Evaluating the role of CRM1-mediated export for adenovirus gene expression

Christoph C. Carter,[1] Reza Izadpanah, and Eileen Bridge*

Department of Microbiology, 32 Pearson Hall, Miami University, Oxford, OH 45056, USA

Abstract

A complex of the Adenovirus (Ad) early region 1b 55-kDa (E1b-55kDa) and early region 4 ORF6 34-kDa (E4-34kDa) proteins promotes viral late gene expression. E1b-55kDa and E4-34kDa have leucine-rich nuclear export signals (NESs) similar to that of HIV Rev. It was proposed that E1b-55kDa and/or E4-34kDa might promote the export of Ad late mRNA via their Rev-like NESs, and the transport receptor CRM1. We treated infected cells with the cytotoxin leptomycin B to inhibit CRM1-mediated export; treatment initially delays the onset of late gene expression, but this activity completely recovers as the late phase progresses. We find that the E1b-55kDa NES is not required to promote late gene expression. Previous results showed that E4-34kDa-mediated late gene expression does not require an intact NES (J. Virol. 74 (2000), 6684–6688). Our results indicate that these Ad regulatory proteins promote late gene expression without intact NESs or active CRM1.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Adenovirus; E1b 55kDa; CRM1; Leptomycin B; E4; RNA export; NES

Introduction

The presence of a nucleus in eukaryotic cells has necessitated the development of transport mechanisms for the movement of proteins and RNAs between the nucleus and the cytoplasm. RNA export mediated by the human immunodeficiency virus (HIV) Rev protein was one of the first export pathways to be studied in molecular detail and has provided important insights into RNA transport mechanisms. HIV Rev binds to a specific Rev responsive element (RRE) present in the introns of viral RNAs. The Rev leucine-rich nuclear export signal (NES) binds to a transport receptor, CRM1, resulting in the export of intron-containing RNAs from the nucleus to the cytoplasm. These unspliced RNAs encode additional viral proteins. Thus, the adapter protein Rev binds to viral RNA and the cellular transport receptor CRM1. CRM1 then transfers intron-containing RNP through the nuclear pore complex (reviewed by Cullen, 2000). CRM1 mediates the nuclear export of numerous cellular proteins with leucine-rich NESs as well as U snRNAs, 5S rRNA, and the 60S and 40S ribosomal subunits (Cullen, 2000; Johnson et al., 2002) in uninfected cells. Rev is a viral protein that is able to shunt unspliced viral RNA into the cellular CRM1-mediated export pathway.

The export of cellular mRNA is less well understood. In current models, the protein TAP is thought to be an important receptor for mRNA export. TAP is a cellular protein that binds a stem loop structure known as the constitutive transport element (CTE) present in the introns of some retroviral RNAs. The CTE/TAP interaction promotes the export of unspliced CTE-containing RNAs, just as Rev and CRM1 promote the export of RRE-containing RNA. TAP is the human ortholog of the yeast Mex67 protein. These proteins shuttle between the nucleus and the cytoplasm and are able to interact with nuclear pore complex proteins. Genetic studies in yeast and microinjection experiments in *Xenopus laevis* oocytes indicate that TAP/Mex67 is a critical export factor for cellular mRNA (reviewed by Izaurralde, 2001; Zenklusen and Stutz, 2001). CTE containing

---

* Corresponding author. Fax: +1-513-529-2431.

E-mail address: BridgeE@muohio.edu (E. Bridge).

[1] Present address: 2965 Taubman Medical Library, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0619.

retroviral RNAs access a cellular mRNA export pathway by directly binding the transport receptor TAP. Thus, the CTE and Rev both mediate the export of unspliced retroviral RNA, but they do so by accessing different cellular transport receptors. Recent evidence suggests that there is a relationship between splicing of cellular mRNA and nuclear export (reviewed by Dreyfuss et al., 2002; Reed and Hurt, 2002; Reed and Magni, 2002). Splicing deposits a complex of proteins upstream of the exon–exon junction (LeHir et al., 2000, 2001). The exon junction complex (EJC) proteins include Aly/REF, which binds TAP and may be important for recruiting TAP to mRNAs (Stutz et al., 2000). While retroviral RNAs containing the CTE can directly access TAP, at least some cellular mRNAs may recruit Aly/REF and subsequently TAP, in a splicing-dependent manner (Reed and Hurt, 2002; Reed and Magni, 2002).

Adenovirus (Ad) gene expression results in the production of viral pre-mRNAs, which are polyadenylated and spliced to mRNAs before they are exported from the nucleus to the cytoplasm. The precise mechanism used by Ad to export its RNAs is not known. Model RNA substrates containing exons of the Ad tripartite leader sequence have been used to identify the location of the EJC after in vitro splicing (Le Hir et al., 2000). It is not known if EJC proteins are present on Ad mRNAs synthesized in vivo, or whether Ad mRNAs use the same export pathway as cellular mRNA. There are at least two Ad early proteins that contain leucine-rich NESs that are similar to the HIV Rev NES. These proteins are the 55-kDa protein produced from early region 1b (E1b-55kDa) and the 34-kDa protein produced from early region 4 ORF 6 (E4-34kDa). The E1b-55kDa and E4-34kDa proteins both shuttle between the nucleus and the cytoplasm (Dobbelstein et al., 1997; Dosch et al., 2001; Kratzer et al., 2000), and previous genetic studies implicated a complex of these two proteins in promoting viral mRNA export while preventing cellular mRNA export (reviewed by Tauber and Dobner, 2001). The E1b-55kDa protein was further shown to possess nonspecific RNA-binding activity (Horridge and Leppard, 1998). These observations led to models for viral RNA export in which the NESs of these proteins would promote Ad RNA export by Rev-like mechanism. It was proposed that the E1b-55kDa/E4-34kDa complex would associate with viral RNA and that either the E4-34kDa or the E1b-55kDa NES would link viral RNA to the export receptor CRM1, thereby mediating the export of viral RNA from the nucleus to the cytoplasm (Dobbelstein et al., 1997; Dosch et al., 2001; Kratzer et al., 2000). If this model for viral late mRNA export is correct, we would predict (i) that the NESs of the E4-34kDa and/or the E1b-55kDa proteins should be important for their function in promoting late gene expression, and (ii) that late gene expression should be inhibited by the cytotoxin leptomycin B, which covalently modifies CRM1 and prevents its activity in nuclear transport (Kudo et al., 1999).

Our previous results suggested that the NES of E4-34kDa was not essential for its function in promoting late gene expression. We also found that leptomycin B had little effect on viral late gene expression (Rabino et al., 2000). However, recent studies demonstrating that E1b-55kDa also has a Rev-like NES (Kratzer et al., 2000), and that E4-34kDa export is not completely blocked by leptomycin B (Dosch et al., 2001), prompted us to reevaluate the role of CRM1-mediated export for Ad gene expression. Here, we have tested the effect of preventing CRM1-mediated export with leptomycin B during both the early and the late phases of viral infection to better define the role of this transport pathway in the viral life cycle. We have also studied the ability of an E1b-55kDa mutant with lesions in its NES to complement a defective virus mutant that lacks the E1b-55kDa gene. The results of these studies show that the E1b-55kDa NES is not critical for complementation and confirm that CRM1-mediated nuclear export is not essential for the expression of viral late genes.

Results

Leptomycin B treatment inhibits the cytoplasmic accumulation of NES- containing proteins without affecting cell viability

If Ad late RNA export relies on the NES of either the E4-34kDa or the E1b-55kDa protein, we would expect that treatment of infected cells with leptomycin B should block CRM1-dependent transport and thereby prevent late mRNA transport. Leptomycin B is known from previous studies to inhibit the cell cycle (Yoshida et al., 1990). To make sure that our preparation of leptomycin B was functional, we studied cell growth in cultures treated with leptomycin B. HeLa cells were cultured in the presence of increasing concentrations of leptomycin B. At 24, 48, and 72 h post-treatment, cells were trypsinized and stained with trypan blue, and the number of viable cells per dish was determined by counting a fraction of the suspension in a hemocytometer. The results are shown in Fig. 1. We find that 20 nM leptomycin B was sufficient to inhibit cell growth over a 24 h period. As little as 4 nM leptomycin B was able to inhibit cell growth over a 48 h period. Cells treated with leptomycin B were viable for at least 24 h at all concentrations of leptomycin B tested. Cell death was observed in cultures treated with leptomycin B at 20 and 100 nM for longer than 24 h. These results indicate that leptomycin B inhibits cell growth as previously described (Yoshida et al., 1990), that leptomycin B concentrations between 4 and 100 nM are inhibitory, and that incubations of 24 h or less at these concentrations do not affect the viability of our cells.

We next studied the cellular location of GFP fusion proteins in leptomycin B treated cells. Constructs producing GFP, or GFP fused to the pyruvate kinase nuclear import signal and a leucine-rich NES from the Epstein–Barr virus Mta protein (pGL56) (Chen et al., 2001), were transfected into HeLa cells and the localization of GFP was examined
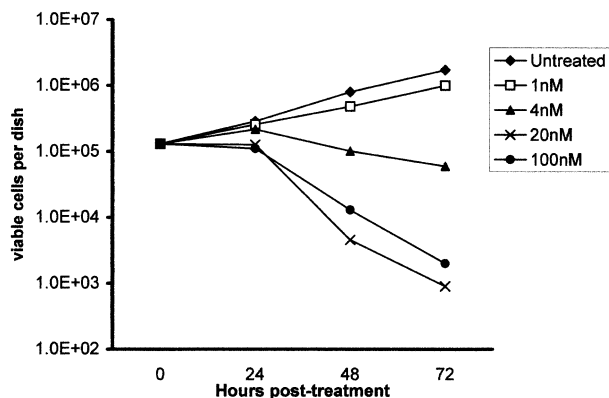
Fig. 1. Leptomycin B inhibits cell growth in a dose-dependent manner. HeLa cells were cultured in the presence of 0 (untreated), 1, 4, 20, and 100 nM leptomycin B. At 0, 24, 48 and 72 h posttreatment, cells were treated with trypsin to release them from the monolayer and stained with trypan blue, and the number of viable cells per dish was determined by counting a fraction of the suspension in a hemocytometer.

after treatment with 20 nM leptomycin B for 12 h. The results are presented in Fig. 2. Leptomycin B had no effect on the localization of GFP. GFP was detected in both the nucleus and the cytoplasm of transfected cells that were and were not treated with leptomycin B (Fig. 2). The NES-containing GFP fusion protein expressed from pGL56 was detected in both the nucleus and the cytoplasm in untreated transfected cells. When these cells were treated with leptomycin B, the NES-containing GFP protein was detected only in the nucleus. The results indicate that leptomycin B treatment inhibited the cytoplasmic accumulation of an NES-containing protein as expected. Identical results were obtained when transfected cells were treated for 2–4 h with leptomycin B (data not shown). Taken together, these results demonstrate that leptomycin B treatment inhibits the nuclear export of a GFP fusion protein containing a leucine-rich NES, and that our cells are viable in the presence of the drug for up to 24 h.

It has been proposed that Ad E1b-55kDa and E4-34kDa proteins may use their NESs to promote viral late mRNA export (Dobbelstein et al., 1997; Dosch et al., 2001). We studied the effect of leptomycin B treatment on the localization of the NES-containing E1b-55kDa protein. HeLa cells were infected with Ad5 at 40 FFU/cell and subsequently were either not treated or treated with 10 nM leptomycin B from 2 to 12 h postinfection (hpi). Cells were fixed and immunostained with antibody against the viral E1b-55kDa protein. In untreated cells infected with Ad5, E1b-55kDa was present in both the nucleus and the cytoplasm. When cells were cultured with leptomycin B E1b-55kDa staining was only observed in the nucleus (Fig. 3A). This result indicates that leptomycin B can prevent localization of E1b-55kDa to the cytoplasm and is consistent with previous reports showing that E1b-55kDa uses its leucine-rich NES to shuttle from the nucleus to the cytoplasm (Kratzer et al., 2000; Dosch et al., 2001). We next determined how long cultures needed to be treated with leptomycin B to inhibit

E1b-55kDa export. Cells infected with Ad5 for 12 h were untreated or treated with 10 nM leptomycin B for the times indicated (Fig. 3B). We found that a 2-h treatment with leptomycin B was sufficient to prevent E1b-55kDa localization to the cytoplasm (see also Dosch et al., 2001).

Leptomycin B treatment does not prevent viral late gene expression

We have found that leptomycin B does not dramatically affect the production of late proteins when added during the
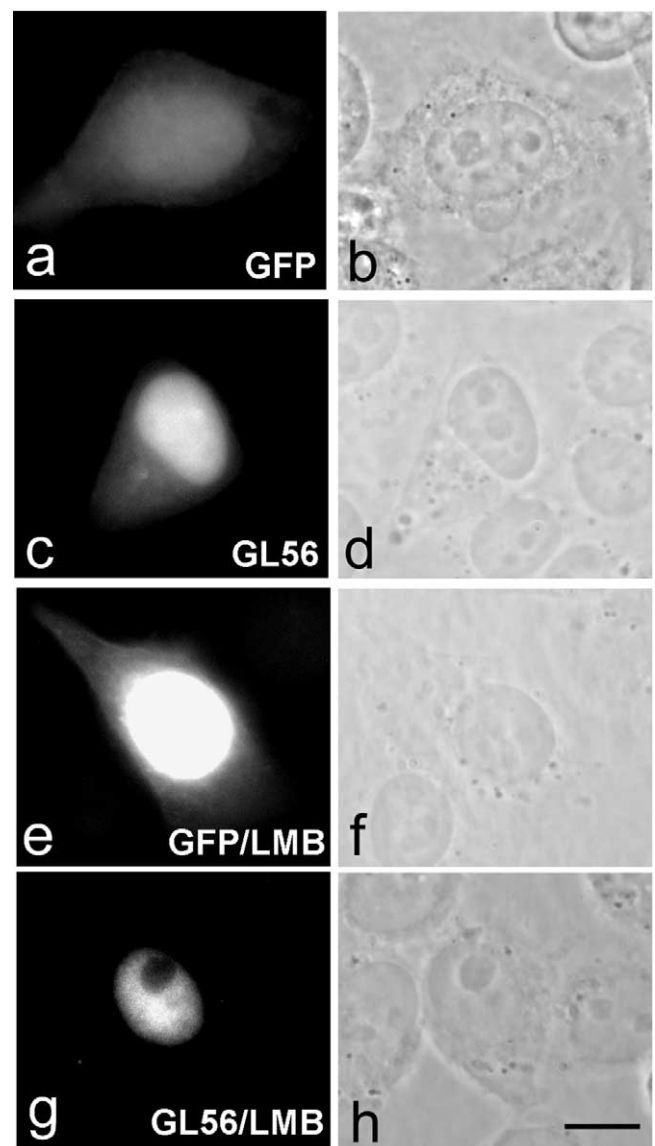

Fig. 2. Leptomycin B treatment prevents the cytoplasmic localization of a GFP protein fused to a leucine-rich NES. HeLa cells were transfected with 2.0 μg pEGFP expressing GFP (a, b, e, and f), and pGL56, which expresses GFP fused to a NLS and a leucine-rich NES (c, d, g, and h). Transfected cells were either not treated (a–d) or treated with 20 nM leptomycin B (LMB) from 36 to 48 h posttransfection (e–h). Transfected cells were fixed with 4% paraformaldehyde and analyzed by microscopy to detect GFP fluorescence (a, c, e, and g). Phase contrast images are shown in b, d, f, and h. Bar, 10 μm.
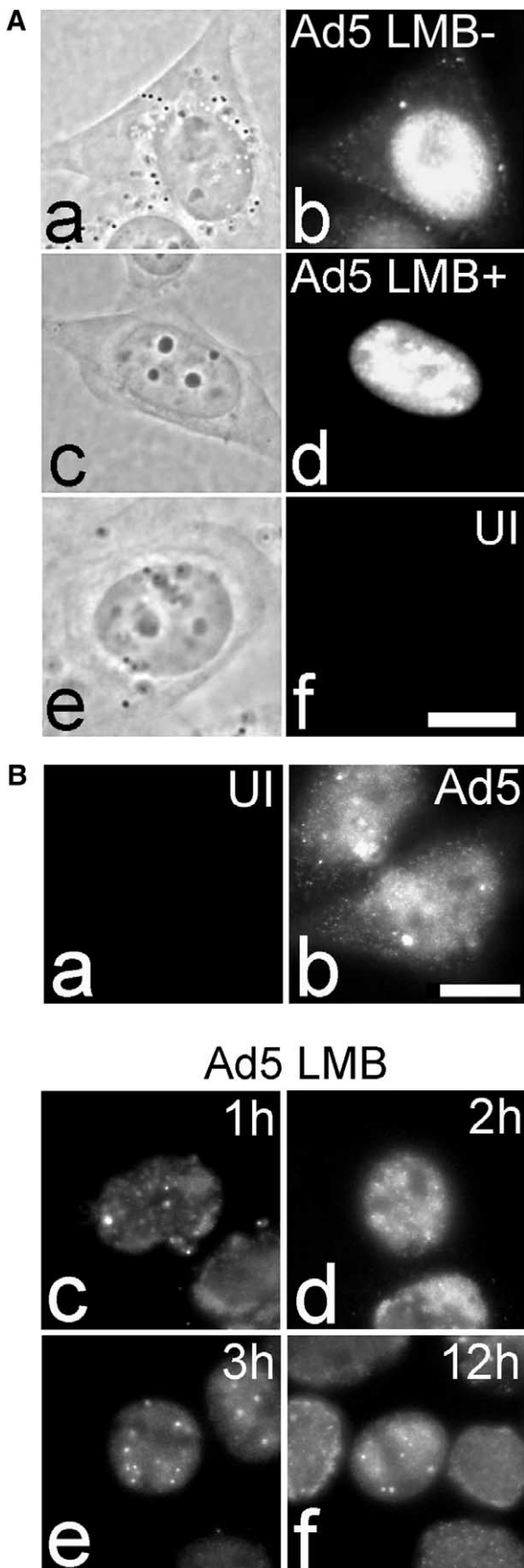
late phase of Ad5 infections (Rabino et al., 2000). These results suggested that CRM1-mediated nuclear export was not critical for late mRNA accumulation in the cytoplasm and the synthesis of late proteins. However, it is known that the E1b-55kDa/E4-34kDa complex has a role in promoting viral replication and late gene expression that is redundant with, and is largely compensated for by, the E4 ORF 3 11-kDa protein (E4-11kDa) (Bridge and Ketner, 1989, 1990; Huang and Hearing, 1989). If E4-11kDa promotes late gene expression independently of the CRM1-mediated export pathway, then it is possible that CRM1-mediated export of E1b-55kDa and/or E4-34kDa would be important for late gene expression in mutants lacking the E4-11kDa gene. We studied the effect of leptomycin B on late gene expression by wild-type Ad5, and E4 mutant H5*il*E4I, which carries the cDNA for the E4-34kDa protein in place of the wild-type E4 coding region. This virus lacks the gene for the E4-11kDa protein, but is similar to wild-type Ad for viral growth (Hemstrom et al., 1988). Cells were infected with Ad5 and H5*il*E4I, and either treated or not treated with 10 nM leptomycin B from 12 to 24 hpi. Extracts were prepared from the infected cells and subjected to Western blotting experiments using an antibody that detects the viral late proteins penton and fiber. We find that leptomycin B had no effect on late protein production in either Ad5 or H5*il*E4I (Fig. 4, top). This result indicates that even in the absence of redundant E4-11kDa functions, inhibiting CRM1-mediated export with leptomycin B has little effect on late gene expression when added during the late phase. In contrast, leptomycin B did affect the accumulation of late proteins of both Ad5 and H5*il*E4I when added to infected cultures from 2 to 12 hpi (Fig. 4, bottom).

We next performed a time course to determine if late gene expression was defective or delayed when infected cells were incubated with leptomycin B throughout the early phase. Cells infected with Ad5 were untreated or treated with 10 nM leptomycin B from 2 hpi to the times indicated. Protein extracts were prepared and subjected to Western blotting. Although late gene expression in leptomycin B treated cultures was reduced at 12 hpi, it had substantially recovered by 16 hpi (Fig. 5A). Southern blotting experiments were then performed to examine the effect of leptomycin B treatment on the accumulation of viral DNA. The results shown in Fig. 5B indicated that viral DNA replication was slightly reduced in cells treated with leptomycin B

Fig. 3. Leptomycin B prevents cytoplasmic accumulation of the E1b-55kDa protein. (A) Cells were infected with Ad5 at 40 FFU/cell and were either untreated (a and b) or treated with 10 nM leptomycin B from 2 to 12 hpi (c and d). Cells were fixed with paraformaldehyde at 12 hpi and immunostained with MAb 2A6 to detect the viral E1b-55kDa protein (b, d and f). Phase contrast images of the cells are shown in a, c, and e. e and f show a cell from an untreated and uninfected (UI) culture. Bar, 10 μm. (B) Uninfected (a) and cells infected with Ad5 at 40 FFU/cell (b-f) were untreated (a and b) or treated with 10 nM leptomycin B (LMB) for the times indicated before fixing at 12 hpi and immunostaining with MAb 2A6 to detect E1b-55kDa.
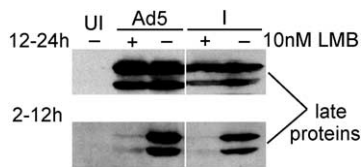
Fig. 4. The affect of leptomycin B treatment on late gene expression by Ad5 and H5il/E4I. Cells were either uninfected (UI) or infected with Ad5 and H5il/E4I (I) at 40 FFU/cell, and subsequently either not treated or treated with 10 nM leptomycin B (LMB) for the times indicated. Extracts were prepared from the infected cells and subjected to Western blotting experiments using an antiserum that detects the viral late proteins penton and fiber.

from 2 to 12 hpi but had recovered completely by 16 hpi. This suggests that leptomycin B treatment during the early phase may delay the onset of the late phase. We think it is likely that once viral DNA replication recovers, late gene expression recovers shortly thereafter.

We examined the levels of the viral early region 2 72-kDa (E2-72kDa) and E1b-55kDa proteins to see if the delay in the onset of late gene expression caused by leptomycin B treatment stemmed from an effect on early protein expression. Cells were infected with Ad5 and were either not treated or treated with leptomycin B from 2 hpi infection to the times indicated. Protein extracts were prepared and subjected to Western blotting using antibodies that detect the E1b-55kDa protein or the viral E2-72kDa DNA-binding protein. We did not observe any dramatic effect on levels of these early proteins when cells were cultured with leptomycin B from 2 to 16 hpi (Fig. 5C). These results suggest that the leptomycin B mediated delay in the onset of the late phase is not the result of reduced early protein levels.

E1b-55kDa proteins with or without an intact NES complement late gene expression of H5dl1016 in the absence of CRM1-mediated export

We previously found that the E4-34kDa NES was not required for late gene expression (Rabino et al., 2000), but the importance of the E1b-55kDa NES for late gene expression has not yet been addressed. We constructed an E1b-55kDa expression plasmid with three leucine-to-alanine changes in its NES (see Materials and methods). We then examined the localization of wild-type and NES-mutant E1b-55kDa proteins transiently expressed in HeLa cells (Fig. 6A). Wild-type E1b-55kDa was found primarily in the cytoplasm with concentration in large perinuclear bodies (Zantema et al., 1985), while localization of the E1b-55kDa NES mutant protein was restricted to the nucleus. This indicated that mutations in the NES prevented the cytoplasmic localization of the protein as was shown previously (Kratzer et al., 2000). When transfected cells were cultured with leptomycin B, both proteins were found in the nucleus, further indicating that the cytoplasmic localization of the wild-type E1b-55kDa protein is the result of CRM1-medi-

ated nuclear export. We next performed complementation experiments in which HeLa cells were first transfected with expression constructs producing either wild-type E1b-55kDa or NES mutant E1b-55kDa and subsequently infected with H5dl1016 (Fig. 6B). H5dl1016 carries mutations affecting the genes of the E1b-55kDa and E4-11kDa proteins. This mutant is defective for the ability to replicate DNA and express its late genes since it lacks the E1b-55kDa partner of the E1b-55kDa/E4-34kDa complex as well as the redundant functions of the E4-11kDa protein (Bridge and Ketner, 1990). We found that the E1b-55kDa NES mutant was able to stimulate late gene expression by H5dl1016 as well as the wild-type protein. A transfected construct producing GFP had no effect on late gene expression by H5dl1016 as expected. Mutant and wild-type E1b-55kDa expression constructs were also able to complement late gene expression in the presence of leptomycin B (Fig. 6B). NES mutant and wild-type E1b-55kDa genes were expressed from transfected constructs in these assays and are therefore not regulated as they would be if they were expressed from the viral genome. Nevertheless, we have ob-
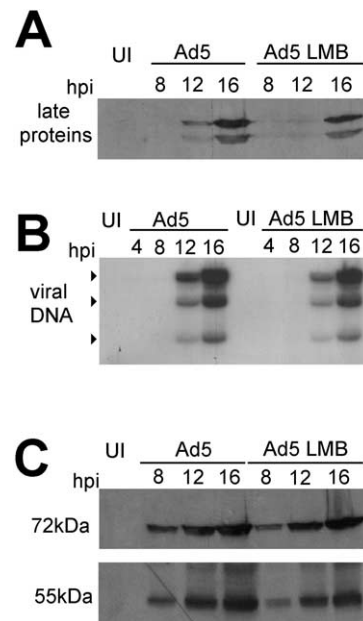

Fig. 5. Leptomycin B treatment delays but does not prevent Ad late gene expression. We performed a time course to study the effect of leptomycin B treatment on the accumulation of viral late proteins (A), viral DNA (B), and viral early proteins (C). Uninfected (UI) cells or cells infected with Ad5 at 40 FFU/cell were untreated or treated with 10 nM leptomycin B (LMB) from 2 hpi to the times indicated. Protein extracts were prepared from a fraction of each culture; total cellular DNA was prepared from the remaining fraction. (A) Western blotting was performed on the protein samples using antiserum raised against the viral late proteins penton and fiber. (B) DNA samples were digested with EcoR1, fractionated on a 1.4% agarose gel, and then transferred to a nylon membrane for Southern hybridization. Random-primed Ad5 DNA was used as a probe. The positions of the DNA fragments corresponding to EcoR1-digested Ad5 are indicated with arrowheads. (C) Western blotting was performed on protein samples using antibodies that detect the E1b-55kDa protein or the viral E2-72kDa DNA binding protein.
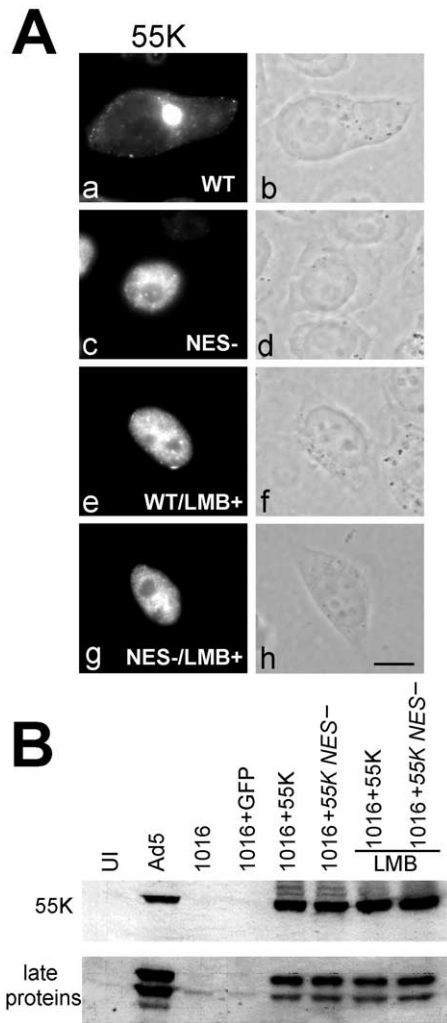
Fig. 6. An E1b-55kDa protein lacking an intact NES promotes viral late gene expression in the presence or absence of leptomycin B. (A) Plasmids expressing wild-type E1b-55kDa or E1b-55kDa with a mutant NES were used to transfect HeLa cells. Immunofluorescence staining was performed with MAb 2A6 to detect the E1b-55kDa protein. The results show the localization of wild-type (WT, a, b, e, and f) and NES mutant (NES-, c, d, g, and h) E1b-55kDa proteins in untreated cells (a–d) and in cells treated from 36 to 48 h posttransfection with 20 nM leptomycin B (LMB, e–h). Bar, 10 μm. (B) A complementation assay was performed by transfecting HeLa cells with constructs producing wild-type E1b-55kDa, a E1b-55kDa NES mutant, or GFP. Transfected cells were subsequently infected with H5dl1016, a defective virus lacking the E1b-55kDa gene, at a multiplicity of 5 FFU/cell. The levels of E1b-55kDa and viral late proteins produced in the transfected/infected cells were measured by Western blotting. MAb 2A6 was used to detect E1b-55kDa and antisera raised against penton and fiber was used to detect viral late proteins. The levels of these proteins detected in extracts from uninfected (UI), Ad5, and H5dl1016-infected cells are shown in the first three lanes. The remaining lanes show E1b-55kDa and late proteins levels in extracts prepared from cells transfected with plasmids expressing the proteins indicated and subsequently infected with H5dl1016. Some transfected/infected cells were treated with 20 nM leptomycin B (LMB) from 12 to 24 hpi where indicated.

served E1b-55K by immunofluorescence microscopy in transfected/infected cells that are positive for late proteins and have complemented late gene expression. We find that the intensity of E1b-55K staining in these cells is similar to that observed in cells infected for 24 h with wild-type Ad5 (data not shown). This suggests that the E1b-55K protein is not dramatically overexpressed in the cells that have complemented late gene expression. Our data show that neither the E1b-55kDa NES nor an active CRM1-mediated export pathway was essential for the ability of the E1b-55kDa protein to stimulate late gene expression in a complementation assay.

Discussion

Our previous results suggested that the NES of the E4-34kDa protein was not critical for its function in complementing late gene expression. These results were confirmed in virus-infected cells by blocking the activity of the CRM1 transport receptor with leptomycin B. Leptomycin B treatment did not dramatically affect Ad late gene expression, suggesting that late gene expression requires neither an intact E4-34kDa NES nor a functional CRM1 export pathway (Rabino et al., 2000). In contrast, Weigel and Dobbelstein (2000) found that the E4-34kDa NES is important for facilitating viral DNA replication, late gene expression, and viral growth. Although Dobbelstein et al. (1997) were able to show that the E4-34kDa NES fused to GST could mediate its export, Dosch et al. (2001) were unable to demonstrate that the E4-34kDa NES could support nuclear export when fused to GFP. Furthermore, Dosch et al. (2001) found that leptomycin B did not prevent E4-34kDa nuclear export. In our experiments, leptomycin B inhibits but does not block E4-34kDa shuttling from the nucleus to the cytoplasm, raising the possibility that E4-34kDa may exit the nucleus by more than one export pathway (Rabino et al., 2000). Finally, the discovery of an NES in the E1b-55kDa protein (Kratzer et al., 2000) raised the possibility that E1b-55kDa might mediate RNA export independently of E4-34kDa (Dosch et al., 2001).

The conflicting reports on the function and activity of the E4-34kDa NES (Bridge, 2000; Tauber and Dobner, 2001), and the observation that the E1b-55kDa protein has an independent NES, prompted us to reexamine the role of CRM1-mediated export for viral replication and late gene expression. Blocking CRM1-mediated export with leptomycin B prevents the cytomplasmic localization of E1b-55kDa and a GFP fusion protein containing a leucine-rich NES (Figs. 2 and 3). During prolonged leptomycin B treatments of 12–24 h, the E1b-55kDa protein remained nuclear (Fig. 3 and data not shown). Leptomycin B treatment delayed but did not block the transition to the late phase when it was added to cultures directly after infection (Figs. 4 and 5). Weigel and Dobbelstein (2000) identified a role for the E4-34kDa NES in the onset of viral DNA replication. It is possible that the delay we observe in the onset of the late phase is the result of inhibiting the nuclear export of the E4-34kDa or E1b-55kDa proteins. However, leptomycin B

treatment can also affect the cell cycle (Yoshida et al., 1990), and experiments by Ornelles and colleagues have suggested that the stage of the cell cycle during infection can affect replication of at least some Ad mutants (Goodrum and Ornelles, 1997, 1999). We do not presently know if the leptomycin B mediated delay in the onset of late gene expression is a direct effect on the export of viral NES-containing proteins, or, if it is a result of blocking the export of other cellular NES-containing proteins, and subsequent effects on the cell cycle and/or other cellular activities.

Despite the initial delay, DNA replication and late gene expression in Ad-infected cells cultured in the presence of leptomycin B had completely recovered by 16 hpi (Fig. 5). Leptomycin B had no effect on the accumulation of late proteins when it was added at the onset of the late phase (Fig. 4), as we have previously shown (Rabino et al., 2000). Significantly, leptomycin B did not inhibit late protein production by H5*il*E4I (Fig. 4). Genetic studies have shown that the functions of the E1b-55kDa/E4-34kDa complex are at least partially redundant with the function of the E4-11kDa protein (Bridge and Ketner 1989, 1990; Huang and Hearing 1989). Since H5*il*E4I lacks E4-11kDa, our results suggest that even in the absence of redundant functions provided by the E4-11kDa protein, the CRM1-mediated export pathway was not essential for continued expression of viral genes during the late phase. Furthermore, since leptomycin B treatment restricts E1b-55kDa localization to the nucleus (Kratzer et al., 2000; Dosch et al., 2001; Fig. 3), our results predict that the export activity of the E1b-55kDa protein should not be critical for its function in late gene expression. We tested the role of the E1b-55kDa NES in complementation studies and found that NES mutations did not affect the ability of E1b-55kDa to stimulate late gene expression of H5*dl*1016 in either the presence or the absence of leptomycin B treatment (Fig. 6B). We have not identified a critical role for the NESs of either the E4-34kDa (Rabino et al., 2000) or the E1b-55kDa protein (Fig. 6B) in complementation studies. A limitation of this assay is that expression of the NES mutant E1b-55kDa and E4-34kDa genes is not regulated by expression from their endogenous promoters within the context of the viral genome. However, the results of the complementation studies are in complete agreement with our observation that inhibiting NES-mediated export with leptomycin B does not dramatically affect viral late gene expression either in the complementation assay (Fig. 6B) or during virus infection (Fig. 4). Taken together, these results strongly support our previous observation that the CRM1-mediated export pathway is not required for viral late gene expression (Rabino et al., 2000) and suggest that other activities of the E4-34kDa and E1b-55kDa proteins are important for their function in promoting viral late gene expression.

E4-34kDa and E1b-55kDa form a complex with a cellular ubiquitin ligase that is able to target specific cellular proteins for proteasome-mediated degradation (Harada et al., 2002; Querido et al., 2001). Known targets of the E1b-55kDa/E4-34kDa complex include the cell-cycle regulatory protein p53 (Querido et al., 2001) and cellular DNA double-strand break repair (DSBR) proteins (Stracker et al., 2002). We have performed a mutational analysis of the E4-34kDa protein and found that the same regions of the protein that are critical for directing proteasome-mediated degradation of p53 are also important for complementing late gene expression of a defective mutant lacking the E4-34kDa gene. We used proteasome inhibitors to demonstrate that complementation depended on the presence of functional proteasomes (K.A. Corbin-Lickfett and E. Bridge, submitted for publication). These results suggest a link between the ability of the E4-34kDa protein to promote late gene expression and its ability to target cellular proteins for proteasome-mediated degradation. However, we also find that E4-34kDa complements late gene expression independently of cellular p53 status. This suggests that degradation of p53 is not the only function of the E1b-55kDa/E4-34kDa complex in promoting late gene expression (K. A. Corbin-Lickfett and E. Bridge, submitted for publication). It is possible that degradation of cellular DSBR proteins by the E1b-55kDa/E4-34kDa complex is important for promoting late gene expression. When the cellular double-stranded break repair system is active, it ligates the ends of linear double-stranded viral DNA to each other, forming genome concatemers. The E1b-55kDa/E4-34kDa complex and the E4-11kDa proteins are able to prevent ligation of viral genomes into concatemers (Boyer et al., 1999; Stracker et al., 2002; Weiden and Ginsberg, 1994). It is not known if the formation of DNA concatemers interferes with subsequent steps in viral late gene expression. Alternatively, additional, and as yet unidentified, proteins targeted for degradation by the E1b-55kDa/E4-34kDa complex could play an important role in selective viral RNA export during the late phase. Numerous cellular RNA-binding proteins are important for the metabolism and export of host RNA (reviewed in Dreyfuss et al., 2002). Although to date no proteins involved in RNA stability or export have been identified as targets for E1b-55kDa/E4-34kDa-mediated degradation, this possibility could provide an attractive explanation for the role of the complex in promoting viral late gene expression while inhibiting the export of newly synthesized host mRNA.

Our results indicate that the CRM1-mediated export pathway is unlikely to be the only export pathway accessed by viral late mRNA. CRM1 exports a variety of shuttling proteins, and some cellular RNAs, but it is not likely to be the major transport receptor used to export cellular mRNA (Gallouzi and Steitz, 2001; Izaurralde, 2001). Ad mRNAs are capped, spliced, and polyadenylated by the host post-transcriptional processing machinery and are therefore structurally very similar to cellular mRNA. How are viral late mRNAs identified and selectively exported from the nucleus during the late phase? One possibility is that the activity of virus gene products shuts down cellular mRNA export pathways, while virus mRNAs are able to access a

separate pathway. Another possibility is that newly synthesized virus mRNAs have exclusive access to cellular mRNA export pathways during the late phase of infection. Ornelles and Shenk (1991) proposed that limiting cellular factors recruited to sites of viral RNA transcription might promote viral mRNA export and be unavailable for promoting cellular mRNA export. In this model, host factors are selectively recruited to newly synthesized viral mRNA. Presumably the activity of viral early proteins would promote the selective interaction of these host factors with viral RNA. Interestingly, Chen et al. (2002) recently showed that the herpesvirus ICP27 protein interacts with, and is able to recruit, the EJC protein Aly/REF to sites of viral transcription. Since Aly/REF interacts with the cellular transport receptor TAP, these results suggest that ICP27 is able to target unspliced herpesvirus RNA to the TAP-mediated cellular export pathway. It remains to be determined whether Ad early proteins will recruit cellular export factors to Ad late transcripts in an analogous manner.

Materials and methods

Cell culture and viruses

HeLa cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in 5% CO_2 at 37°C. Wild-type Ad5 was originally from J. Williams. H5*il*/E4I carries a cDNA expressing the E4 ORF6 34-kDa protein in place of the E4 region (Hemstrom et al., 1988), and therefore, lacks the gene for the E4-11kDa protein. H5*dl*1016 (Bridge and Ketner, 1990) lacks the genes coding for E1b-55kDa and E4-11kDa. H5*dl*1016 was propagated in monolayers of HEK 293 cells. Virus titers, expressed as fluorescence-forming units (FFU)/ml, were determined on W162 cells for Ad5 and H5*il*/E41, as described (Philipson, 1961). Titers of Ad5 and H5*dl*1016 were determined in HEK 293 cells.

Plasmids and transfections

The GFP expressing control plasmid, pEGFP, was from Clontech. pGL56 expresses GFP fused to an NLS from pyruvate kinase and an NES derived from the Epstein–Barr virus Mta protein (Chen et al., 2001) and was a gift from S.D. Hayward. The wild-type Ad2 E1b-55kDa gene cloned into pcDNA3.1 was a gift from Goran Akusjarvi (Punga and Akusjarvi, 2000). We used PCR-based mutagenesis to construct a vector that expresses the E1b-55kDa protein carrying a mutation in its NES. We amplified a fragment of the wild-type clone using a 3′ primer 5′-TAGAAGGCA-CAGTCGAGG-3′ that overlapped with the reverse priming site of pcDNA3.1 located downstream of the 3′ end of the gene. The 5′ mutagenic oligonucleotide primer 5′-GAATGTTGTACAGGTGGCTGAAGCGTATCCGGAAG-CGAGACGCATTGCGAC-3′ contains the *Bsr*GI restriction

site at base-pair (bp) 230 and the NES at bp 246–279 of the E1b-55kDa gene. The sequence of the primer was designed to convert three leucines in the NES (L83, L87, and L91) to alanines. The fragment amplified by PCR was digested with *Xho*I, which cuts just downstream of the gene in the pcDNA3.1 multiple cloning site, and *Bsr*GI and cloned back into the wild-type E1b-55kDa vector cut with *Xho*I-*Bsr*GI. Clones containing NES mutation in the E1b-55kDa gene were identified by the presence of a *Bsp*EI restriction site introduced into the mutagenic oligo. The sequence of the cloned fragment with the expected mutations was confirmed by nucleotide sequence analysis using an automated DNA sequencer (ABI PRISM). Expression constructs were transfected into HeLa cells using the Effectene transfection reagent according to instructions provided by the manufacturer (Qiagen).

Immunostaining

Infected or transfected HeLa cells grown on coverslips for the times indicated were fixed by incubating them with 1% paraformaldehyde in PBS for 3–5 min, extracting with 0.5% Triton X-100 in PBS for 15 min, and postfixing for 10 min in 4% paraformaldehyde in PBS. GFP distribution was analyzed directly after fixing the cells in 4% paraformaldehyde. The intracellular distribution of E1b-55kDa was analyzed by immunostaining of the fixed cells using anti-E1b-55kDa monoclonal antibody (MAb) 2A6, which recognizes an epitope located within the N-terminal 180 amino acids of the protein (Sarnow et al., 1984) and FITC-conjugated anti-mouse Ig as the secondary antibody (Southern Biotechnology Associates Inc.).

Western blotting

Infected, transfected, or transfected/infected cultures were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in 250–300 μL of lysis buffer [150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 0.15% (vol/vol) Nonidet P-40, 0.1 mM DTT, and 5 μg/ml aprotinin and leupeptin]. Cell lysates were sonicated and total protein was measured by Bradford assay using Coomassie Plus protein reagent (Pierce) according to the manufacturer's specifications. Equal amounts of total protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12% polyacrylamide gels. Proteins were transferred to enhanced chemiluminescence (ECL) nitrocellulose (Amersham Pharmacia Biotech) overnight and the membranes were probed with primary antibodies diluted in 5% nonfat dry milk. Primary antibodies used included rabbit antiserum against viral late proteins penton and fiber (Ulf Pettersson), E2-72kDa DNA binding protein (Tomas Linné), and MAb2A6 against E1b-55kDa (from A. Levine). A 1:1500 dilution of peroxidase-coupled anti-mouse or anti-rabbit secondary antibody (Amersham Pharmacia Biotech) was used for detection. The membranes were incubated with ECL Western Blotting Detection Re-

agents according to the manufacturer's instructions (Amersham Pharmacia Biotech) and exposed to autoradiography film.

Southern blotting

Total DNA was prepared from infected and uninfected cells (Bridge and Ketner, 1989). Ten micrograms of each sample DNA was digested with *Eco*RI, and the resulting fragments were separated according to size by electrophoresis through an agarose gel. The fractionated DNA was then transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech) for Southern blotting. Purified Ad5 DNA was used for random priming to generate ^{32}P-labeled probe. The viral DNA fragments hybridizing to the probe were visualized by autoradiography.

Drug treatment

Leptomycin B was a gift from Barbara Wolff and was used for the times and concentrations indicated in the figures.

Acknowledgments

References

Boyer, J., Rohleder, K., Ketner, G., 1999. Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. Virology 263, 307–312.

Bridge, E., 2000. The nuclear export signal within the adenovirus E4orf6 protein contributes to several steps in the viral life cycle. J. Virol. 74, 12000–12001.

Bridge, E., Ketner, G., 1989. Redundant control of adenovirus late gene expression by early region 4. J. Virol. 63, 631–638.

Bridge, E., Ketner, G., 1990. Interaction of adenoviral E4 and E1b products in late gene expression. Virology 174, 345–353.

Chen, I.H., Sciabica, K.S., Sandri-Goldin, R.M., 2002. ICP27 interacts with the RNA export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the TAP export pathway. J. Virol. 76, 12877–12889.

Chen, L., Liao, G., Fujimuro, M., Semmes, O.J., Hayward, S.D., 2001. Properties of two EBV Mta nuclear export signal sequences. Virology 288, 119–128.

Cullen, B.R., 2000. Nuclear RNA export pathways. Mol. Cell. Biol. 20, 4181–4187.

Dobbelstein, M., Roth, J., Kimberley, W.T., Levine, A.J., Shenk, T., 1997. Nuclear export of the E1b 55-kDa and E4 34kDa adenoviral oncopro-

teins mediated by a Rev-like signal sequence. EMBO J. 16, 4276–4284.

Dosch, T., Horn, F., Schneider, G., Kratzer, F., Dobner, T., Hauber, J., Stauber, R.H., 2001. The adenovirus type 5 E1B-55K oncoprotein actively shuttles in virus-infected cells, whereas transport of E4orf6 is mediated by a CRM1-independent mechanism. J. Virol. 75, 5677–5683.

Dreyfuss, G., Kim, V.N., Kataoka, N., 2002. Messenger-RNA-binding proteins and the messages they carry. Nat. Rev. Mol. Cell. Biol. 3, 195–205.

Gallouzi, I.E., Steitz, J.A., 2001. Delineation of mRNA export pathways by the use of cell-permeable peptides. Science 294, 1895–1901.

Goodrum, F.D., Ornelles, D.A., 1997. The early region 1B 55-kilodalton oncoprotein of adenovirus relieves growth restrictions imposed on viral replication by the cell cycle. J. Virol. 71, 548–561.

Goodrum, F.D., Ornelles, D.A., 1999. Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication. J. Virol. 73, 7474–7488.

Harada, J.N., Shevchenko, A., Shevchenko, A., Pallas, D.C., Berk, A.J., 2002. Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. J. Virol. 76, 9194–9206.

Hemström, C., Nordqvist, K., Pettersson, U., Virtanen, A., 1988. Gene product of region E4 of adenovirus type 5 modulates accumulation of certain viral polypeptides. J. Virol. 62, 3258–3264.

Horridge, J.J., Leppard, K.N., 1998. RNA-binding activity of the E1B55-kilodalton protein from human adenovirus type 5. J. Virol. 72, 9374–9379.

Huang, M.M., Hearing, P., 1989. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. J. Virol. 63, 2605–2615.

Izaurralde, E., 2001. Friedrich Miescher Prize awardee lecture review. A conserved family of nuclear export receptors mediates the exit of messenger RNA to the cytoplasm. Cell Mol. Life Sci. 58, 1105–1112.

Johnson, A.W., Lund, E., Dahlberg, J., 2002. Nuclear export of ribosomal subunits. Trends Biochem. Sci. 27, 580–585.

Kratzer, F., Rosorius, O., Heger, P., Hirschman, N., Dobner, T., Hauber, J., Stauber, R.H., 2000. The adenovirus type 5 E1B-55K oncoprotein is a highly active shuttle protein and shuttling is independent of E4orf6, p53 and Mdm2. Oncogene 19, 850–857.

Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E.P., Wolff, B., Yoshida, M., Horinouchi, S., 1999. Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proc. Natl. Acad. Sci. USA 96, 9112–9117.

Le Hir, H., Gatfield, D., Izaurralde, E., Moore, M.J., 2001. The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. EMBO J. 20, 4987–4997.

Le Hir, H., Izaurralde, E., Maquat, L.E., Moore, M.J., 2000. The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon-exon junctions. EMBO J. 19, 6860–6869.

Ornelles, D.A., Shenk, T., 1991. Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein. J. Virol. 65, 424–429.

Philipson, L., 1961. Adenovirus assay by the fluorescent cell counting procedure. Virology 15, 263–268.

Punga, T., Akusjarvi, G., 2000. The adenovirus-2 E1B-55K protein interacts with a mSin3A/histone deacetylase 1 complex. FEBS Lett. 476, 248–252.

Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W.G., Conaway, R.C., Conaway, J.W., Branton, P.E., 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev. 15, 3104–3117.

Rabino, C., Aspegren, A., Corbin-Lickfett, K., Bridge, E., 2000. Adenovirus late gene expression does not require a Rev-like nuclear RNA export pathway. J. Virol. 74, 6684–6688.

Reed, R., Hurt, E., 2002. A conserved mRNA export machinery coupled to pre-mRNA splicing. Cell 108, 523–531.

Reed, R., Magni, K., 2001. A new view of mRNA export: separating the wheat from the chaff. Nat. Cell Biol. 3, E201–E204.

Sarnow, P., Hearing, P., Anderson, C.W., Halbert, D.N., Shenk, T., Levine, A.J., 1984. Adenovirus early region1B 58,000-dalton tumor antigen is physically associated with an early region 4 25,000-dalton protein in productively infected cells. J. Virol. 49, 692–700.

Stracker, T.H., Carson, C.T., Weitzman, M.D., 2002. Adenovirus oncoproteins inactivate the Mre11 Rad50 NBS1 DNA repair complex. Nature 418, 348–352.

Stutz, F., Bachi, A., Doerks, T., Braun, I.C., Seraphin, B., Wilm, M., Bork, P., Izaurralde, E., 2000. REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export. RNA 6, 638–650.

Tauber, B., Dobner, T., 2001. Molecular regulation and biological function of adenovirus early genes: the E4 ORFs. Gene 278, 1–23.

Weiden, M.D., Ginsberg, H.S., 1994. Deletion of the E4 region of the genome produces adenovirus DNA concatemers. Proc. Natl. Acad. Sci. USA 91, 153–157.

Weigel, S., Dobbelstein, M., 2000. The nuclear export signal within the E4orf6 protein of adenovirus type 5 supports virus replication and cytoplasmic accumulation of viral mRNA. J. Virol. 74, 764–772.

Yoshida, M., Nishikawa, M., Nishi, K., Keiichi, A., Horinouchi, S., Beppu, T., 1990. Effects of leptomycin B on the cell cycle of fibroblasts and fission yeast cells. Exp. Cell Res. 187, 150–156.

Zantema, A., Fransen, J.A., Davis-Olivier, A., Ramaekers, F.C., Vooijs, G.P., DeLeys, B., Van der Eb, A.J., 1985. Localization of the E1B proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies. Virology 142, 44–58.

Zenklusen, D., Stutz, F., 2001. Nuclear export of mRNA. FEBS Lett. 498, 150–156.