Inhibition of Nuclear Import Mediated by the Rev-Arginine Rich Motif by RNA Molecules†

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Received October 8, 2002; Revised Manuscript Received December 2, 2002

ABSTRACT: The HIV-1 Rev protein plays a pivotal role in viral replication, and therefore, inhibition of its function should block the progression of the virus-induced immune deficiency syndrome (AIDS). Here, RNA molecules have been shown to inhibit import of the HIV-1 Rev protein into nuclei of permeabilized cells. Nuclear uptake of biotinylated recombinant His-tagged Rev-GFP was assessed in nuclear extracts from digitonin-permeabilized cells by binding to either importin β-receptors or nickel molecules immobilized on a microtiter plate. Using this method together with fluorescence microscopy, we determined that nuclear import of Rev is inhibited by the addition of a reticulocyte lysate which routinely is used as a source of nuclear import receptors. This inhibition was released by treatment with the RNase enzyme. Also t-RNA molecules and the oligoribonucleotide RRE IIB, namely, the second stem structure of the Rev responsive element (RRE) of the viral RNA, inhibit Rev nuclear import. Similar results were obtained when BSA molecules with covalently attached Rev-arginine rich motif (ARM) peptides were used as a nuclear transport substrate, indicating that the nuclear import inhibition of the Rev protein is due to the presence of the ARM domain. Binding experiments revealed that the RNA molecules inhibit the interaction between the ARM region and importin β, implying that the RNA prevents the formation of the import complex. The implication of our results for the regulation of the nuclear import of Rev as well as for the use of RNA molecules as antiviral drugs is discussed.

Nucleocytoplasmic transport is an essential process occurring in all eukaryotic cells. Nuclear proteins, including regulatory proteins and transcription factors, are imported from the cytoplasm into the cells' nuclei, while various RNA molecules and RNA−protein complexes are exported from the nucleus to the cytoplasm (*1*, *2*). These trafficking processes proceed via nuclear pore complexes (NPC),[1] macromolecular structures of ∼125 MDa that perforate the nuclear envelope (reviewed in ref *3*). Shuttling of large molecules is an active process mediated by specific cellular receptors, including the importin $\alpha-\beta1$ heterodimer, and requires the participation of the Ran GTPase system (*4*). Binding of the shuttling proteins to the corresponding import and export receptors is mediated by specific amino acid sequences, the nuclear localization signal (NLS) and the nuclear export signal (NES), respectively (*4*).

Nucleocytoplasmic transport is subjected to a diverse array of regulatory mechanisms. For example, binding of the NLS of the SV-40 T-antigen (*5*) and the NLS of *Drosophila morphogen* Dorsal protein (*6*) to the importin $\alpha-\beta1$ heterodimer receptor is enhanced by phosphorylation of the transport substrates. The NLS sequences of NF-κB, STAT factors, and the mammalian glucocorticoid receptor are masked by specific molecules (*7*, *8*). Signal-mediated removal of these inhibitory molecules results in the exposure and subsequent binding of NLSs to their corresponding nuclear receptors. The resulting complexes are then imported into the cells' nuclei (reviewed in ref *2*). p53 regulation involves phosphorylation-dependent masking of its nuclear

† This work was supported by grants from the German Israeli Foundation (Grant I-590-105.09/98 to A.L. and A.G.) and from the Israel Science Foundation (Grant 107/00 to A.L.). This work was also supported by grants from the National Health and Medical Research Council (NHMRC) to D.A.J. (Grant 128821).

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[1] Abbreviations: NPC, nuclear pore complexes; NLS, nuclear localization signal; NES, nuclear export signal; ARM, arginine rich notif; BSA, bovine serum albumin; GFP, green fluorescent protein; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside, dioxane free; Rho, rhodamine; TCEP, tris(2-carboxyethyl)-phosphine hydrochloride; DB, dissolving buffer; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; RRE, Rev response element; GTP-γ-S, guanosine 5-*O*-(3-thiotriphosphate).

10.1021/bi0206199 CCC: $25.00 © 2003 American Chemical Society
Published on Web 02/14/2003

export signal (*9*). Inert intracellular structures are also capable of inhibiting nucleocytoplasmic transport. For example, the influenza virus nuclear protein NP binds to actin filaments (*10*); the myc-interacting zinc-finger protein-1 MIZ-1 binds to the cellular microtubules (*11*), and the β-catenin binds to the cell's plasma membrane (*12*). Homeostatic factors, including osmotic stress, can modulate the yeast osmolarity glycerol response protein HOG1 by initiating the NLS-dependent translocation into nuclei (*13*). Nucleocytoplasmic trafficking of karyophilic molecules can, therefore, be regulated by the following general mechanisms: (i) modulation of the binding affinity of the karyophilic proteins for their corresponding receptors, (ii) competition between nuclear receptors and other cellular components for binding of the transport substrate, (iii) masking of the NLS and NES transport signals by specific inhibitors that prevent the formation of an active transported complex, and (iv) restriction of the intracellular mobility of the transport substrates by its binding to intracellular structures and/or macromolecules (reviewed in refs *14* and *15*).

Nucleocytoplasmic shuttling of the HIV-1 Rev protein offers a convenient experimental system for studying the regulation of the import−export process. The HIV-1 Rev protein is a small, 13 kDa, RNA-binding phosphoprotein that contains at least three functional domains (*16*−*19*). The region between amino acids 38 and 49, termed the arginine rich motif (ARM), contains eight arginine residues, and its sequence is homologous with the sequences of nucleic acid binding domains of other proteins, including HIV-1 Tat (*20*), HTLV-1 Rex (*21*), and a large number of cellular proteins such as the STAT factor and the histone deacetylase-4 (*22*, *23*). The Rev protein also contains a nuclear export signal (NES) (*17*, *24*) and multimerization domains (*25*, *26*). The presence of both nuclear import and export signals causes Rev to shuttle continuously between the nucleus and cytoplasm (*25*, *27*). The ARM has been shown to mediate both the nuclear import and nucleolar localization of the Rev protein (*17*). Using an in vitro import assay, it has been demonstrated that importin β alone is sufficient for mediating the import of the Rev protein, thus bypassing the requirement for importin α to mediate nuclear import (*20*). Interestingly, the ARM also mediates binding of the Rev protein to a 240-base region of RNA within the viral transcript (*25*, *28*) called the Rev response element (RRE). It was well established that the Rev-ARM binds to a single high-affinity binding site within the second stem−loop portion of the RRE (RRE IIB) (*29*, *30*). Rev then polymerizes along the length of the RRE via protein−protein and protein−RNA interactions, and facilitates the export of unspliced and singly spliced viral RNA (*16*). Regulation of the nuclear import of the HIV-1 Rev protein has recently been studied in vivo in astrocytes. Production of HIV by infected astrocytes is less efficient than that in T cells or microglial cells (*31*, *32*). Utilizing cellular expression of Rev-GFP, the transport of Rev into the cell nuclei was compared to that of other karyophilic proteins in astrocytes and HeLa cells. Indeed, these studies indicate that the extent of nuclear import of Rev is significantly reduced in astrocytes, suggesting the presence of a cytosolic inhibitor that specifically interacts with the Rev-ARM domain (*33*).

In this paper, we show that cytosolic extract obtained from reticulocytes specifically inhibits nuclear import mediated by the Rev-ARM. Our results show that this is due to the effects of ribonucleic acids present in the reticulocyte extract. Utilizing fluorescence microscopy and a quantitative assay, we also show that tRNA molecules promote partial inhibition of Rev nuclear import. In addition, the binding site for the Rev-ARM within the RRE, namely, RRE IIB oligoribonucleotide, strongly inhibits nuclear import of recombinant Rev-GFP molecules as well as of Rev-ARM−BSA conjugates. Our results provide evidence for a general regulatory role of RNA in the nucleocytoplasmic transport of the Rev protein.

MATERIALS AND METHODS

Materials

Purified RRE IIB, an oligoribonucleotide bearing 46 nucleotides (*16*), was obtained from Dharmacon Research Inc. (RNA Technologies, Lafayette, CO). Its proper folding was promoted exactly as described previously (*34*).

Lyophilized t-RNA-type X-SA was from baker's yeast (powder), and DNA (calf thymus) was obtained from Sigma. RNase inhibitor from human placenta was obtained from Roche Diagnostics, GmbH (Mannheim, Germany), and bovine RNase A was obtained from Fluka. Untreated reticulocyte lysate was obtained from Promega (Madison, WI).

All other chemicals and reagents were of a highly purified grade.

Cultured Cells

A monolayer of HeLa cells or a suspension of human Colo-205 cells was grown in DMEM or RPMI, respectively. The growth medium was supplemented with 10% FCS, 0.3 g/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Beit Haemek). Cells were incubated at 37 °C in a 5% CO_2 atmosphere and recultured every 4 days as described previously (*35*).

Synthesis of Peptides

Peptides bearing the SV40 large T-antigen NLS (PKKKRKV), as well as those bearing the HIV-1 Rev-ARM (RQARRNRRR) or the ARM (RRRNRRAQR) in reverse order, were synthesized exactly as described previously (*35*).

Expression and Purification of Recombinants Rev-GFP and Importin β

The expression vector pET28-hIMPb1 was kindly provided by V. Citovsky (State University of New York, Stony Brook, NY) and was expressed in *Escherichia coli* strain BL21-(DE3). Histidine-tagged (His-tagged, Qiagen) fusion proteins were expressed and purified by standard protocols following the growth at 37 °C and induction of the *E. coli* strains at 25 °C.

The Rev-GFP-expressing construct was derived by initially generating a 294 bp fragment by PCR using the *Nhe*I site-containing primers 5′-CTAGCTAGCGGGATGGCAGGAA-GAAG-3′ and 5′-CAGGCTAGCAGTTCCACAATCCTCGT-3′ (bottom strand) and dsRev$^-$ of HIV-1 strain NL4-3$^-$ [kindly provided by M. Hatanaka, Department of Molecular Virology and Research Center for Immunodeficiency Virus, Institute for Virus Research, Kyoto University, Kyoto, Japan

(*36*)] as a template. The fragment was digested with restriction endonuclease *Nhe*I and ligated into *Nhe*I-restricted and dephosphorylated plasmid vector pTRCA-GFP (*37*). Expression of Rev(1−92)-GFP was induced with 1 mM IPTG in *E. coli* strain M15 (pREP4) and purified using nickel chromatography as described previously in detail (*37*, *38*).

Nuclear Import in Permeabilized Cells

Transport Substrates. Linear peptides bearing the ARM sequence of the HIV-1 Rev-GFP protein were covalently attached either to Lissamine rhodamine-labeled bovine serum albumin (Rho-BSA) or to biotinylated BSA (Bio-BSA, Sigma) molecules using sulfo-SMCC as a cross-linker to give a Rho-BSA−Rev-ARM or biotinylated BSA−Rev-ARM conjugate, respectively, as described previously (*35*).

Recombinant Rev-GFP was biotinylated as follows. For reduction of disulfide bonds, a solution of tris(2-carboxyethyl)phosphine (TCEP) in dissolving buffer (DB) was added to a solution of Rev-GFP (5−10 mL in dissolving buffer) to give final concentrations of 1.2 and 120 mM, respectively. Subsequently, a solution of biotin-bound maleimide (Sigma) in DMSO was added to give a final concentration of 2 mM, and the solution that was obtained was incubated either for 2 h at 25 °C or overnight at 4 °C. Following the incubation period, the solution containing biotinylated Rev-GFP was dialyzed against 2−3 L of dissolving buffer at 4 °C.

Fluorescent Microscopy Observations. HeLa cells were cultivated for 2 days on 10 mm coverslips to a subconfluent density and then permeabilized with digitonin (*35*). The cells were then incubated with either recombinant Rev-GFP (4−8 μg) or the Rho-BSA−Rev-ARM (15−40 μg) conjugates, and nuclear import was followed by fluorescence microscopy using experimental conditions identical to those described previously (*35*). Each experiment has been repeated at least three times giving the same results.

Quantitative Estimation. (1) Nuclear Import of Biotinylated BSA−Rev-ARM Conjugates through Binding to Anti-BSA Antibody-Coated Microtiter Plates. Nuclear import was quantitatively determined using an ELISA-based assay system, essentially as described previously (*35*). Briefly, a suspension of Colo-205 cells was permeabilized with digitonin, and nuclear import was initiated by the addition of the biotinylated BSA−Rev-ARM conjugate (1−1.5 μg). All the subsequent steps of washing and neutralization of the non-nuclear biotinylated BSA−Rev-ARM conjugate as well as the disruption of loaded nuclei by Triton X-100 and the attachment of the of the biotinylated BSA−Rev-ARM conjugate to anti-BSA antibody-coated microtiter plates (Nunclon) were exactly as described previously (*35*, *39*). The attached surface of the biotinylated BSA−Rev-ARM conjugate was estimated by the addition of the streptavidin−HRP conjugate (Roche Diagnostic, GmbH) (*35*, *39*). The results given are averages of three independent measurements where the standard deviation (SD) was not greater than 20% the value of the mean.

(2) Quantitation of Nuclear Import of Biotinylated Rev-GFP through Binding to Importin β or Nickel-Coated Microtiter Plates. Recombinant biotinylated Rev-GFP (1.5−4.0 μg) in DB was added to a suspension of digitonin and permeabilized Colo-205 cells as described for the biotinylated BSA−Rev-ARM conjugate above and previously (*35*). All subsequent steps of promoting nuclear import washing and neutralization of non-nuclear biotinylated Rev-GFP as well as the disruption of loaded nuclei by Triton as described above for the biotinylated BSA−Rev-ARM conjugate have been described previously (*35*).

Increasing volumes of nuclear lysate (2−20 μL) were added to 96-well plates (Nunc Inc.) coated with either importin β (prepared as described below) or nickel [Ni−NTA HisSorb plates, white (Qiagen, GmmbH)]. Each well contained 200 mL of 5% BSA in PBS. Following incubation for 2 h at 37 °C (or overnight at 4 °C), the plates were washed three times with PBS and the attached surface of biotinylated Rev-GFP was estimated using the streptavidin−HRP conjugate as described above and previously (*35*). It should be noted that since the nickel-coated plates are not transparent, the colored solution obtained following the use of the streptavidin−HRP conjugate (250 μL) was aspirated and added to transparent wells before the absorbance was estimated at OD_{490}. The results given are averages of triplicate determination where the SD was not greater than 20% of the value of the mean.

(3) ARM-Mediated Binding of the Biotinylated BSA−Rev-ARM Complex to Surface-Attached Importin β. For coating of the 96-well plates (Nunc Inc.) with importin β, 200 mL of a solution containing recombinant importin β [30 mg/mL in $NaHCO_3/Na_2CO_3$ buffer (pH 9.6)] was added to each well and incubated overnight at 4 °C. Following the incubation period, the plates were washed three times with PBS, and then 200 μL of a solution of PBS containing BSA (5%) was added to each well for 1 h at 37 °C. The plates were washed again three times in PBS, and increasing concentrations (see Figure 6) of the biotinylated BSA−Rev-ARM complex (in DB) were added to the 96 importin β-coated wells, each well containing 200 μL of 5% BSA in PBS. Following incubation for 2 h at 37 °C (or overnight at 4 °C), the wells were washed three times with PBS. The amount of surface-bound biotinylated BSA−Rev-ARM conjugate was estimated following the use of the streptavidin−HRP conjugate as described above and previously (*35*). The results given are averages of triplicate determinations where the SD was not greater than 20% of the value of the mean.

Binding of Rev Fl to RRE IIB and tRNA^{Phe}. A fluorescent Rev peptide ($_{suc}$TRQARRNRRRRWRERQRAAAAC$_{am}$-fluorescein, 10 nM) in buffer [30 mM HEPES (pH 7.5), 100 mM KCl, 10 mM sodium phosphate, 20 mM NH_4OAc, 20 mM guanidinium hydrochloride, 2 mM $MgCl_2$, 20 mM NaCl, 0.5 mM EDTA, and 0.001% Nonidet P-40] was thermoregulated at 22 °C, and a concentrated stock of either RRE IIB or tRNA^{Phe} was titrated until saturation was reached. The fluorescence anisotropy of the peptide was monitored with a Perkin-Elmer LS-50B fluorimeter (maximum slit widths, excitation at 490 nm, emission at 530 nm, 2 s integration time, and six readings averaged per concentration) (*40*). The fractional change in anisotropy is taken as the fraction of Rev Fl bound by RNA.

RESULTS

RNA but Not DNA Inhibits the Nuclear Import of Rev-GFP: Fluorescence Microscopy Observations. Digitonin-permeabilized HeLa cells and fluorescence microscopy have been employed to analyze Rev-GFP nuclear import in vitro.

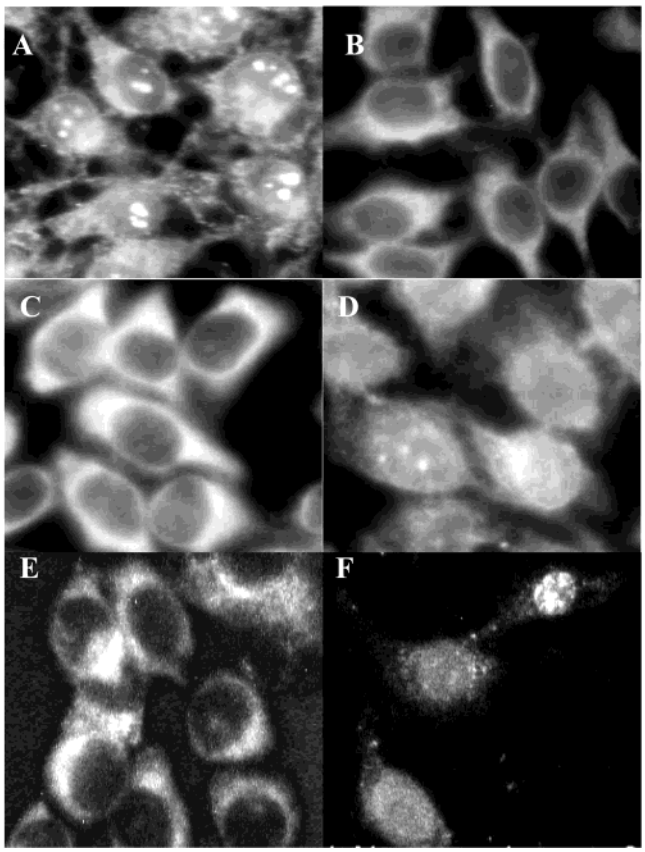

FIGURE 1: Microscopic observations of the active import of Rev-GFP into nuclei of permeabilized HeLa cells and its inhibition by RRE IIB molecules. Rev-GFP was imported into nuclei of permeabilized HeLa cells: (A) 37 °C, (B) 4 °C, (C) 37 °C and in the presence of free Rev-ARM peptide (200:1 Rev-ARM peptide: Rev-GFP molar ratio), (D) 37 °C with the permeabilized cells incubated with pancreatic RNase, (E) 37 °C and in the presence of RRE IIB molecules (4:1 RRE IIB:Rev-GFP molar ratio), and (F) 37 °C with the RRE IIB molecules incubated prior to their addition to the permeabilized cells with pancreatic RNase for 15 min at 25 °C. All other experimental conditions were as described in Materials and Methods. Please note that in panels A, D, and F the intranuclear space is fluorescently labeled, while in panels B, C, and E, the nuclei are empty.

Results (Figure 1A and Table 1) were consistent with previous observations (*20*) that the nuclear import of Rev is an active process, being ATP-dependent (Table 1) and inhibited in the cold (Figure 1B) and by the nonhydrolyzable GTP analogue GTP-γ-S (Table 1). This view was further supported by our results showing that peptides bearing the Rev-ARM (NLS) sequence competitively inhibited nuclear import of Rev-GFP (Figure 1C). However, in our experiments, nuclear and/or nucleolar accumulation of Rev-GFP did not require the addition of exogenous cytosolic factors, implying that residual importin β molecules, which are required to mediate nuclear import of Rev, are still present in these cells (*20*). Surprisingly, the addition of cytosolic extract (reticulocyte lysate), which is required for nuclear import mediated by the SV40 large T-antigen NLS (*39*) or other conventional importin α/β-recognized NLSs, significantly inhibited Rev-GFP nuclear import (Table 1). Interestingly, the inhibition obtained by the reticulocyte extract could be released by incubating the extract with an active RNase (Table 1). Indeed, the addition of RNase-treated extract did not cause any inhibition of nuclear import and practically slightly stimulated it (Table 1). Consistent with these observations, slight stimulation of nuclear import was also observed when the permeabilized cells themselves had been treated with an active RNase (Figure 1D). Inactivated RNase did not have any effect either when incubated with the reticulocyte extract or when added to the permeabilized cells (not shown; see results in Figure 2B, j), clearly indicating that the stimulatory effect was due to RNase-promoted hydrolysis of putative RNA molecules. Attempts to study the effect of DNase as well as of protease treatment have failed because incubation with each of these enzymes caused disintegration of the nuclei envelopes, precluding studies on nuclear import (not shown).

Table 1: Effect of RNase Treatment on the Active Import of Rev-GFP into Nuclei of Permeabilized Cells and Its Partial Inhibition by Reticulocyte Extract[a]

experimental conditions	nuclear import of Rev-GFP
(i) in the absence of cytosol	+++
(ii) in the absence of cytosol but with hexokinase (ATP-depleted)	−[b]
(iii) in the absence of cytosol but in the presence of cytosolic extract	±
(iv) in the absence of cytosol but in the presence of cytosolic extract after RNase treatment[c]	++++[d]
(v) n the absence of cytosol but with GTP-γ-S	−
(vi) n the absence of cytosol but with free SV40 T-antigen NLS peptide	+++
(vii) n the absence of cytosol but with free Rev-ARM peptide	−
(viii) n the absence of cytosol but with free peptide with Rev-ARM in reverse order	±

[a] Rev-GFP was imported into nuclei of digitonin-permeabilized HeLa cells, and following incubation and fixation, the cells were observed by fluorescence microscopy as described in Materials and Methods (see the legend of Figure 1). GTP-γ-S was added at a final concentration of 1.8 mM. Free peptides, SV40 T-antigen, Rev-ARM, and Rev-ARM in reverse order were added at a peptide:Rev-GFP molar ratio of 200:1. All other experimental conditions were as described in Materials and Methods. [b] Only the cytosol compartment is fluorescently labeled (see Figure 1B). [c] For treatment with RNase, 20 mg of the enzyme was added to each system, and following incubation for 15 min at 25 °C, 0.3 μL of RNase inhibitor was added to terminate the enzymatic reaction. [d] All the cells in the microscopic fields exhibit nuclear import (nuclei are fluorescently labeled) (see Figure 1F).

The above-described microscopic observations raise the possibility that polyoligonucleotide (RNA and possibly DNA) molecules may affect the nuclear import of the Rev-GFP. It was therefore of interest to study the effect of ribooligonucleotide RRE IIB on nuclear import. As mentioned, the interaction between RRE IIB and Rev-ARM was well-established (*29*); however, its effect on nuclear import was never studied. The results depicted in Figure 1E show that the addition of RRE IIB caused total inhibition of Rev-GFP nuclear import. This inhibitory effect could be completely released by treatment of the RRE IIB preparation with RNase (Figure 1F). Inhibition by the RRE IIB molecules was highly efficient, and even when RRE IIB was added at the same molar concentration as the transport substrate Rev-GFP, significant inhibition was observed (not shown). On the other hand, the addition of sonicated DNA molecules (calf thymus type I highly polymerized and sonicated) did not cause any inhibition (not shown; see Figure 3B, k) and

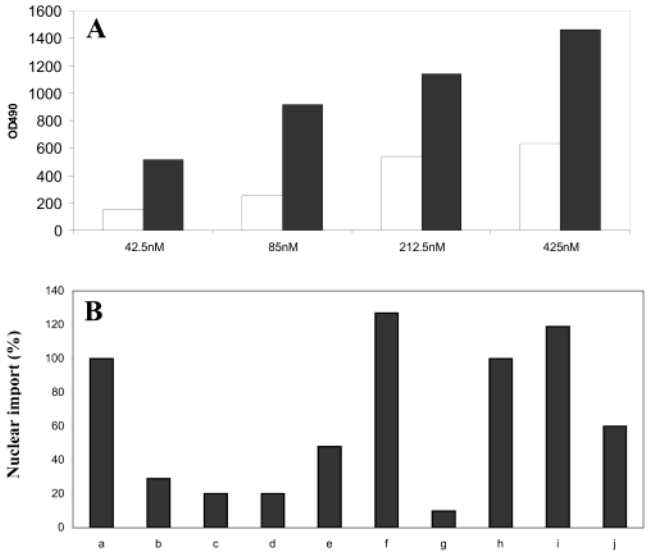

FIGURE 2: Quantitative estimation of Rev-GFP nuclear import. Binding to importin β and inhibition by reticulocyte extract and RNA molecules. (A) Specific binding of biotinylated Rev-GFP to importin β-coated plates. Biotinylated purified recombinant Rev-GFP was added to uncoated (□) and importin β-coated (■) plates to give the indicated final concentrations. All other experimental conditions such as biotinylation of Rev-GFP, coating of the plastic plates with recombinant importin β, and estimation of the amount of surface-bound biotinylated Rev-GFP were as described in Materials and Methods. (B) Inhibition of Rev-GFP import into nuclei of permeabilized Colo-205 cells by reticulocyte extract and RRE IIB. Effect of RNase treatment. Biotinylated recombinant Rev-GFP was imported in the absence of reticulocyte extract into nuclei of untreated (a) or ATP-depleted (b) cells in the presence of GTP-γ-S (1.8 mM) (c) or at 4 °C (d) as described in Materials and Methods. Nuclear import was also performed in the presence of untreated (e) or RNase-treated (f) reticulocyte extract as well as of untreated (g) or RNase-treated (h) RRE IIB or into RNase-treated permeabilized cells (i). Part j like part f but in the presence of an RNase inhibitor. The amount of intranuclear biotinylated Rev-GFP was estimated following its attachment to importin β-coated plates as described in Materials and Methods The RRE IIB:Rev-GFP molar ratio was 15:1. Nuclear import observed in the untreated control cells was considered to be 100%.

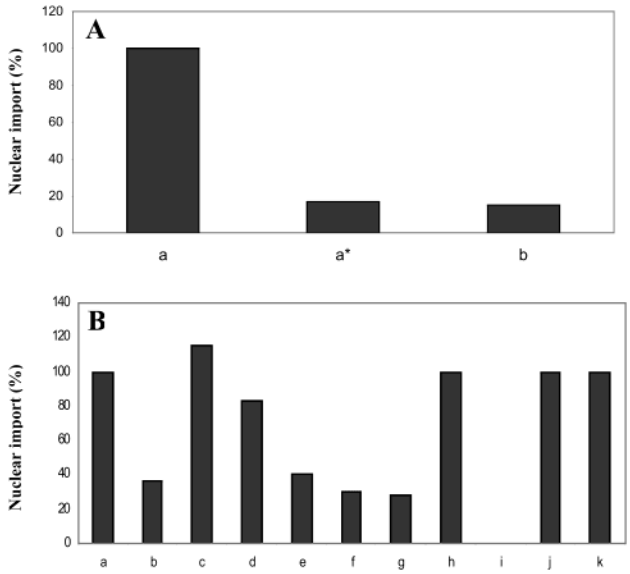

FIGURE 3: Quantitative estimation of Rev nuclear import using Ni-coated microtiter plates. (A) His tag-mediated binding of intranuclear biotinylated Rev-GFP to Ni-coated plates. Rev-GFP was imported into nuclei of untreated (a) or ATP-depleted (b) permeabilized cells as described in the legend of Figure 2B. For part a*, the nuclear lysate of the control untreated cells (a) was incubated with the Ni-coated plates in the presence of 1 M imidazole. All experimental conditions of nuclear import, lysis of loaded nuclei, incubation of nuclear lysate with Ni-coated plates, and estimation of the amount of plate-attached Rev-GFP as described in the legend of Figure 2 and in Materials and Methods. (B) Inhibition of Rev-GFP nuclear import by reticulocyte extract, RRE IIB, and t-RNA. Quantitative estimation using Ni-coated plates. Recombinant Rev-GFP was imported into the nuclei of permeabilized cells in the absence (a) or presence of untreated (b) or RNase-treated (c) reticulocyte extract. Part d like part c but in the presence of an RNase inhibitor. In the presence of untreated (e−g) or RNase-treated (h) t-RNA as well as untreated (i) or RNase-treated (j) RRE IIB molecules. The t-RNA:Rev-GFP molar ratios were 10:1 (e), 50:1 (f), and 100:1 (g), while the RRE IIB:Rev-GFP molar ratio was 15:1 (i). For part k, 30 mg of sonicated DNA molecules was added to the nuclear import assay system.

the nuclear import of Rev-GFP observed in its presence or absence was the same.

Inhibition of the Nuclear Import of Rev-GFP by RNA Molecules: Quantitative Estimation. To better characterize the inhibitory effects observed above, a previously published quantitative assay system (*35*) was modified to allow estimation of nuclear import of biotinylated karyophilic proteins (see Materials and Methods). Briefly, after the transport process, the nuclear lysate is added to plates coated with the appropriate importin receptor, and finally, the streptavidin−HRP conjugate is added for quantitation. Fluorescence microscopic examination indicated that biotinylation did not affect the karyophilic properties of Rev-GFP (not shown), and it was able to bind to importin β-coated microtiter plates 2−4-fold more efficiently than to uncoated control plates (Figure 2A).

Figure 2B summarizes results showing that active nuclear import of biotinylated Rev-GFP can be quantified using this method. This should be inferred from the results demonstrating that the amount of nuclear Rev-GFP in ATP-depleted (Figure 2B, b) or GTP-γ-S-treated (Figure 2B, c) permeabilized cells was small and reached only 30 and 20%, respectively, of that found in the nuclei of control untreated cells (Figure 2B, a), indicating 70−80% inhibition. Similarly, very little Rev-GFP was detected in the nuclei of cells incubated at 4 °C (Figure 2B, d). The use of this assay system allowed us to demonstrate and to confirm the microscopic observations (see Table 1) that the addition of the reticulocyte extract inhibited nuclear import of Rev-GFP by ∼50% (Figure 2B, e). On the other hand, an extract that was treated with pancreatic RNase not only failed to cause any inhibition of nuclear import but also slightly stimulated it (Figure 2B, f). This quantitative assay system also confirms the microscopic observations (see Figure 1) showing that the addition of an excess of RRE IIB caused almost complete inhibition of nuclear import (Figure 2B, g), which however could be reversed by treatment of RRE IIB with RNase (Figure 2B, h). Interestingly, treatment of the permeabilized cells themselves with RNase caused stimulation of nuclear import over that observed in the control untreated cells (Figure 2B, i). The view that the stimulation of the observed nuclear import (Figure 2B, i) is indeed due to the RNase activity can be inferred from the results showing that in the presence of RNase inhibitor the incubation with the RNase did not cause any stimulation (Figure 2B, j).

To further confirm these results, an alternative assay was used in which the Rev-GFP molecules have been pulled out

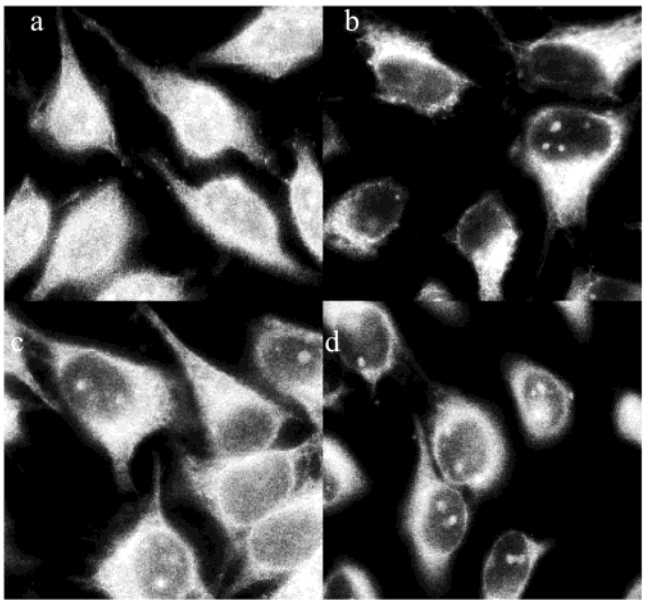

FIGURE 4: Active import of Rev-ARM−BSA conjugates into the nuclei of permeabilized HeLa cells as observed by fluorescence microscopy. BSA−Rev-ARM conjugates were prepared as described in Materials and Methods and incubated with digitonin-permeabilized HeLa cells in the absence (a, c, and d) or presence (b) of cytosolic extract. In panel c, ATP-depleted permeabilized cells were used, and in panel d, a 200-fold molar excess of unlabeled free Rev-ARM peptide was included. All other experimental conditions were as described in the legend of Figure 1 (*40*).

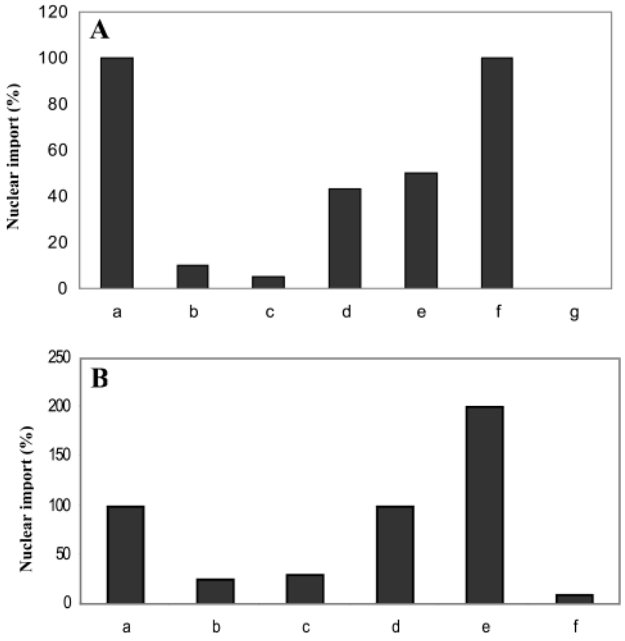

FIGURE 5: Quantitative estimation of nuclear import of BSA−Rev-ARM conjugates (A) and inhibition by RNAs (B). The Rev-ARM was cross-linked to biotinylated BSA, and the extent of nuclear import of the resultant conjugate was estimated using anti-BSA antibody-coated microtiter plates as described in Materials and Methods (*40*). (A) The extent of nuclear import was estimated in the absence of cytosol at 25 °C (a, b, and d−g) or at 4 °C (c) with the following additions: (b) GTP-γ-S, (d) Rev-ARM, (e) Rev-ARM in reverse sequence, (f) a peptide comprising the SV40-T-Antigen-NLS (*40*), and (g) an anti-importin β antibody. Unlabeled peptides were added at a 200-fold molar excess, while the anti-importin β-antibody was added at a 4-fold molar excess relative to the BSA−Rev-ARM conjugate. (B) The extent of nuclear import was estimated in the absence of cytosol at 30 °C with the following additions: (a) none, (b) GTP-γ-S, (c) cytosolic extract, (d) cytosolic extract but following pretreatment of the permeabilized cells with RNase, (e) none but following pretreatment of the extract with RNase, and (f) RRE IIB. The RRE IIB:BSA−Rev-ARM molar ratio was 15:1.

by Ni-coated microtiter plates. As can be seen in Figure 3A, the amount of Ni-bound His-tagged biotinylated Rev-GFP in the nuclei of ATP-depleted permeabilized cells (Figure 3A, b) was ∼5-fold smaller than that found in control untreated cells (Figure 3A, a). Specific binding of the Rev-GFP present in the nuclear lysate to the surface-attached nickel can be inferred from the fact that 1 M imidazole decreased the level of binding more than 90% (Figure 3A, a*).

The results in part b of Figure 3B show that the extent of inhibition of Rev-GFP nuclear import by the reticulocyte extract, as quantified using the Ni-coated plates, was >60% and could be released by treatment with RNase, like what has been observed using the importin β-coated plates (see Figure 2B). Evidently, the presence of an RNase inhibitor almost completely prevented the RNase effect (Figure 3B, d). The finding that RNase-treated extract failed to inhibit nuclear import clearly indicated the presence of inhibitory ribonucleotides. Indeed, t-RNA molecules were found to inhibit nuclear import (Figure 3B, e−g), and at a 50-fold molar excess, they caused ∼55% inhibition (Figure 3B, f). Treatment with RNase completely reversed the t-RNA inhibitory effect. However, RRE IIB [which was shown to firmly attach to the Rev-ARM (*16*)] was found to be the most potent inhibitor, and at a 15-fold molar excess, it caused almost complete inhibition of nuclear import (Figure 3B, i). Treatment of RRE IIB with RNase reversed the inhibitory effect (Figure 3B, j). Sonicated DNA did not exhibit any inhibitory activity (Figure 3B, k) indicating a certain specific effect of the RNA molecules.

Cytosolic Extract and RNA Molecules Inhibit Nuclear Import of BSA−Rev-ARM Conjugates. In view of the observations described above, it was of interest to determine whether the inhibition of nuclear import caused by the cytosolic extract and by the RNAs is due to specific interaction with the Rev-ARM (NLS) or with other domains of the Rev-GFP protein. By using BSA−Rev-ARM conjugates as transport substrates (ref *35* and see Materials and Methods), we were able to test the specific involvement of the ARM domain directly using both fluorescence microscopic and quantitative analyses. The results depicted in Figures 4 and 5 show that the BSA−Rev-ARM conjugates were similar to Rev-GFP in terms of translocating into the nuclei of permeabilized cells. Rev-ARM-mediated nuclear import (Figure 4a) was inhibited in ATP-depleted cells (Figure 4c) or at 4 °C (Figure 5A, c) and was inhibited by GTP-γ-S (part b of Figure 5A and part b of Figure 5B), clearly indicating an active process. Further, it was also inhibited ∼50% by excess free Rev-ARM peptide (Figure 4d and Figure 5A, d). Interestingly, the same level of inhibition was obtained when a peptide carrying the ARM sequence in a reversed order was added (Figure 5A, e; see also below). The specificity of the inhibition exerted by the ARM peptides is indicated by the fact that an excess SV40 large T-antigen NLS peptide did not exert any inhibitory effects (Figure 5A, f). Importantly, anti-importin β antibodies strongly blocked Rev-ARM-mediated nuclear import, dem-

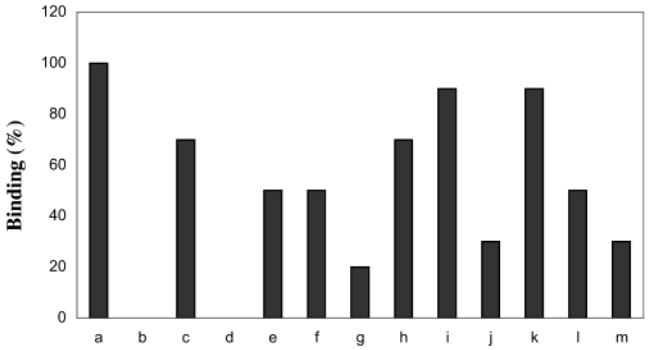

FIGURE 6: Importin β binding of BSA−Rev-ARM conjugates. Inhibition by RNAs. The extent of binding of Bio-BSA−Rev-ARM conjugates to importin β was estimated using a plate binding assay as described in Materials and Methods. Before the addition of the Bio-BSA−Rev-ARM conjugates, the importin β-microtiter plates were incubated for 15 min at 25 °C with the following: (a) none, (b) biotinylated BSA, (c and d) Rev-GFP at 1- and 10- fold molar excesses, respectively, relative to the biotinylated BSA−Rev-ARM conjugate, (e) unlabeled Rev-ARM peptide, (f) unlabeled Rev-ARM peptide but a peptide carrying the ARM sequence in reverse order, (g) 5 mL of reticulocyte lysate, (h) 5 mL of reticulocyte lysate but with RNase-pretreated reticulocyte lysate, (i) RNase, (j) RRE IIB in a 3-fold molar excess relative to the biotinylated BSA−Rev-ARM conjugate, (k−m) t-RNA at 20-, 200-, and 2000-fold molar excesses, respectively, relative to the biotinylated BSA−Rev-ARM conjugate. The various peptides were used at a 500-fold molar excess relative to the biotinylated BSA−Rev-ARM conjugate.

onstrating the role of importin β in the process (Figure 5A, g; see also below).

Nuclear import of the BSA−Rev-ARM conjugates, as for to the Rev-GFP, did not require external cytosolic extract, and again its addition strongly inhibited it (Figure 4b and Figure 5B, c). Also as for Rev-GFP, the inhibitory effect of the cytosolic extract could partially be released by RNase treatment (Figure 5B, d). Inhibition of nuclear import was also observed by the addition of t-RNA (not shown) or RRE IIB (Figure 5B, f). An increase in the level of nuclear import above that of control untreated cells was obtained when the biotinylated BSA−Rev-ARM conjugates were added to RNase-treated permeabilized cells (Figure 5B, e).

Cytosolic Extract and RNAs Block Binding of the ARM Peptide to Importin β. A likely explanation for the results described above is that the cytosolic extract and the RNA molecules inhibit nuclear import by interfering with the binding of the Rev-ARM to the importin β receptor (*20*). Plate binding assays were accordingly performed to assess this directly, with the results, summarized in Figure 6, supporting this idea. As expected, biotinylated BSA−Rev-ARM conjugates bound to the plate (Figure 6, a), the specificity of binding being demonstrated by the fact that biotinylated BSA lacking the ARM peptide did not bind (Figure 6, b). Furthermore, competition experiments between BSA−Rev-ARM conjugates and either Rev-GFP or unlabeled Rev-ARM suggest that the BSA−Rev-ARM conjugate indeed binds specifically to immobilized importin β (Figure 6, a−e). Correlating well with the inhibition observed of the nuclear import of the conjugates (Figure 5A, e), a peptide bearing the ARM sequence in a reverse order also inhibited the biotinylated BSA−Rev-ARM−importin β binding (Figure 6, f). The presence of cytosolic extract also strongly blocked (∼80%) the biotinylated BSA−Rev-ARM−importin β interaction (Figure 6, g), with significantly less inhibition

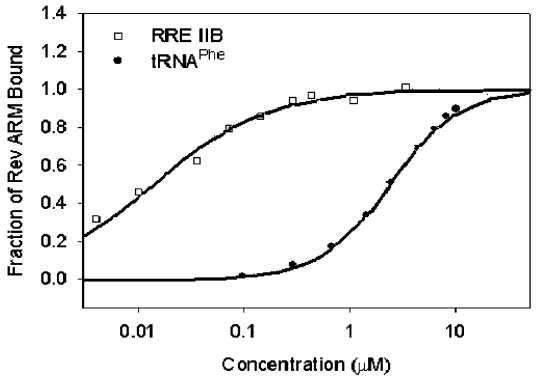

FIGURE 7: Association of a fluorescein-labeled Rev-ARM peptide with either RRE IIB or tRNA^{Phe}. The fluorescence anisotropy of the peptide "Rev Fl" was monitored as a function of RNA concentration and used to determine the fraction of Rev Fl bound a each point (see Materials and Methods for experimental details).

(∼30%) observed with RNase-pretreated cytosolic extract (Figure 6, h), again indicating the involvement of the RNA molecule in inhibition. The RNase enzyme itself did not have any affect (Figure 6, i). The addition of t-RNA as well as of RRE IIB also caused a significant inhibition of the biotinylated BSA−Rev-ARM−importin interaction (Figure 6, j−m), although the inhibitory effect of RRE IIB was greater than that of t-RNA. Indeed, our experiments revealed that the affinity of the Rev-ARM for RRE IIB is significantly higher than that for t-RNA (Figure 7).

DISCUSSION

The most significant finding of this work is the ability of RNA molecules to specifically block nuclear import mediated by the Rev-ARM domain. This is based on our observations that t-RNA as well as RRE IIB molecules inhibited nuclear import and on the fact that the inhibition exerted by the reticulocyte extract could be released by treatment with RNase. Our finding that t-RNA molecules, and most likely also RNA molecules present in the cytosolic extract, inhibit ARM-mediated nuclear import raises the interesting possibility that the interactions between cellular RNA and the ARM domain may play an important role in regulating nuclear import, especially of karyophilic proteins with positively charged NLSs. On the other hand, the inhibitory effect of the RRE IIB molecules probably does not have any biological relevance but can lead to the development of efficient and specific inhibitors of nuclear import with therapeutic potential.

Nuclear import is routinely studied by the use of fluorescently labeled karyophilic substrates and fluorescence microscopy (*40*). Also, a number of important studies using confocal laser scanning microscopy to quantitative nuclear import kinetics have been reported (*6*, *37*, *38*). However, in the case of the HIV-1 Rev protein, fluorescence microscopy studies may be misleading. The Rev protein tends to undergo oligomerization and to form insoluble fibril-like structures (*41*). Thus, the possibility that the fluorescence of the Rev-based transport substrates may undergo self-quenching within the intranuclear space cannot be excluded, leading to distortion of the microscopic observation and misrepresentation of the extent of nuclear transport. Thus, a quantitative assay which allows the accurate estimation of nuclear transport based on the simultaneous use of a large number

of cells and not on fluorescence molecules as a marker is needed. Previously, we and others have used such an assay, but it allowed quantitation of nuclear import only with BSA−NLS conjugates as the transport substrate (*40*, *42*, *43*). In this assay, anti-BSA antibodies were employed to pull down biotin-labeled conjugates present within nuclear extracts obtained from permeabilized cells (*40*, *42*). Clearly, the principles of this assay system could also be applied to follow the nuclear import of intact proteins, provided that specific antibodies are available for each specific karyophilic protein. To overcome the need for specific antibodies, in the study presented here, we have used immobilized recombinant importin β molecules to pull down biotin-labeled Rev-GFP molecules from nuclear extracts after the transport process, importin β being used since nuclear import of the HIV-1 Rev protein is mediated specifically by this cellular receptor (*20*, *27*). Alternatively, recombinant karyophilic proteins bearing a specific ligand can be pulled down from nuclear extracts by using the appropriate immobilized binding molecule as is demonstrated here using nickel-coated plates to pull down His-tagged recombinant Rev-GFP that accumulated in the nucleus.

Our microscopic observations and the quantitative studies demonstrated that the reticulocyte cytosolic extract as well as the RNA molecules specifically blocked or modulated ARM-mediated nuclear import. Nuclear import mediated by other NLSs such as that of the SV40 large T-antigen NLS not only is not inhibited but also is absolutely dependent on the addition of exogenous cytosol which serves as a source of importin α. Furthermore, no inhibition of T-antigen−NLS-mediated nuclear import was observed upon addition of t-RNA (not shown).

The bifunctionality of the Rev-ARM domain has been well-established; it mediates the binding to importin β as well as to the RRE region of the viral transcript (*16*). Indeed, direct interaction between a peptide bearing the Rev-ARM domain and RRE IIB has been demonstrated by us and others (*30*, *40*). Therefore, it is likely that the observed inhibition of nuclear import is due to specific binding and thus masking of the ARM domain within Rev by RRE IIB preventing interaction with its cellular receptor, importin β. This indeed was demonstrated by direct binding experiments. Interestingly, the same effect, namely, inhibition of the ARM−importin interaction, was exerted by the t-RNA molecules and probably by RNA molecules present in the reticulocyte extract as was monitored using our ELISA-based assay (see Figure 6). The direct binding experiments confirm our nuclear import experiments, demonstrating that the inhibition exerted by t-RNA as well as cytosolic extract was less efficient than that obtained by RRE IIB. It appears that, in addition to electrostatic attractions, the three-dimensional arrangement of the oligonucleotide molecules is important for the binding between the positively charged ARM and the negatively charged RNA. This can be inferred from our results showing no inhibition of nuclear import was observed following the addition of the negatively charged DNA molecules.

Further support for the conclusion that the inhibition by RNA is due to interference of the ARM-mediated nuclear import and not to stimulation of nuclear export can be inferred from the experiments using the BSA−Rev-ARM conjugates as a binding or transport substrate. While the Rev-GFP protein bears an NLS as well as NES sequences, the BSA conjugates carry only an NLS sequence and thus can be only imported but not exported even after being introduced into the cell nucleus by microinjection (not shown).

In contrast to the extensive effort spent during the past few years to elucidate the mechanism of active nuclear transport, little attention has been given to better understanding its regulation (*14*, *15*). NLS masking through binding to a specific molecule to prevent interaction with importins is a mechanism that controls nuclear import of transcription factor NF-κB through the I-κB protein (*44*). Direct interaction between I-κB and the NF-κB NLS has been demonstrated from analysis of the crystal structure of the I-κB−NF-κB complex (*45*). Signal-mediated phosphorylation of the I-κB molecule leads to its proteolytic degradation, unmasking the NF-κB NLS and leading to nuclear import of the NF-κB p65 subunit (*46*). It is not inconceivable to assume that regulation of nuclear import via reversible masking of the NLS domains of certain karyophilic proteins can be exerted not only by polypeptides but also by nucleic acids and especially by cytoplasmic RNA molecules. In this regard, it should be mentioned that in most of the nuclear RNA and DNA binding proteins the nucleic acid binding domains overlap with the NLSs of these proteins (*47*), with the closeness of these motifs purported to allow coordinate regulation. Supporting this idea are observations that DNA binding on the part of the yeast transcriptional activator GAL4 (*48*) and chromatin remodeling factor SRY (*38*) competes with importin β1 binding, and TATA site-containing DNA is similarly able to enhance dissociation of the TATA-binding protein from its nuclear import receptor Kap114p (*49*). The competition of specific promoter sequences for importin β binding has been postulated to play a role in nuclear import substrate release within the nucleus (*38*).

In this context, our observation that RRE IIB blocks ARM-mediated nuclear import is obviously of interest but, because of the bifunctionality of the ARM domain, perhaps not unexpected. Competition between the importin β and the RRE for occupancy of the Rev-ARM domain may be the basis of the opposing dual roles of the ARM domain. Within the intranuclear space, binding of RanGTP to importin β liberates Rev, and the Rev-ARM in particular, from importin β to enable it to interact with the RRE on nascent viral transcripts (*16*). Following export of the Rev−RNA complex to the cytoplasm, the opposite occurs whereby the Rev-ARM releases the RRE site of the viral transcript to be able to interact with importin β. The results showing inhibition of nuclear import by t-RNA, and possibly by polyribonucleotides present in the cytosolic extract, may reflect a new kind of nuclear import regulation. Thus, in the case of HIV-1 Rev, and possibly also Tat, the extent and probably the rate of its nuclear import may be governed by the relative cytoplasmic concentrations of its cellular receptor (importin β) and RNA molecules. The low affinity of the ARM domain for t-RNA may be compatible with such a model; high affinity would lead to total inhibition, rather than regulation of nuclear import. Although not proven, inhibition by astrocyte-specific RNAs may explain the retarded nuclear import of Rev recently observed in these cells (*33*).

The possibility that the results presented here may have potential therapeutic applications cannot be ignored. Reduc-

tion of the extent of nuclear import of certain viral karyophilic proteins whose nuclear presence is crucial to the completion of the HIV life cycle should undoubtedly lead to inhibition of the infection process (*50*). On the basis of previous work (*51*, *52*) as well as on the basis of this study, it is clear that metabolically stable, cell permeable derivatives of the RRE IIB molecule should possess antiviral properties.

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BI0206199