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Adenoviral E1B-55kDa protein inhibits yeast mRNA export and perturbs nuclear structure

[adenovirus/poly(A)$^+$ RNA/yeast nucleus]

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ABSTRACT **The mechanisms of export of RNA from the nucleus are poorly understood; however, several viral proteins modulate nucleocytoplasmic transport of mRNA. Among these are the adenoviral proteins E1B-55kDa and E4-34kDa. Late in infection, these proteins inhibit export of host transcripts and promote export of viral mRNA. To investigate the mechanism by which these proteins act, we have expressed them in *Saccharomyces cerevisiae*. Overexpression of either or both proteins has no obvious effect on cell growth. By contrast, overexpression of E1B-55kDa bearing a nuclear localization signal (NLS) dramatically inhibits cell growth. In this situation, the NLS-E1B-55kDa protein is localized to the nuclear periphery, fibrous material is seen in the nucleoplasm, and poly(A)$^+$ RNA accumulates in the nucleus. Simultaneous overexpression of E4-34kDa bearing or lacking an NLS does not modify these effects. We discuss the mechanisms of selective mRNA transport.**

During the late phase of infection by adenovirus, most host mRNA export from the nucleus stops while viral mRNAs are efficiently exported (1–5). The two early gene products, E1B-55kDa and E4-34kDa, which are necessary for this differential mRNA export, form a complex (4, 6–8). Differential export does not appear to reflect inhibition of splicing of host mRNAs or to depend on the 5′ tripartite leader sequence which is characteristic of late viral transcripts (2, 9, 10). The activity of the two viral proteins correlates with their location: they are not active early in infection when E1B-55kDa is in the cytoplasm, whereas later, when both proteins are demonstrable in the nucleus, they are active (8, 11, 12). It is not known whether they directly inhibit transport of host mRNAs, but the effect on late viral mRNAs has been attributed to facilitation of an intranuclear step of transport (13). Neither protein has characteristic RNA-binding motifs. E1B-55kDa is also associated with kinase activity (14) and with the tumor suppressor p53 (15, 16).

The dramatic differential effects of the viral proteins provide an opportunity to investigate mRNA export, which is key for gene expression. Due to the complexity of the virus–host cell interaction and mRNA processing in animal cells, we have turned to the yeast *Saccharomyces cerevisiae* to evaluate the effect of the viral proteins on export of cellular mRNA. Since internal methylation may influence mRNA export (17), it is of interest that yeast mRNA, unlike animal cell mRNAs, does not exhibit internal methylation (18).

METHODS

Plasmids and Cells. The inducible promoter of pYeDP60/2 (19) was replaced with that of pLGSD5 (20) to produce pYeSL (a 2μ-based vector containing the *GAL10/CYC1* promoter, the first three codons of the *CYC1* gene, the transcription termination sequence of the *PGK* gene, and both *URA3* and a truncated *ADE2* markers). Between the promoter and terminator regions, there are *Bam*HI, *Cla* I, *Sma* I, *Kpn* I, and *Sac* I sites in order. The *URA3* gene of pYeSL was inactivated by filling in the *Apa* I site to produce pGu, and the plasmid pGa was obtained by deleting a *Bgl* II–*Bst*XI fragment of the *ADE2* gene from pYeSL. A *Bam*HI-compatible synthetic sequence encoding a 12-amino acid sequence (FLEPPKKKRKVE) of the simian virus 40 tumor antigen (SV40T) nuclear localization signal (NLS) was fused in-frame to the first three amino acids derived from the *CYC1* gene of pGu and pGa to give pGuN and pGaN. This fusion retains a *Bam*HI site. The open reading frames of E1B-55kDa and E4-34kDa from serotype-2 adenovirus were PCR amplified from CMV55 or pilE41 (21, 22), digested by both *Bcl* I and *Bam*HI, and inserted separately into the unique *Bam*HI site of these cassettes to give pGu-55/pGuN-55 and pGa-34/pGaN-34. Equivalent constructs [pLGSD5 (20), pNLS-lacZ] include the coding region of β-galactosidase without or with the N-terminal NLS. The sequences derived from PCR amplification were verified by DNA sequencing.

Wild-type diploid strain YPH501 (23) was transformed with each of the plasmids or plasmid combinations and grown at 30°C on uracil or adenine drop-out synthetic solid medium supplemented with 2% glucose (24). Individual colonies were streaked on appropriate drop-out solid media supplemented with 2% glucose or galactose. For measuring growth rate quantitatively, cells were grown to early logarithmic phase in drop-out liquid medium containing 2% raffinose. One-tenth volume of 20% galactose was added to initiate transcription from the *GAL10/CYC1* hybrid promoter. Samples were taken at various times for optical density measurement at 600 nm.

Morphology. Fixation, spheroplasting, and mounting were performed as described (25). Immobilized cells were treated 5 min in prechilled methanol at −20°C. Indirect immunofluorescence staining was performed with the mouse anti-E1B-55kDa monoclonal antibody 2A6 (6), the affinity-purified rabbit anti-nucleoporin NSP1 antibody EC10-2 (26), or a commercial anti-β-galactosidase antibody. Fluorescein isothiocyanate (FITC)- or rhodamine-conjugated goat anti-mouse or anti-rabbit antibodies (The Jackson Laboratory) were used as secondary antibodies as 1:200 and 1:100, respectively. DNA was detected by 4′,6-diamidino-2-phenylindole (DAPI) staining.

To detect poly(A)$^+$ RNA by *in situ* hybridization, immobilized cells were dehydrated by serial ethanol treatments, hybridized with a biotinylated oligo(dT), and stained with FITC-avidin as described (25). For electron microscopy, we prepared cells and thin sections as described (25).

RESULTS

We have subcloned the coding regions of E1B-55kDa (22) and E4-34kDa (21) into galactose-inducible high-copy-number ex-

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Abbreviations: NLS, nuclear localization signal; SV40T, simian virus 40 tumor antigen; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; ORF, open reading frame.

pression vectors to produce pGu-55 and pGa-34, respectively (Fig. 1*A*). Additional constructs include a SV40T NLS (27) at the N terminus of each ORF. The corresponding inducible plasmids are pGuN-55 and pGaN-34 (Fig. 1*A*). Equivalent constructs code for β-galactosidase with or without NLS.

Cells transformed with pGu-55, pGa-34, pGaN-34, or NLS-β-galactosidase grow at similar rates on solid medium containing either 2% glucose or 2% galactose (Fig. 1*B* and not shown). By contrast, the growth of cells expressing pGuN-55 is dramatically reduced in the presence of galactose (Fig. 1 *B* and *C*). Equivalent experiments with cells expressing NLS-E1B-55kDa based on a low copy expression cassette show less severe inhibition of growth (not shown). Thus, the inhibition of E1B-55kDa depends on the level of expression and the NLS.

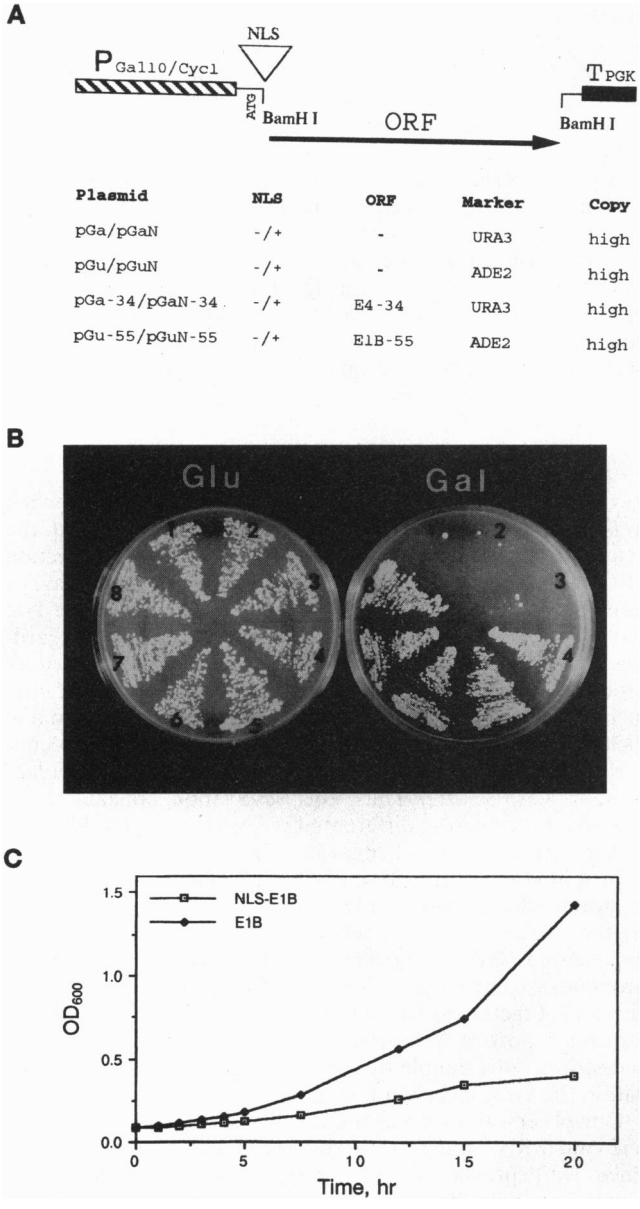

FIG. 1. (*A*) Inducible-expression cassettes. All these plasmids are derived from 2μ-based pYeSL [a derivative of pYeDP60/2 (19)]. Either of the markers (*URA3* and *ADE2*) of pYeSL was inactivated to produce pGu (which retains an *ADE2* marker) or pGa (which possesses a *URA3* marker). A synthetic NLS or SV40T was inserted into the *Bam*HI site to produce pGaN and pGuN separately. PCR-amplified open reading frames (ORFs) were inserted in frame into these expression cassettes. (*B* and *C*) Effect of adenoviral protein overexpression on the growth of yeast. (*B*) Comparison of growth on solid medium supplemented with glucose (Glu) or galactose (Gal). YPH501 (23) was cotransformed by plasmid combinations: 1, pGuN-55/pGa; 2, pGuN-55/pGa-34; 3, pGuN-55/pGaN-34; 4, pGuN-55/pGa; 5, pGu-55/pGa-34; 6, pGu-55/pGaN-34; 7, pGu/pGa-34; and 8, pGa/pGaN-34. Note that growth is severely inhibited in cells transformed with plasmids 1–3. (*C*) Comparison of growth in liquid culture of cells expressing NLS-E1B-55kDa or E1B-55kDa (plasmid combinations 1 or 4 as indicated in *B*). Cells expressing NLS-E1B-55kDa continue to grow slowly for at least 15 hr.

In animal cells, E1B-55kDa is complexed with E4-34kDa late in infection. To explore the possible consequences of coexpression the two proteins in yeast, we compared growth of cells transformed with pGu-55 and pGa-34 or pGaN-34 in the presence of galactose. Growth of cells in either situation is quite normal (Fig. 1*B*). Coexpression of pGuN-55 with either pGa-34 or pGaN-34 reduced cell growth to an extent comparable to pGuN-55 alone. Thus, any association of the two proteins in yeast does not potentiate the activity of E1B-55kDa.

The NLS-E1B-55kDa fusion protein is actually localized to the yeast nucleus. Cells cultured in raffinose give no detectable immunofluorescent signal with an anti-E1B antibody, but after 3 hr of galactose induction, cells give a punctate signal largely coincident with the nuclear pore protein NSP1 (26) (Fig. 2). After longer induction, the staining of NLS-E1B-55kDa forms a continuous yet irregular annulus at the nuclear periphery and sometimes occupies a considerable volume of the nucleus, as is the case for E1B-55kDa in infected animal cells (11, 28). Cells expressing E1B-55kDa without an NLS show only a cytoplasmic signal (not shown), and cells expressing β-galactosidase show a cytoplasmic or nuclear signal according to whether the construct lacks or bears an NLS (Fig. 2).

The nucleus undergoes a dramatic change in cells expressing pGuN-55, although nuclear pores persist. Cells begin to accumulate seemingly fibrillar, often somewhat curved, material in the nucleoplasm after 5 hr of induction (Fig. 3 *Top* and *Middle*). This material, which invades up to half of the nucleoplasm after 15 hr of induction, occasionally appears continuous with the inner nuclear membrane, and therefore may be membranous. It does not, however, obviously stain with the

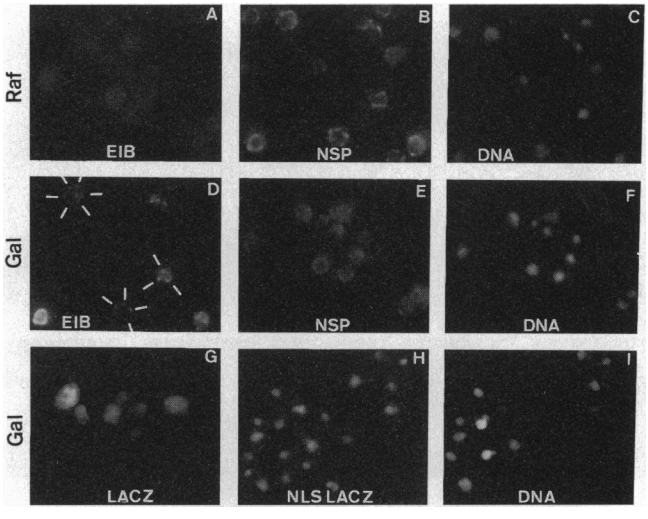

FIG. 2. Localization of NLS-E1B-55kDa, nuclear pores, β-galactosidase, and NLS-β-galactosidase. YPH501 (23) was transformed with pGuN-55, pLGSD5, or pNLS-lacZ. Noninduced (raffinose-grown, *A–C*) or 3-hr galactose-induced (*D–F*) cells were triple-stained to detect E1B-55kDa (*A* and *D*), nuclear pores (*B* and *E*), or DNA (*C* and *F*). (*G* and *H*) Distribution of β-galactosidase in cells transformed with pLGSD5 (i.e., without an NLS) after 3-hr induction (*G*), or transformed with pNLS-lacZ after 3-hr galactose induction (*H*). (*I*) DAPI image corresponding to *H*. Note the accumulation of NLS-E1B-55kDa at the nuclear periphery (*D*) and the accumulation of NLS-lacZ within the nucleus (*H*). (×1000.)

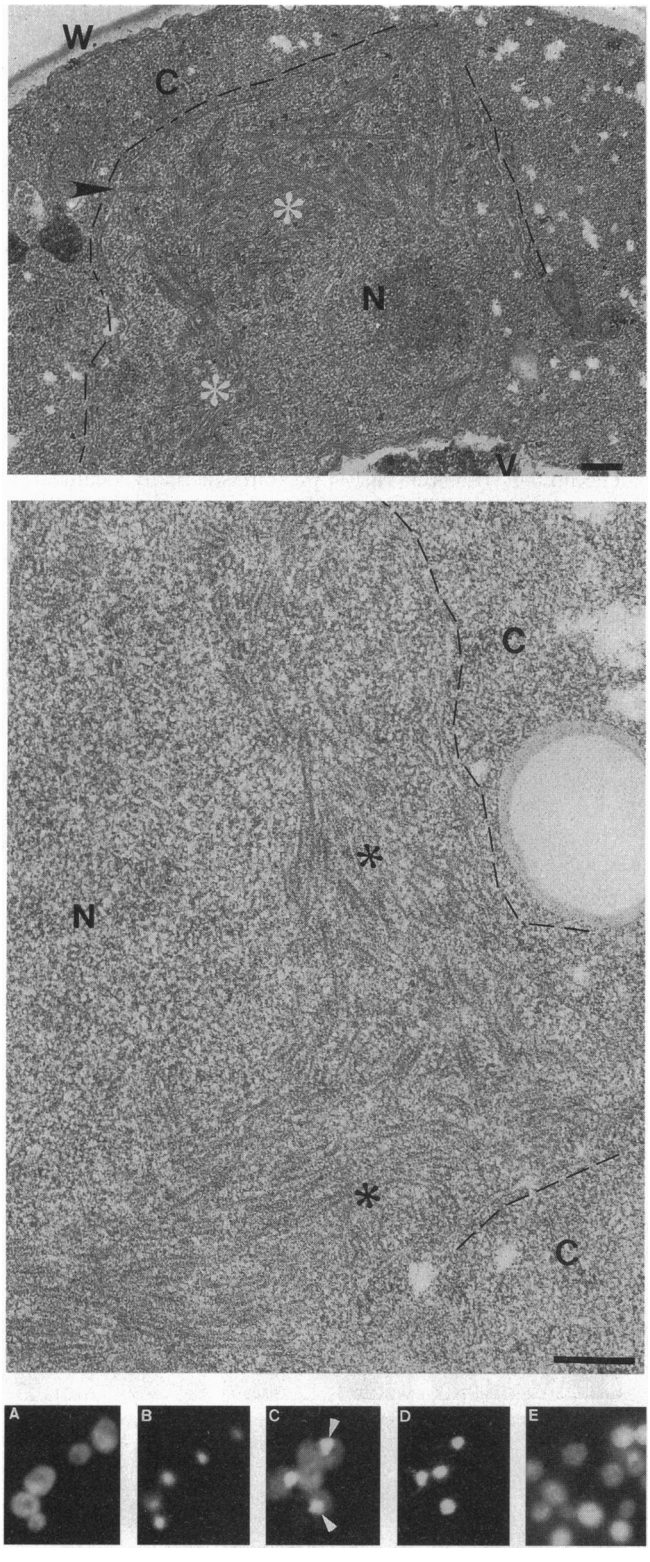

FIG. 3. NLS-E1B-55kDa (pGuN-55) perturbs the nucleus and causes nuclear accumulation of poly(A)$^+$ RNA. (*Top* and *Middle*) Electron micrographs of cells expressing NLS-E1B-55kDa for 15 hr. The wild-type haploid strain YPH258 (23) was transformed with pGuN-55 and used for this experiment. The nuclear envelope is indicated by the dashes. Note the unusual filamentous/tubular structures in the nucleoplasm (asterisks). The large arrowhead indicates the apparent continuity of filamentous material with the inner nuclear membrane. N, nucleoplasm; C, cytoplasm; W, cell wall; V, vacuole. (Bars = 0.1 μm.) (*Bottom*) Distribution of poly(A)$^+$ mRNA in YPH501 expressing NLS-E1B-55kDa or NLS-β-galactosidase. (*A, C,* and *E*) Staining of poly(A)$^+$ RNA by *in situ* hybridization. (*B* and *D*) Staining of DNA by DAPI. (×1000.) Expression of NLS-E1B-55kDa was induced for 0 hr (*A* and *B*) or 15 hr (*C* and *D*). Expression of NLS-β-galactosidase (*E*) was induced for 3 hr. Similar observations were made after 15-hr induction. Note that only NLS-E1B-55kDa leads to a nuclear accumulation of poly(A)$^+$ RNA (*C*, arrowheads). Biotinylated oligo(dA) and RNase treatment were used as negative controls (not shown).

membrane stain 3,3′-dihexyloxacarbocyanine ($DiOC_6$) (unpublished results). In adenovirus-infected animal cells, the nuclear envelope becomes characteristically convoluted and many alterations of the nucleoplasm are seen (29, 30). The present observations show that a possibly related perturbation of the nucleus occurs when nuclear E1B-55kDa is expressed in yeast.

To determine whether nuclear E1B-55kDa blocks mRNA export, a biotinylated oligo(dT) probe was used to detect poly(A)$^+$ RNA by *in situ* hybridization (25). Upon induction of NLS-E1B-55kDa (pGuN-55), nuclear accumulation of yeast poly(A)$^+$ RNA is conspicuous (Fig. 3 *Bottom*). The signal appears to fill the nucleoplasm. It can be detected as early as 3 hr and as late as 15 hr after induction, is sensitive to nonspecific RNase, and is not seen with an oligo(dA) probe (not shown). This nuclear accumulation of poly(A)$^+$ RNA is reminiscent of that in yeast temperature-sensitive mutants that are defective in mRNA export (25, 31). Inhibition of mRNA splicing does not yield such a signal in yeast (32). Expression of E4-34kDa bearing or lacking an NLS, β-galactosidase bearing or lacking an NLS, or E1B-55kDa lacking an NLS does not lead to the accumulation of nuclear poly(A)$^+$ RNA (not shown).

DISCUSSION

In yeast, E1B-55kDa can be detected in the nucleus only when it is equipped with an NLS. These observations and the cytoplasmic distribution of E1B-55kDa during early infection of animal cells indicate that this protein does not have a functional NLS or that its NLS is masked. During the late phase of infection, the protein does enter the nucleus, regardless of whether E4-34kDa is active; however, E4-34kDa does promote the association of E1B-55kDa with the nuclear viral inclusion bodies. These are the sites of viral transcription and include heterogeneous nuclear ribonucleoprotein C proteins, certain small nuclear ribonucleoproteins, and "nucleolar" proteins (11, 33–35). Thus, this association appears to be essential for achieving differential mRNA export. E4-34kDa is also known to affect splicing (36, 37).

In animal cells, the differential effects of adenovirus on viral vs. host mRNA export might be explained in any of three ways: (*i*) the two effects are independent of each other but both depend on E1B-55kDa; (*ii*) inhibition of export of host mRNA promotes export of late viral mRNAs, perhaps by liberating key export factors to be used by the viral mRNAs; or (*iii*) the massive export of late viral mRNAs inhibits export of host transcripts, for example by sequestering key transport factors within the viral inclusion bodies.

Our observations make the third model unlikely, since the one vital mRNA that is transcribed is not a late viral transcript, since overexpression of other mRNAs with a galactose promoter is not inhibitory, and since no viral inclusion bodies are present in yeast. Consistent with the idea that late viral mRNAs are exported because they are transcribed at these inclusion bodies, it is of interest that when foreign genes are engineered into the major late transcriptional unit of the viral chromosome, their transcripts are also efficiently expressed (38, 39). It has also been reported that hsp70, β-tubulin, and influenza mRNAs can be efficiently expressed during adenoviral infection even if they are not transcribed from the viral

chromosome (9, 10). These latter observations prove that efficient export does not require transcription from the adenoviral major later promoter or the presence of a 5′ tripartite leader. The genes in question may well home to the viral inclusion bodies.

We therefore favor the first or second model and suggest (*i*) that E1B-55kDa itself inhibits a key protein(s), possibly part of the nuclear pore complex, which is needed for most mRNA transport and (*ii*) that late viral mRNAs which escape this block require the presence of E1B-55kDa in the viral inclusion bodies, where they rely on export factors which concentrate at these sites. If these export factors are made available due to the inhibition of export of host mRNAs, the second model may be the most accurate. It is not known to what extent this alternate export path matches the normal mechanisms for export of mRNA; however, E1B-55kDa also blocks export of rRNA (40). Studies of *Xenopus* oocytes imply that there are several at least somewhat distinct export paths for RNA (41).

mRNA transport can also be regulated by several other viruses. For example, the influenza M1 protein promotes export of its ribonucleoproteins from the nucleus (42), and the influenza NS1 protein binds to poly(A) and inhibits both splicing and export of mRNA (43, 44). Human immunodeficiency virus rev protein and the human T-lymphotropic virus type 1 rex protein enhance export of unspliced viral mRNAs which include a "rev-response element" (45, 46). Although a weak rev-like phenomenon has been reproduced in yeast (47), the mechanisms by which these several viral proteins regulate mRNA transport in animal cells are unknown.

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