lanes 7 and 8), thus suggesting that this residual BSA–TF conjugate was not competent for nuclear export. Importantly, labeled unconjugated BSA remained in the nucleus of all injected oocytes (lanes 1, 3, 5, and 7).

DISCUSSION

Whereas the Rev and Rev-like proteins encoded by many complex retroviruses have been extensively studied, it has remained unclear whether eukaryotic cells contain proteins that display a comparable ability to induce the sequence-specific nuclear export of cellular RNA species (34). One candidate for this role is TFIIIA, which binds not only to the 5S rRNA gene but also to the resultant 5S rRNA transcript (26–29) and has been proposed to play an important role in the nuclear export of 5S rRNA in amphibian oocytes (30). Here, we show that a TFIIIA sequence element that displays homology to the essential Rev activation domain (Fig. 1), not only can replace this domain in mediating HIV-1 mRNA export from the nucleus (Figs. 2 and 3) but also shares with the Rev activation domain the ability to function as a NES (Fig. 4).

It is important to note that the data presented in this manuscript do not directly address whether the NES present in amphibian TFIIIA (Fig. 1) plays a role in nuclear 5S rRNA export equivalent to that played by the Rev activation domain/NES in late HIV-1 RNA export. However, indirect evidence in support of this hypothesis has been provided by the finding that the microinjection into frog oocytes of peptides encoding the leucine-rich activation domain of Rev results in specific inhibition not only of Rev-dependent RNA export but also of 5S rRNA export (15). In contrast, mRNA and tRNA export from the oocyte nucleus were found to be unaffected. These data imply that Rev-dependent RNA export and 5S rRNA export are occurring via RNA export pathways that depend on identical, or overlapping, cellular cofactors. Our finding that Rev and TFIIIA contain functionally equivalent NES elements is clearly consistent with the hypothesis that these two proteins access similar, or identical, RNA export pathways.

Based on this latter hypothesis, one might also predict that TFIIIA should interact with the nucleoporin-like human protein that was recently shown to specifically bind to the Rev activation domain/NES (16, 17). However, initial attempts to demonstrate binding of the Rev activation domain binding (Rab) protein (17), also termed the human Rev interacting protein (hRIP) (16), to the *B. americanus* TFIIIA NES have been unsuccessful (data not shown). While this negative result may simply reflect the inadequacy of the assays used thus far, it also raises the possibility that the TFIIIA NES, despite its close functional similarity to the Rev NES, may nevertheless act via a cellular cofactor distinct from Rab/hRIP. It is, in any event, clear that amphibian TFIIIA represents only the second cellular gene product, after human protein kinase inhibitor α (24), to be shown to contain an autonomous NES. However, based on the critical role played by intracellular transport in the life of the eukaryotic cell, it appears probable that NES elements may soon be as familiar as other eukaryotic intracellular transport signals.

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