

Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence

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The E1B 55-kDa and E4 34-kDa oncoproteins of adenovirus type 5 (abbreviated here as E1B-55kD and E4-34kD) promote the export of viral mRNA and inhibit the export of most cellular mRNA species. We show that the intracellular complex containing E1B-55kD and E4-34kD continuously shuttles between the nucleus and the cytoplasm, and may thus serve as a nucleocytoplasmic transporter for viral mRNA. We present evidence that within this complex, it is the E4-34kD protein that directs both nuclear import and nuclear export. E4-34kD contains a functional nuclear export signal similar to corresponding sequences found in the retroviral proteins rev and rex. This sequence element is required for nuclear export of the complex, and it can function autonomously when fused to a carrier protein and microinjected in HeLa cell nuclei. When E4-34kD is expressed alone, a portion of the protein that contains a predicted arginine-rich amphipathic α -helical structure mediates nuclear retention of the protein. This retention, however, can be abolished by the association with E1B-55kD or by a specific point mutation within the arginine-rich motif. The export of E4-34kD can be blocked by an HTLV-rex derived competitive inhibitor and overexpressed E4-34kD inhibits rev-mediated transport, suggesting that the export pathways accessed by the adenoviral and retroviral proteins share components. The interplay between two polypeptides as well as the involvement of a dominant nuclear retention domain are novel features that might contribute to the efficiency and regulation of the adenovirus export system.

Keywords: adenovirus/HIV/mRNA export/
nucleocytoplasmic transport/viral oncogenes

Introduction

The export of RNA from the nucleus to the cytoplasm appears to involve distinct pathways for different classes of RNA (Jarmolowski *et al.*, 1994). Some of these pathways are utilized by viruses to allow efficient nucleo-

cytoplasmic transport of viral RNA. The human retroviruses HIV and HTLV encode the gene products rev and rex that mediate the export of their genomic RNA. These proteins interact with specific viral RNA elements, and the complex of RNA and protein is carried from the nucleus to the cytoplasm. This transport is mediated by a short sequence termed the nuclear export signal (NES) (Simos and Hurt, 1995; Gorlich and Mattaj, 1996). The NES from the HIV rev protein has been shown to mediate transport on a pathway overlapping at least in part with that supporting the export of 5S ribosomal RNA but not mRNA (Fischer *et al.*, 1994; Fischer *et al.*, 1995). The mechanism of this export is currently under intense investigation, and several candidate protein-transport mediators have been identified (Bogerd *et al.*, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995; Bevec *et al.*, 1996).

During the late phase of infection with adenovirus type 5, the nuclear export of most cellular mRNA species is inhibited while viral mRNA accumulates efficiently in the cytoplasm (Beltz and Flint, 1979; Babiss *et al.*, 1985; Pilder *et al.*, 1986). The effect depends on two viral gene products: The E1B 55-kDa protein (E1B-55kD) and the E4 34-kDa protein (E4-34kD, or E4 orf 6) (Babiss *et al.*, 1985; Halbert *et al.*, 1985; Pilder *et al.*, 1986; Shenk, 1996). The two adenoviral proteins seem to associate not only within infected cells (Sarnow *et al.*, 1984), but also when transiently expressed (Goodrum *et al.*, 1996). E1B-55kD localizes mainly in the cytoplasm when expressed alone, whereas E4-34kD localizes in the nucleus. However, when coexpressed, both proteins are found predominantly in the nucleus, indicating that E4-34kD redirects the location of E1B-55kD, presumably by complex formation. Within the infected cell nucleus, the E4-34kD protein directs the E1B-55kD protein to the periphery of the viral replication/transcription centers during the late phase of infection (Ornelles and Shenk, 1991). It is not known how these proteins affect the export of both viral and cellular mRNA, but it has been suggested that the complex containing E1B-55kD and E4-34kD proteins binds a cellular factor required for mRNA export and relocates it to the vicinity of viral replication/transcription centers, limiting the availability of this factor largely to viral mRNA (Ornelles and Shenk, 1991).

Both E1B-55kD (Sarnow *et al.*, 1982; Yew and Berk, 1992; Yew *et al.*, 1994) and E4-34kD (Dobner *et al.*, 1996) bind and inactivate the tumor suppressor gene product p53 and contribute to the transformation of cells (Moore *et al.*, 1996). To date, it is unclear why the same proteins are employed by the virus to modulate mRNA export and cell proliferation, and whether there is a link between these two activities.

We now show that the complex of E1B-55kD and E4-34kD protein shuttles between the nucleus and cytoplasm. The export is mediated at least in part by a nuclear export

signal on the E4-34kD protein. In addition, E4-34kD contains a previously undescribed domain that mediates nuclear retention of the protein. E1B-55kD abolishes this retention and allows nuclear export of the complex. The adenoviral export pathway overlaps, at least in part, with the pathway mediating the export of HIV rev and HTLV rex proteins. As predicted from this overlap, over-expressed E4-34kD is an inhibitor of rev activity.

Results

The complex containing E1B-55kD and E4-34kD shuttles between the nucleus and the cytoplasm

Since the E1B-55kD and E4-34kD proteins from adenovirus type 5 modulate the export of RNA, we asked whether the complex containing both proteins shuttles between the nucleus and the cytoplasm. When both proteins were transiently expressed in HeLa cells, virtually all E4-34kD and the vast majority of E1B-55kD were found in the nucleus (data not shown), consistent with previous results (Goodrum *et al.*, 1996). To assay for nucleocytoplasmic shuttling, the transfected HeLa cells were fused to murine Balb-c 3T3 cells using polyethylene glycol, to produce heterokaryons. Cycloheximide was added 15 min prior to the fusion to prevent protein synthesis. The cells were fixed and stained, 2 h after the fusion, with a monoclonal antibody against E4-34kD. Simultaneously, the cells were stained with an antibody that recognizes human but not murine nuclear Ku-antigen, an autoantigen that is part of the DNA-dependent kinase DNA-PK (Gottlieb and Jackson, 1993), thus distinguishing the nuclei of the different species from each other. Nucleocytoplasmic shuttling was indicated by the detection of E4-34kD in the murine nuclei, as previously shown in this assay for some of the hnRNP proteins (Pinol-Roma and Dreyfuss, 1992). As shown in Figure 1a–c, the E4-34kD protein shuttles between nucleus and cytoplasm when coexpressed with E1B-55kD. The same observation was made when these cells were stained for E1B-55kD instead of E4-34kD (data not shown). Thus, both E1B-55kD and E4-34kD shuttle continuously between nucleus and cytoplasm when coexpressed. In contrast, E4-34kD did not migrate from the human to the murine nuclei in the absence of E1B-55kD (Figure 1d–f), indicating that it is not efficiently exported from nuclei when expressed alone. This was observed in >200 heterokaryons and three independent experiments. Since transiently expressed E1B-55kD is localized in the cytoplasm in the absence of E4-34kD (cf. Figure 3B and Goodrum *et al.*, 1996), export characteristics of the isolated protein could not be assessed in a heterokaryon assay. Therefore, we transiently expressed E1B-55kD fused to a nuclear localization signal (NLS) from SV40 T antigen. The stability of NLS-tagged E1B-55kD was similar to E1B-55kD, as judged by immunoprecipitation from transfected and radiolabeled cells (data not shown). The NLS sequence efficiently directed E1B-55kD to the nucleus, and the protein accumulated in a nucleolar pattern (Figure 1g, and our unpublished results) resembling transiently expressed HIV rev (Meyer and Malim, 1994). However, in contrast to rev, NLS-tagged E1B-55kD was unable to shuttle to the cytoplasm in a heterokaryon assay (Figure 1g–i). When coexpressed with E4-34kD, NLS-tagged E1B-55kD localized in the

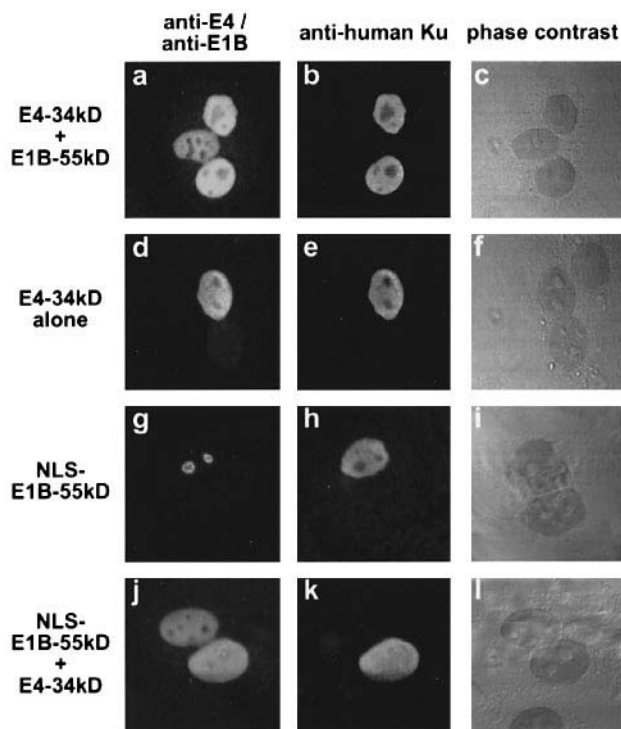


Fig. 1. Nucleocytoplasmic shuttling of E1B-55kD and E4-34kD. HeLa cells were transfected with expression plasmids for E1B-55kD and E4-34kD (a–c), E4-34kD alone (d–f), NLS-E1B-55kD (g–i) or NLS-E1B-55kD and E4-34kD (j–l). After heterokaryon formation with murine cells, the cells were incubated for 2 h at 37°C. Then, the heterokaryons were fixed and stained for E4-34kD (a and d) or E1B-55kD (g and j) using the monoclonal antibodies RSA3 and 9C10, respectively. This was followed by an FITC-conjugated secondary antibody subclass-specific for mouse IgG 2a or specific for rat IgG, respectively. Simultaneously, the cells were stained with a monoclonal antibody recognizing human but not murine Ku antigen, followed by Texas Red-coupled anti-mouse IgG1 (b, e, h and k). In parallel, the cells were photographed using phase contrast optics (c, f, i and l). Ku-negative (i.e. murine) cell nuclei staining positive for adenoviral antigens and localized within heterokaryons containing transfected HeLa nuclei are indicative of nucleocytoplasmic shuttling.

nucleoplasm, and efficient shuttling could be observed (Figure 1j–l). Hence, the two proteins must be present and presumably form a complex for nuclear export, and neither of the proteins can be efficiently exported when expressed alone.

The E4-34kD protein contains a signal sequence mediating nuclear export

The E4-34kD protein contains a 10 amino acid sequence similar to characteristic arrays of hydrophobic amino acids (Figure 2A) shown previously to be crucial for export and termed nuclear export signal (Gorlich and Mattaj, 1996; Murphy and Wente, 1996). This sequence is 100% conserved between the E4-34kD proteins from types 2 and 5 of adenovirus (not shown). We therefore tested whether this sequence might be involved in the nuclear export of the E1B-55kD–E4-34kD protein complex by mutating two of the hydrophobic amino acids, L90 and I92, to alanine (Figure 2A, bottom line). An analogous change in amino acid sequences has been observed to inhibit the function of the rev NES (Fischer *et al.*, 1995). This change in the E4-34kD sequence greatly reduced shuttling of the complex (Figure 2B, compare g–i with a–c), even though

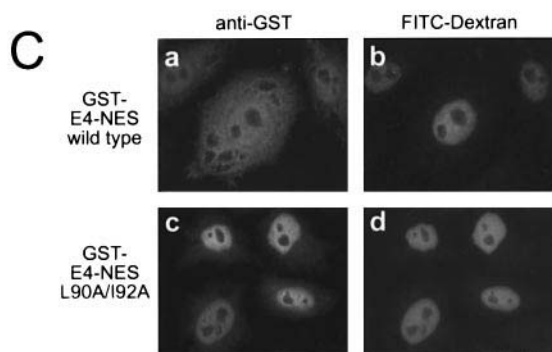
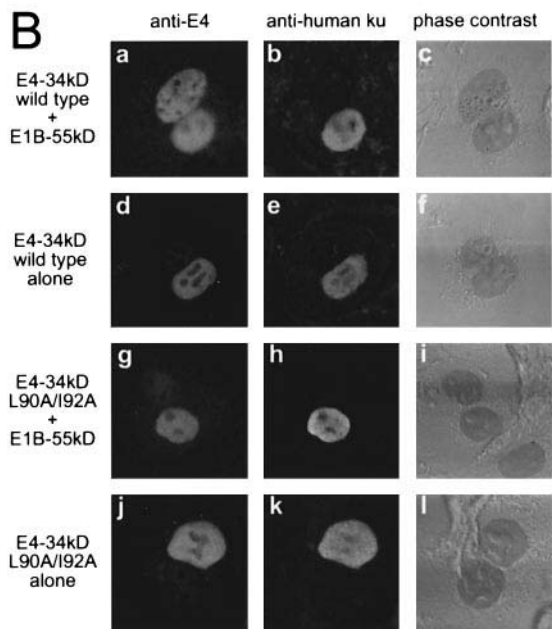
complex formation by E4-34kD and E1B-55kD was apparently unaffected (see below). Like the wild-type E4-34kD protein (Figure 2B, d–f), the mutant form did not shuttle in the absence of E1B-55kD (Figure 2B, j–l). Furthermore, when fused to a carrier protein (glutathione *S*-transferase, GST), the E4-34kD-derived NES mediated nuclear export after intranuclear microinjection (Figure 2C, a and b), as was previously observed with the rev NES (Meyer *et al.*, 1996). As anticipated, the majority of the protein remained in the nucleus, when the NES sequence was mutated at two of the hydrophobic regions as before (L90A and I92A; Figure 2C, c and d). Taken together, these results indicate that the E4-34kD protein contains an NES that is both necessary and sufficient to mediate efficient nuclear export.

In the absence of E1B-55kD, E4-34kD is retained in the nucleus by a C-terminal domain

If E4-34kD contains a functional NES, why is it unable to shuttle in the absence of E1B-55kD? We tested the hypothesis that E4-34kD might be actively retained in the nucleus. In a search for a domain in the protein that might mediate this retention, our attention was drawn to a sequence within the C-terminus of the protein, containing arginine residues (positions 234–251, cf. Figure 3A, top panel) that can be predicted to locate on the hydrophilic side of an amphipathic α -helical structure (J.S.Orlando and D.A.Ornelles, personal communication). To assess a possible role for this structure in nuclear retention, we mutated one of these arginine residues to glutamic acid. This mutation enabled E4-34kD to shuttle even in the absence of E1B-55kD (Figure 3A, a–c). When the NES mutation was introduced in addition, no shuttling was observed (Figure 3A, d–f), further demonstrating the presence of a functional NES within E4-34kD. Both single mutants, as well as the double mutant, retained the ability to relocalize E1B-55kD, as shown by cotransfection and double immunostaining (Figure 3B). These results show that an amino acid within an amphipathic α -helical domain near the C-terminus of E4-34kD is required for the active retention of the protein in the nucleus. This retention can be overcome by either a point mutation or by the association of E4-34kD with E1B-55kD.

A

E4-34 kD	83	MV . . LTREELVI	92
HIV-1 rev	75	LPP . LER . . LTL	83
VMV rev	106	MVG . MEN . . LTL	114
EIAV rev	36	LESQWCRVLRQSL	49
HTLV-1 rex	82	LSAQLYSS . LTL	92
Gle1	351	LP . . LGK . . LTL	358
PKI	37	LALKLAG . . LDI	46
TFIIIA	330	LPV . LEN . . LTL	338
E4-34 kDmt	83	MV . . LTREEAVA	92



The export pathway for E4-34kD includes components that also mediate the export of HIV rev and HTLV rex

To probe the relationship of the pathways mediating the nuclear export of mRNAs encoded by adenoviruses and complex retroviruses, we coexpressed the E4-34kD protein with an N-terminus-truncated form of HTLV rex fused to the nuclear localization signal of simian virus 40 (SV40) T antigen (Figure 4A). When the rex construct is over-expressed, it can inhibit the nuclear export of RNA by

Fig. 2. (A) A sequence element from E4-34kD is compared with known nuclear export signals (NESs). The hydrophobic residues within the NESs believed to be critical for export function are shown in bold. Dots are inserted to equalize the pairing between conserved hydrophobic residues in the different motifs. A mutant version of E4-34kD was created with the sequence shown in the bottom line. Numbers indicate the position of the amino acids within the proteins. The references for the NES elements were: human immunodeficiency virus 1 (HIV-1) rev (Fischer *et al.*, 1995); human T cell lymphotropic virus 1 (HTLV-1) rex (Palmeri and Malim, 1996); visna-maedi virus (VMV) rev (Tiley *et al.*, 1991); equine infectious anemia virus (EIAV) rev (Mancuso *et al.*, 1994); yeast mRNA transport protein Gle1 (Murphy and Wentz, 1996); cAMP-dependent protein kinase inhibitor (PKI) (Wen *et al.*, 1995); transcription factor TFIIIA (Fridell *et al.*, 1996). (B) Two amino acid changes (L90A and I92A) within the putative E4–NES element inhibit shuttling of the complex containing E1B-55kD and E4-34kD. The cells were transfected to express E4-34kD as wild type (a–f) or mutant (g–l) either together with E1B-55kD (a–c and g–i) or alone (d–f and j–l), and the heterokaryons were stained for E4-34kD (a, d, g and j) and human Ku (b, e, h and k) and photographed in phase contrast (c, f, i and l). (C) The E4 NES element can direct nuclear export of a carrier protein. The NES-like sequences were fused to GST, and the resulting proteins were microinjected into the nuclei of HeLa cells. Two hours after injection, the cells were fixed and stained against GST, using a Texas Red-coupled secondary antibody (a and c). FITC-dextran was coinjected to verify the nuclear injection site (b and d). The NES-like sequence from E4-34kD was thus assayed for export activity in wild type (a and b) and mutant (L90A and I92A) form (c and d).

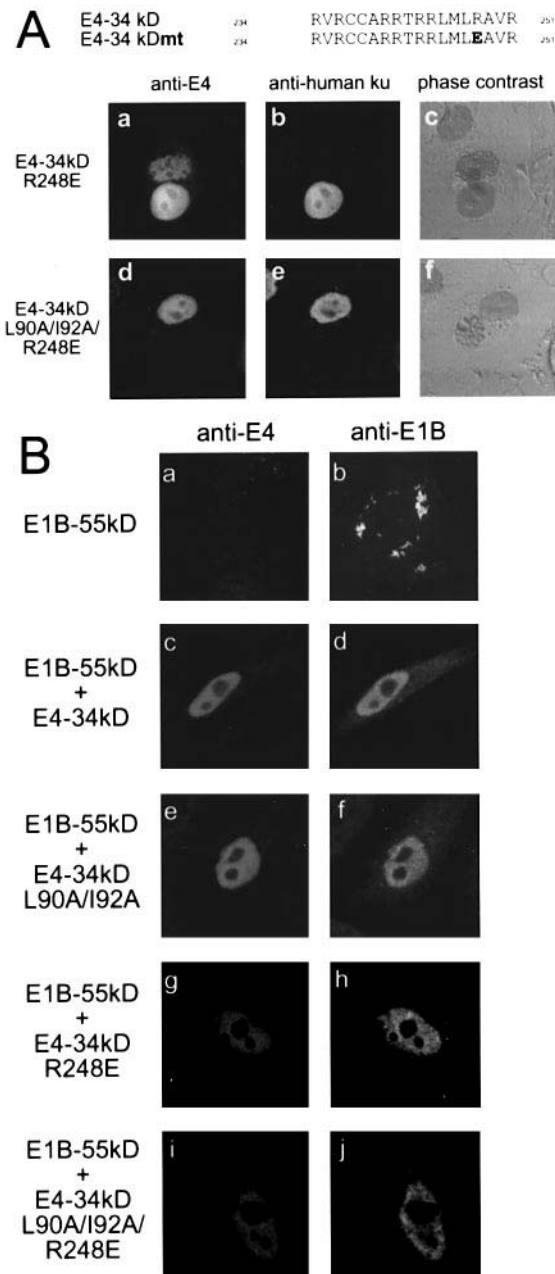


Fig. 3. (A) Effect of a mutation within the arginine-rich amphipathic helical domain of E4-34kD (R248E) on nuclear retention. The mutant protein was expressed in HeLa cells and subjected to a heterokaryon assay (a–c). A double mutant of E4-34kD having the NES-mutation (cf. Figure 2A) in addition to R248E was assayed in parallel (d–f). **(B)** Effect of mutations within E4-34kD on E1B-55kD translocation from the cytoplasm to the nucleus. E1B-55kD was expressed in HeLa cells by transient transfection in the absence (a and b) or presence of E4-34kD expression plasmid (c–j). Either wild type E4-34kD (c and d) or the indicated mutants of it (e–j) were expressed. The cells were fixed 18 h after electroporation, and simultaneously stained for E4-34kD (a, c, e, g and i) and E1B-55kD (b, d, f, h and j) as described in the legend to Figure 1.

the rev protein in a dominant fashion (Katahira *et al.*, 1995). The inhibitory effect presumably results from rapid shuttling by the rex protein in a ‘short circuit’ across the nuclear membrane that exhausts the cellular machinery mediating this type of export. Coexpression of the rex inhibitor greatly reduced shuttling by the E4-34kD pro-

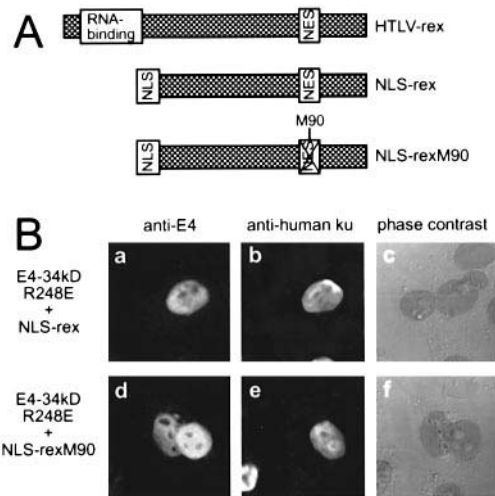


Fig. 4. (A) Schematic depiction of fusion proteins that inhibit the rev export pathway. The RNA binding domain of HTLV-1 rex was replaced by the nuclear localization signal (NLS) from SV40 T antigen. This fusion protein still retains the rex nuclear export signal. A point mutation within the NES, M90, renders this fusion protein inactive for export and competition, thus serving as a negative control. **(B)** Effect of an inhibitor of rev-mediated export on nucleocytoplasmic shuttling of E4-34kD. A mutated form of E4-34kD carrying an amino acid exchange (arginine 248 to glutamic acid) to allow shuttling in the absence of E1B-55kD was coexpressed with NLS-rex (plasmid pCFNrex, a–c), or with an inactive rex fragment carrying a point mutation at amino acid 90 of rex (pCFNrexM90, d–f). Shuttling assays were performed as described in the legend to Figure 1.

tein mutated in the putative retention domain (Figure 4B, a–c). When an inactive form of the inhibitor carrying a mutation (Figure 4A) within the rex NES region (Katahira *et al.*, 1995) was used, no such effect was observed (Figure 4B, d–f). Taken together, these results suggest that the nuclear export of E4-34kD occurs on a pathway that overlaps with the pathway supporting export of HIV rev and HTLV rex.

We also asked whether overexpressed E4-34kD protein can inhibit rev function. To this end, HeLa cells were cotransfected with a rev expression plasmid (pRSVrev) and a reporter construct that contained the chloramphenicol acetyl transferase (CAT) coding region within an intron that also contains the rev responsive element (RRE) of HIV RNA (pDM138RRE) (Hope *et al.*, 1991). In this assay, export of non-spliced RNA by rev is assayed by the accumulation of CAT activity (McDonald *et al.*, 1992). In each experiment, a reporter construct expressing luciferase independently of rev was cotransfected as an internal reference for normalization. The rev protein efficiently mediated nuclear export of RRE-containing RNA (Figure 5, compare lanes 1 and 8). However, when E4-34kD was coexpressed, CAT expression was reduced in a dose-dependent manner (Figure 5, lanes 2 and 3). In contrast, neither the E1B-55kD expression construct nor the empty expression vector had a detectable effect on CAT expression (Figure 5, lanes 4–6). NLS-rex inhibited rev-dependent CAT expression (Figure 5, lane 7), as predicted from previous results (see above). These results strongly suggest that E4-34kD can inhibit nuclear export mediated by the HIV rev protein.

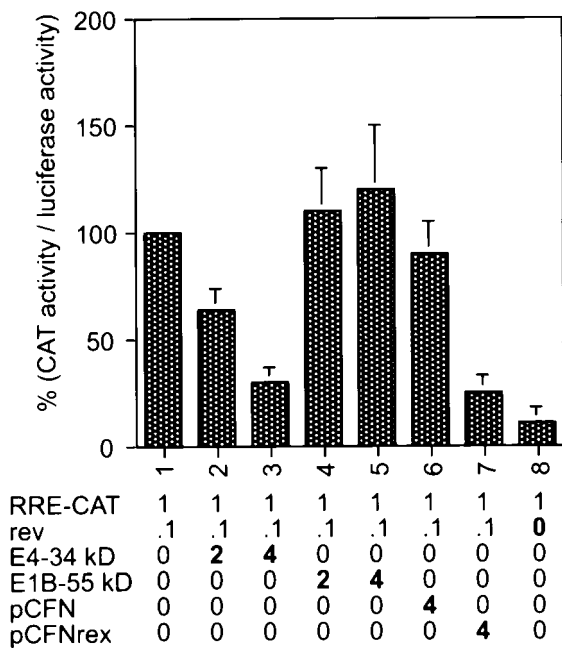


Fig. 5. E4-34kD as an inhibitor of rev-mediated RNA export. The ability of the rev-protein to export non-spliced mRNA was assayed by cotransfecting a rev expression plasmid (pRSVrev; 0.05 µg) with a reporter plasmid containing the CAT coding sequence within an intron and along with the rev responsive element from genomic HIV RNA (pDM138RRE; 0.5 µg), and the ability of rev to export RNA was quantified by the CAT activity observed. pDM138RRE was transfected into HeLa cells in the absence (lane 8) or presence (lanes 1–7) of pRSVrev. Increasing amounts of the E4-34kD expression plasmid were cotransfected (lanes 2 and 3), and so was the E1B-55kD expression plasmid (lanes 4 and 5). As a positive control, a known inhibitor of rev-export was cotransfected (pCFNrex, lane 7) and compared with the cotransfection of its empty expression vector (pCFN, lane 6). In all experiments, the total amount of cotransfected expression plasmid was kept at 3 µg by adding empty expression vector pCMVneoBam; 0.5 µg of a luciferase expression construct (pGL3control, Promega) were cotransfected as an internal standard. The ratios of CAT and corresponding luciferase activities are shown (average and standard deviation of at least three independent experiments).

Discussion

Our data show that the adenoviral modulators of mRNA export, E1B-55kD and E4-34kD, shuttle between the nucleus and cytoplasm on a rev-like pathway. The E4-34kD protein (Figure 6A) appears to mediate both import (Goodrum *et al.*, 1996) and export of the complex consisting of E1B-55kD, E4-34kD, and possibly cellular factors (Figure 6B). The export is mediated by an NES, and competition experiments (Figures 4 and 5) indicate that the E4-34kD NES interacts with the same cellular factors as the rev and rex proteins from human retroviruses. This competition leads us to infer that the adenovirus and retrovirus mRNA transport systems operate through the same or overlapping transport pathways. It is intriguing to note that in *Xenopus* oocytes peptides comprising the rev NES inhibit the nucleocytoplasmic transport of 5S ribosomal RNA and spliceosomal U1 small nuclear RNAs (snRNAs) but not mRNA, tRNA or ribosomal subunit RNAs (Fischer *et al.*, 1995). This result has been interpreted to suggest that rev-mediated viral mRNA transport utilizes a 5S RNA/snRNA pathway rather than a cellular mRNA pathway, and the same may be true of the adenovirus mRNA transport system. Consistent with this

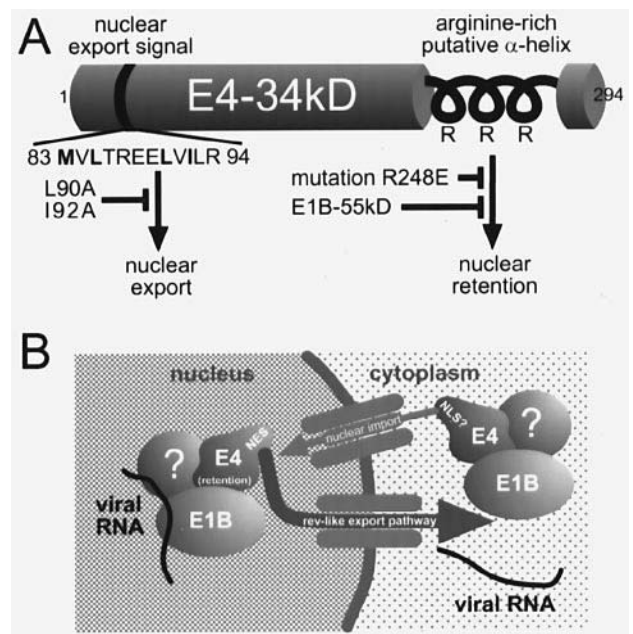


Fig. 6. (A) Domains on E4-34kD that play a role in nuclear export. An NES motif (amino acids 83–92) is required for export, and its mutation (L90A and I92A) abolishes nucleocytoplasmic shuttling. A C-terminal arginine-rich sequence (amino acids 234–251) predicted to fold into an amphipathic α helix mediates nuclear retention, unless mutated (R248E) or inactivated by association with E1B-55kD. (B) Model for adenovirus-mediated RNA-export. E1B-55kD and E4-34kD form a complex that might contain cellular protein(s) as well. E4-34kD directs nuclear import and nuclear export of this complex. Viral mRNA may be exported by direct or indirect interaction with this complex. Tethering of cellular factors essential for mRNA transport to this complex may lead to the observed block in the export of most cellular mRNA species during adenovirus infection.

proposal, 5S rRNA and U1 snRNA have been shown to continue to accumulate in the cytoplasm of adenovirus-infected cells where mRNA transport is blocked (Smiley *et al.*, 1995). However, it remains possible that 5S RNA, snRNA and cellular mRNAs utilize the same pathway; the competition experiments might detect differences in the affinity of various NES motifs for an RNA transport machine that carries all classes of transported RNA to the cytoplasm. If this latter proposal is correct and the E4 NES exhibits a relatively high affinity for components of a common transport pathway, then the E4 protein could redirect the transport system from cellular to viral mRNAs as it accumulates in the periphery of viral replication/transcription centers.

The E4 NES sequence contains some dissimilarities when compared with the corresponding sequences from rev and rex but it fits reasonably well within the context of the growing family of naturally occurring NES motifs (Figure 2A) and the functional NES elements derived from an *in vivo* randomization-selection assay (Bogerd *et al.*, 1996). A relatively broad range of sequences can mediate the export function when placed in a suitable context. The rev NES has been reported to interact with several different cellular factors, including the protein termed Rab (Bogerd *et al.*, 1995) or hRIP (Fritz *et al.*, 1995). The similarity in the E4 and rev NES motifs (Figure 2A) and the ability of the two proteins to compete for one or more limiting cellular factors (Figure 4 and 5)

suggest that rev-binding factors such as Rab/hrIP also interact with the adenovirus E4-34kD protein.

E4-34kD does not shuttle when it is expressed alone in the nucleus of HeLa cells (Figure 1). This is in contrast with a previous report suggesting that E4-34kD alone is a shuttling protein (Goodrum *et al.*, 1996). The apparent discrepancy can be explained by the assumption that the nuclear retention is specific for primate cells. In our experiments, the viral proteins were expressed in human cells that were fused to rodent cells. In the previous report, E4-34kD was transiently expressed in rodent cells before cellular fusion and movement from rodent to human nuclei. The protein not only shuttled but also accumulated in the human nuclei to an extent exceeding the amount found in the rodent nuclei. This is consistent with the hypothesis that E4-34kD is retained in primate but not rodent nuclei. The mechanism of nuclear retention is currently not understood. Our results indicate that the arginine-containing amphipathic α helix within E4-34kD is important for nuclear retention (Figure 3). Perhaps the motif can interact with the nuclear matrix or a matrix-associated factor that holds the protein in the nucleus. Since E4-34kD does not shuttle in the absence of E1B-55kD, it is clear that the 'retention domain' is dominant over export signaled by the NES. E1B-55kD might sterically block the E4-34kD retention signal when the two proteins interact to activate shuttling.

Our data suggest that E4-34kD does not require the presence of E1B-55kD to inhibit rev function (Figure 5). Cotransfection of E1B-55kD does not significantly change the inhibitory effect of E4-34kD on rev, and E4-34kD with the R248E mutation seems to work equally as well as wild-type E4-34kD to inhibit rev function (our unpublished observations). Therefore, E4-34kD apparently does not need to be liberated from retention to block the export activity of rev. Perhaps a factor necessary for rev-mediated RNA transport is bound and sequestered by E4-34kD regardless of its retention or precise intranuclear location.

E4-34kD might contain a nuclear retention signal so that it can be retained in the nucleus to perform non-transport-related functions when it is not associated with E1B-55kD. Since the retention signal in E4-34kD is rich in arginine, one might speculate that the motif functions as a nuclear localization signal as well. Indeed, a portion of E4-34kD comprising the putative amphipathic α helix (residues 230–283) can mediate nuclear import when fused to β -galactosidase, but the same is true for a fusion protein containing the R248E mutation (our unpublished observations). Therefore, nuclear localization and nuclear retention seem to be distinct activities that can be separated by a point mutation. Further, the complex of E4-34kD and E1B-55kD fused to the NLS from SV40 T antigen can exit the nucleus (Figure 1, j–l), suggesting that nuclear retention is mediated by an activity different from nuclear import. The heterogeneous ribonucleoprotein (hnRNP) C proteins also contain a nuclear retention domain that overlaps the NLS region and is rich in arginine residues (Nakielny and Dreyfuss, 1996). It is tempting to speculate that E4-34kD and the hnRNP C proteins might be retained in the nucleus by the same mechanism.

Our results are consistent with a model (Figure 6B) in which E4-34kD functions as a transporter and E1B-55kD

mediates the interaction of the transporter with RNA. Although such an E1B-55kD–RNA interaction has not been demonstrated, there are several lines of evidence consistent with such a role for E1B-55kD. First, E1B-55kD does not seem to mediate nuclear export in the absence of E4-34kD, yet it is required for mRNA export during infection, consistent with the view that it provides a link between the E4-34kD transporter and the mRNA to be exported. Secondly, the expression of E1B-55kD protein fused to a nuclear localization signal blocks the export of mRNA in yeast (Liang *et al.*, 1995), arguing that E1B-55kD may have the capability to interact directly or indirectly with mRNA. Thirdly, NLS-tagged E1B-55kD localizes in discrete nucleolar structures in a pattern similar to that observed when transiently expressing HIV rev; and in rev, the nucleolar localization maps to the same sequence elements as the interaction with RNA (Cochrane *et al.*, 1990; Venkatesh *et al.*, 1990; D'Agostino *et al.*, 1995). The nucleolar localization of NLS-tagged E1B-55kD is sensitive to low concentrations of actinomycin D that block the synthesis of ribosomal RNA in the nucleolus (W.T.Kimberley, unpublished data), as has been reported for HIV rev (Richard *et al.*, 1994; D'Agostino *et al.*, 1995). The RNA binding domain of rev can interact either with the RRE or with the nucleolar B23 protein, but not with both (Fankhauser *et al.*, 1991). Similarly, the domain within NLS-tagged E1B-55kD that attaches to a nucleolar structure might associate with RNA in the context of an adenoviral infection. Finally, the E1B-55kD protein contains a domain at residues 249–288 homologous to the RNP domains that mediate direct interaction with RNA (J.Horridge and K.Leppard, personal communication).

It still remains to be determined why the same adenoviral proteins that mediate RNA export each also bind directly to the tumor suppressor p53, and antagonize its activity. Intriguingly, the hdm2 oncoprotein, which binds to the same region on p53 and inhibits its activity as does E1B-55kD (Lin *et al.*, 1994), also shuttles between the nucleus and the cytoplasm (J.Roth, M.Dobbelstein, D.A.Freedman, T.Shenk and A.J.Levine, unpublished results); and hdm2 has been shown to have RNA binding properties as well (Elenbaas *et al.*, 1996). E1B-55kD, E4-34kD and hdm2 are the first oncogene products shown to undergo nuclear export, raising the possibility that rev-like nuclear export might contribute to the regulation of cellular growth and oncogenic transformation.

The formation of a E1B-55kD–E4-34kD complex cannot be observed in most rodent cells. In those cells, E1B-55kD is not relocalized to the nucleus when co-expressed with E4-34kD. Fusion of these rodent cells with human cells apparently leads to complex formation, as judged by relocalization of E1B-55kD (Goodrum *et al.*, 1996). This suggests that a primate cellular factor is required for the complex formation between the two viral proteins. Perhaps a cellular protein forms a complex with both viral proteins simultaneously. Even though both adenovirus proteins have been shown to interact with p53 (Dobner *et al.*, 1996), the presence of p53 is not required for the relocalization of E1B-55kD by E4-34kD, since this phenomenon can also be observed in Saos-2 and H1299 cells, which both lack a p53 gene (not shown). Therefore, p53 is not a necessary partner to form a complex containing both viral proteins. Another possibility is that a factor specifically found in

primate cells can post-translationally modify one or both of the viral proteins, thus enabling them to interact efficiently with each other. This could, for example, be achieved through a cellular kinase. Indeed, at least E1B-55kD is known to be a phosphoprotein, and the mutation of some phosphorylation sites can reduce the protein's efficiency to inactivate p53 (Teodoro *et al.*, 1994). However, these mutations did not show an observable effect on the virus's ability to shut down cellular protein synthesis (Teodoro *et al.*, 1994), indirectly suggesting that the modulation of mRNA export, and hence the association of E1B-55kD with E4-34kD, were not affected.

Should a cellular factor (or several of them) form a 'bridge' between the two viral proteins, then such a factor may function as part of a complex that mediates mRNA export in the absence of virus, and may be recruited away from cellular mRNA during adenovirus infection, explaining the observed block in the export of most, though not all (Moore *et al.*, 1987), cellular mRNA molecules (Ornelles and Shenk, 1991). An important goal for future investigations will be the identification of these putative cellular factors.

In conclusion, the adenoviral strategy to influence mRNA export comprises several unique features that are not found in retroviral RNA export: (i) the use of at least two different viral proteins that cooperate to modulate mRNA export; (ii) the growth-regulating and transforming activity of these proteins; (iii) the involvement of a dominant nuclear retention domain in addition to an NES in one of these components; (iv) the species-specificity of the proteins' function and (v) the simultaneous down-regulation of cellular and upregulation of viral mRNA export. An enhanced understanding of these viral proteins will potentially reveal how the cell regulates the transport of its mRNA from the nucleus to the ribosome and how this in turn regulates other cellular processes.

Materials and methods

Cells and antibodies

All cell lines were obtained from ATCC and propagated in DMEM containing 10% FBS. Monoclonal antibodies were obtained from hybridoma cultures and used as crude supernatants, unless stated otherwise.

Plasmids

Expression plasmids for the E1B-55kD protein were constructed as follows. The E1B-55kD coding sequence and 3' non-coding region (adenovirus type 5 nucleotides 2019–4107) were excised from the plasmid pDCRE1B (Dobner *et al.*, 1996) using *Bam*HI. The expression vectors pCGN and pCFN containing the cytomegalovirus major immediate early promoter (Zhu *et al.*, 1995) were linearized with *Xma*I. The fragments were filled in with Klenow DNA polymerase fragment and ligated to each other to yield pCGN-E1B and pCFN-E1B, respectively. The proteins expressed from these constructs were both N-terminally fused to an HA-tag sequence that can be recognized by the monoclonal antibody 12CA5 (Boehringer Mannheim). pCFNE1B additionally contains an N-terminal nuclear localization signal derived from SV40 T antigen. Plasmids containing E1B-55kD coding sequences were routinely repropagated in *Escherichia coli* strain SURE (Stratagene) lacking several recombinase enzyme complexes, and each newly prepared batch was checked by restriction analysis, since the presence of these sequences in a plasmid appeared to slow bacterial growth and increase the risk that rearranged forms of the plasmids might accumulate (our unpublished observations).

pCMVE4-34kD (Dobner *et al.*, 1996) was mutated within the putative NES coding region according to the instructions of the QuikChange kit (Stratagene) for site-directed mutagenesis, using the complementary

oligonucleotides GGTCTAACCCGGGAGGAGGCTGTAGCCCTG-AGGAAGTG and CACTTCCTCAGGGCTACAGCCTCTCCCGGGT-TAGAACC. This resulted in a mutation of the amino acids L90 and 192 to alanine residues. Similarly, the arginine residue 248 was changed to glutamic acid using the oligonucleotides CAAGGCGCCTTATGCT-CGAGGCGGTGCGAATCATC and GATGATTCGCACCGCCTCG-AGCATAAGGCGCCTTG. A double mutant was created by performing the two procedures serially.

To construct pCFNrex and pCFNrexM90, the 3' portion of the rex coding region was PCR-amplified using the primers TGCTTAGACC-CACTTCCAGGGTTTGGACAGA and GCGGGATCCTCACGTG-GGGCAGGAGGGGCCA from the plasmids pSR α TagRex and pSR α TagRexM90, respectively (Katahira *et al.*, 1995) (generous gift from H.Shida). The PCR products were cloned into pCFN using the enzymes *Xba*I and *Bam*HI.

The plasmids pGexNESE4 and pGexNESE4mt were each constructed by hybridizing two oligonucleotides to each other, performing a fill-in reaction with Klenow DNA polymerase fragment, and treating with the restriction enzymes *Bam*HI and *Eco*RI. The vector pGex3x (Pharmacia) was treated with the same enzymes, and then ligated to the fragments. These plasmids were used to express NES sequences fused to the C-terminus of GST. The oligonucleotide CGGATCCCTGCTT-CCATGGTTCTAACGCGGGAGGAG was hybridized to each of the following two oligonucleotides: GGAATTCCTCAGGATTACAAGCT-CCTCCCG (for pGexNESE4) and GGAATTCCTCAGGGCTACA-GCCTCCTCCCG (for pGexNESE4mt).

Heterokaryon assays

Nucleocytoplasmic shuttling was detected by using a heterokaryon assay (Pinol-Roma and Dreyfuss, 1992). HeLa cells were transfected with the indicated expression plasmids by electroporation as described by Dobbelstein and Shenk (1996). Briefly, 10 μ g of the expression plasmid was mixed with 400 μ l of a HeLa cell suspension (3×10^6 cells per ml) in DMEM/10%FBS and subjected to a voltage pulse in a cuvette of 0.4-cm gap width using a Bio-Rad Gene Pulser with capacitance extender at 230 V and 950 μ F. Immediately after transfection, the cells from one cuvette were seeded on a 3.5-cm tissue culture dish containing a 2.2 \times 2.2 cm glass cover slip together with 1×10^6 (murine) Balb-c 3T3 cells. After 18 h, the cells were treated with DMEM/10%FBS containing 50 μ g/ml cycloheximide (Sigma) for 15 min. The cells on the cover slip were then covered with a solution of 50% (w/v) polyethylene glycol 8000 (Sigma) in DMEM for 2 min at 37°C, to induce cell fusion. Subsequently, the cover slips were transferred back to DMEM/10%FBS containing cycloheximide and further incubated at 37°C. The cells were fixed 2 h later with 4% paraformaldehyde in PBS for 15 min, followed by permeabilization with 0.2% Triton X-100 in PBS. During and after fixation, all incubations were done at room temperature. The cells were incubated with a blocking solution (10% FBS in PBS with 0.01% w/v sodium azide) for 30 min, followed by primary antibodies [RSA3 mouse monoclonal IgG2a anti-E4-34kD (Ornelles and Shenk, 1991) hybridoma supernatant 1:10 or 9C10 rat IgG anti-E1B-55kD (Oncogene Science) 1: 500, in blocking solution containing 1:40 000 diluted anti-Ku-86 mouse monoclonal IgG1, Santa Cruz J15] for 1 h, three washes for 5 min in PBS, secondary antibodies [FITC-conjugated goat anti-mouse IgG2a or anti-rat IgG, depending on the primary antibody, and Texas Red-conjugated goat anti-mouse IgG1 (Caltag), both diluted 1:500 in blocking solution] for 1 h, then three washes as above followed by mounting in Aquamount solution (Lerner). The cells were examined by fluorescence microscopy using a confocal microscope in a blinded fashion.

Microinjection

GST fusion proteins were prepared from *Escherichia coli* strain DH10B containing the appropriate pGex-derived construct as described previously by Dobbelstein and Shenk (1995). The proteins were adjusted to a concentration of 3 mg/ml in PBS containing FITC-conjugated dextran 150 000 (Sigma FD 150) at 3 mg/ml. This solution was microinjected, as described by Dobbelstein *et al.* (1992), into the nuclei of HeLa cells that were seeded in a semiconfluent monolayer onto glass cover slips coated with poly-lysine (Becton Dickinson) using an automated injection system (AIS, Zeiss/Eppendorf) and an Axiovert 35M microscope (Zeiss). The cells were fixed and stained 2 h after injection with a monoclonal mouse antibody against GST (Santa Cruz) and a Texas Red-conjugated antibody against murine IgG (Jackson) as described above, and evaluated by confocal microscopy in a blinded fashion.

Reporter assays

HeLa cells were transfected with the indicated plasmids by electroporation as described above. In all experiments, the total amount of cotransfected expression plasmid was kept at 3 µg by adding empty expression vector pCMVneoBam (Baker *et al.*, 1990), and 5 µg of pGem7Zf(+) (Promega) were added as a carrier; 0.5 µg of a luciferase expression construct (pGL3control, Promega) were cotransfected as an internal standard. The cells were harvested in reporter buffer (Promega) 18 h after transfection; luciferase and CAT assays were performed using the appropriate reagents (Promega) according to the manufacturer's instructions. Luciferase activities were determined using a luminometer (Analytical luminescence laboratory, Monolight 2010), and CAT assays were quantified after thin layer chromatography using a PhosphorImager (Molecular Dynamics).

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