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Adenovirus inhibition of cellular protein synthesis is prevented by the drug 2-aminopurine

(adenovirus translation/double-stranded RNA-activated inhibitor/protein kinase)

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ABSTRACT **Adenovirus infection results in the suppression of cellular protein synthesis, but the mechanism has not been established. In this report we demonstrate that the shut-off of cellular protein synthesis by adenovirus is prevented in cells by treatment with the drug 2-aminopurine. Treatment with 2-aminopurine is shown to prevent suppression of cellular translation without disrupting the normal viral block in the transport of cellular mRNAs from the nucleus to the cytoplasm. We show that viral suppression of cellular protein synthesis occurs concomitant with activation of the interferon-induced double-stranded RNA-activated inhibitor (DAI), a protein kinase, and phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α), but that prevention of host cell shut-off by 2-aminopurine occurs without a decrease in kinase activity or a dephosphorylation of eIF-2α. Results are presented that indicate that activation of DAI kinase and phosphorylation of eIF-2α may be required but are not sufficient to achieve inhibition of cellular protein synthesis during adenovirus infection. We suggest that other events, in particular the modification of additional initiation factors, are likely involved in viral inhibition of cellular translation.**

Adenoviruses (Ad) mediate a complex series of metabolic alterations at late times after infection, suppressing fundamental host processes such as the continued transport and translation of cellular mRNAs while promoting the translation of late viral messages (1, 2). The mechanism by which Ad dominates cellular protein synthesis is only poorly understood. However, recent progress by O'Malley *et al.* (3) has demonstrated that Ad cannot inhibit host protein synthesis in cells that express very low levels of the double-stranded RNA-activated inhibitor (DAI), a protein kinase. DAI kinase is thought to function in the antiviral response because it is induced by interferon. More importantly, its activation after infection by a variety of viruses results in inhibition of translation through the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α; reviewed in ref. 4). However, wild-type (wt) Ad is immune to the activation of DAI kinase (5), whereas Ad mutant strain H5dl331 (dl331), which is unable to synthesize an abundant small virus-associated I transcript called VAI RNA, evokes an enormous activation of DAI kinase and results in the global inhibition of both viral and cellular protein synthesis (6). VAI RNA may counter the host antiviral response during Ad infection because it binds to DAI kinase (7, 8), blocks its activation (9, 10), and preserves the function of eIF-2 (10, 11). Nevertheless, partial activation of DAI and phosphorylation of eIF-2α molecules occur in some cell lines infected by wt Ad (3, 12).

This report investigates the suppression of host cell protein synthesis by Ad and the role for limited activation of DAI kinase and phosphorylation of eIF-2α. We demonstrate that the drug 2-aminopurine (2AP), an agent that inhibits the *in vitro* activity of a number of protein kinases, including DAI (13, 14), prevents the normal shut-off of cellular translation induced by wt Ad as well as the global inhibition of protein synthesis observed in mutant Ad dl331-infected cells. Since 2AP did not prevent the activation of DAI kinase and the phosphorylation of eIF-2α that mostly occurs after viral infection, our results indicate that these changes are not sufficient for suppression of host cell protein synthesis. Instead, we suggest that the inhibition of cellular protein synthesis during Ad infection is mediated by an additional event, very likely the modification of a second initiation factor, which may be prevented by the action of 2AP.

MATERIALS AND METHODS

Viruses and Cells. wt Ad type 5 (Ad5) strain H5wt300 (wt300) is a plaque-purified stock obtained from H. Ginsberg. Mutant Ad dl331 contains a deletion within the intragenic control region of the *VAI* gene and therefore does not produce VAI RNA (6). 293 cells are a human embryonic kidney cell line transformed with the *E1* region of Ad5 (15). Cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum. Unless otherwise specified, cells were infected with high multiplicities of 50 plaque-forming units of virus. Stock solutions of 200 mM 2AP (Sigma) were freshly prepared by heating in 50 mM NaOH to 70°C with mixing until dissolved. 2AP was diluted 1:20 into culture medium to 10 mM final concentration.

Analysis of Polypeptides and RNAs. Cells were labeled for 1–2 hr with 50 μCi (1 Ci = 37 GBq) of *trans*-[^{35}S]methionine (ICN) per ml in DMEM (lacking methionine) supplemented with 2% calf serum. Equal numbers of cells were used for preparation of extracts, immunoprecipitation analysis, and sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis as described (16). Fluorography was performed with EN^{3}HANCE (New England Nuclear). Cytoplasmic poly(A)$^+$ RNAs were prepared as described (16), subjected to RNA blot-hybridization (Northern) analysis in formaldehyde/agarose gels, and transferred to nitrocellulose paper (17). DNA probes for Northern analysis were prepared by using deoxynucleotide [α-^{32}P]triphosphates (18) and correspond to the human actin gene (pHF1; ref. 19), Ad5 *E2A* gene [*Bgl* II 63.6 map units (m.u.)–*Sma* I 68.0 m.u.], Ad *L3* gene (*Sma* I 52.6 m.u.–54.8 m.u.), and Ad *L5* gene (*Hpa* I 89.0 m.u.–*Sma* I 91.9 m.u.). RNA transport studies were performed as described (20). Briefly, cells were labeled for 3 hr with 200 μCi of [^{3}H]uridine per ml, and poly(A)$^+$ and poly(A)$^-$ RNAs

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Abbreviations: DAI, double-stranded RNA-activated inhibitor; Ad, adenovirus(es); Ad5, Ad type 5; eIF-2α, α subunit of eukaryotic initiation factor 2; wt, wild type; VAI RNA, virus-associated I RNA; 2AP, 2-aminopurine; wt300 and dl331, Ad strains H5wt300 and H5dl331; DBP, DNA-binding protein.

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were prepared. Equal amounts of poly(A)$^-$ RNA were resolved in formaldehyde/agarose gels, rRNAs were visualized by fluorography, and levels were quantitated by densitometry of autoradiograms. Analysis of cellular mRNAs was performed by exhaustive hybridization of poly(A)$^+$ RNA to filters containing single-stranded Ad5 DNA. The labeled RNAs remaining in solution represent cellular transcripts newly transported to the cytoplasm (20).

Analysis of eIF-2α Phosphorylation. Preparation of extracts and *in vitro* phosphorylation assays (21) have been described (10). For *in vivo* analysis of eIF-2α, whole-cell proteins were resolved by isoelectric focusing gels as described by Scorsone *et al.* (22) incorporating the modifications of O'Neill and Racaniello (23). Cell lysis in urea/Ampholine mixture, prefocusing of gels, and focusing of polypeptides were carried out as described (23). Proteins were transferred to nitrocellulose for 15 hr at 400 mA as described (22). Immunoblotting was performed without glutaraldehyde fixation by using a monoclonal antibody to eIF-2α (a gift from E. Henshaw, University of Rochester) followed by ^{125}I-labeled goat anti-mouse sera (Amersham). Densitometry of autoradiograms was performed with an LKB Ultrospec II.

RESULTS

2AP Prevents the Ad-Induced Inhibition of Host-Cell Translation. Monolayers of 293 cells were infected with either wt300 or dl331 (VAI$^-$) viruses and were labeled with [^{35}S]-methionine 18 hr after infection; polypeptides were resolved by SDS/PAGE and fluorography (Fig. 1). Infection with wt300 Ad (Fig. 1*A*, lane 3) resulted in suppression of cellular translation and the synthesis of predominantly late viral polypeptides, whereas infection with mutant (VAI$^-$) virus dl331 resulted in a global inhibition of translation (Fig. 1*A*, lane 5) because of the massive activation of DAI kinase (6, 9–12). To study the effects of 2AP on translation shut-off by Ad, cells were exposed to the drug after infection and

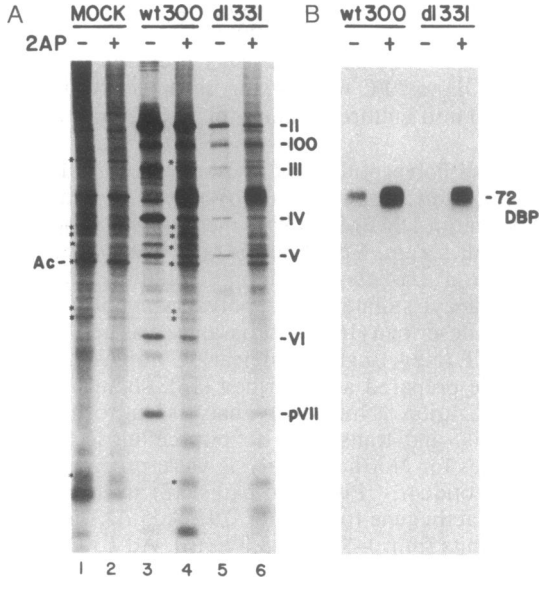

FIG. 1. Effect of 2AP on synthesis of polypeptides in 293 cells late after infection with wt300 or dl331 Ad. Duplicate plates of cells were infected with wt300 or dl331 Ad, 2AP was added to one set 2 hr after infection (lanes 4), cells were labeled for 2 hr with [^{35}S]methionine 18 hr after infection, and extracts were prepared. (*A*) Equal numbers of cells were resolved by SDS/PAGE as described (16). Asterisks mark prominent cellular polypeptides. Late Ad polypeptides are identified on the right side. Ac, actin. (*B*) Immunoprecipitation analysis of cell extracts was performed with monoclonal antibodies to the early Ad 72-kDa DBP.

throughout the period of labeling. The most optimal effect was observed when 2AP was added between 1 and 5 hr after infection, although it weakly prevented the shut-off of cellular translation when added as late as 12 hr after infection (data not shown). In all experiments presented in this study, 2AP was added to cells 2 hr after infection. Whereas 2AP was found to alter only slightly the pattern of protein synthesis in uninfected cells (Fig. 1*A*, compare lanes 1 and 2), it maintained the translation of host mRNAs in cells infected with wt300 virus (Fig. 1*A*, compare lanes 3 and 4). The labeling of both prominent and diffuse host background protein bands demonstrated that treatment of infected cells with 2AP restored cellular protein synthesis to levels approaching that of the corresponding uninfected cells. Surprisingly, 2AP was found to preserve partially the translation of both viral and cellular mRNAs in cells infected with mutant dl331 virus, thereby preventing the global inhibition of protein synthesis typically observed. Accumulation of viral and cellular polypeptides is clearly enhanced (Fig. 1*A*, compare lanes 5 and 6) but not restored to levels observed in wt300-infected cells (Fig. 1*A*, lane 4). Thus, 2AP treatment does not fully correct the translation defect in dl331-infected cells. Synthesis of most late viral polypeptides in cells infected with wt300 virus was only slightly reduced (<50%) after 2AP treatment, demonstrating that suppression of cellular protein synthesis is not required for the enhanced translation of late viral mRNAs.

It also was observed that treatment of infected cells with 2AP resulted in the overproduction of a 70-kDa polypeptide, which was shown by immunoprecipitation analysis to be the Ad 72-kDa DNA-binding protein (DBP, Fig. 1*B*). DBP is produced maximally at intermediate times after infection from mRNAs that lack the viral tripartite leader, a common 5′ noncoding region found on most late viral transcripts. Northern analysis showed that the enhanced production of DBP (10- to 20-fold) caused by 2AP occurred without any significant increase in the level of the corresponding mRNA (see Fig. 3). Thus, it can be concluded that 2AP prevents the normal suppression of cellular translation during wt300 Ad infection and the global inhibition of protein synthesis in mutant dl331-infected cells, the latter previously shown to result from massive activation of DAI kinase (reviewed in ref. 4). Whether all Ad early mRNAs are translationally repressed at late times after infection as are cellular mRNAs is an important issue for investigation.

Effect of 2AP on Viral Growth and Abundance of mRNAs. The growth of wt300 virus was analyzed in the presence and

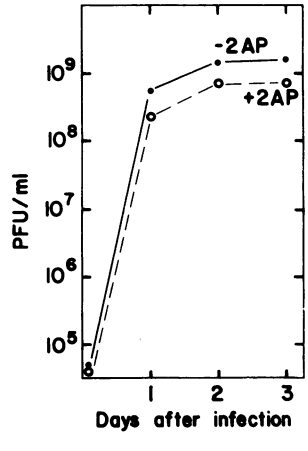

FIG. 2. Growth kinetics of wt300 virus during incubation with 2AP. 293 cells were infected at a multiplicity of 3 plaque-forming units (PFU) per cell, and virus yield was measured by plaque assay. One set of cultures received 2AP throughout the time of infection (3 days). ●, No 2AP; ○, with 10 mM 2AP.

absence of 2AP (Fig. 2). It was found that 2AP did not alter viral growth kinetics and only slightly reduced final growth yields (≈50%), in good agreement with the results of drug treatment on late viral protein synthesis. In addition, 2AP was not toxic to 293 cells during the time course of these experiments, although it did cause cellular degeneration when exposure was extended past 3 days (data not shown).

It was determined whether the maintenance of cellular translation by treatment with 2AP is related to changes in the cytoplasmic levels of either viral or cellular mRNAs. Northern analysis was used to determine the steady-state levels of a number of different cytoplasmic mRNAs at late times after infection with wt300 virus. Levels of cellular and early viral mRNAs remained unchanged during treatment with 2AP (Fig. 3), despite a large increase in the synthesis of new polypeptides. Transcripts analyzed included mRNAs for actin and Ad E2A (Fig. 3) and ribosomal protein L32 and Ad E1a (data not shown). It was also found that treatment with 2AP did not relieve the normal viral block in the transport of cellular RNAs during late Ad infection. RNAs were labeled with [^{3}H]uridine during late (16–18 hr) infection and fractionated into poly(A)$^+$ and poly(A)$^-$ fractions, and the effect of drug treatment on transport was determined (Table 1). Quantitation of labeled 18S rRNA demonstrated a 95% reduction in cytoplasmic accumulation of newly synthesized species as observed (20), which was unaltered by 2AP treatment. The cytoplasmic accumulation of newly synthesized cellular mRNAs was repressed by >70% in late-viral-infected cells to levels close to those reported previously (20) and, most importantly, was also unaltered by treatment with 2AP. The levels of late viral mRNAs, such as hexon L3 and fiber L5 (Fig. 3) generally decreased by factors of 3 to 4 after treatment with 2AP. Similar trends were also found for L2 and L4 transcripts and in dl331-infected cells (data not shown). However, the decreased abundance of late viral transcripts is unlikely to account for the 2AP-mediated effect on cellular protein synthesis for several reasons. First, the reduction in late viral mRNA was not reflected by a similar drop in late polypeptide synthesis. Thus, there was no selective discrimination against late viral mRNAs. Second, early during the onset of the late cycle (13–15 hr after infection), cytoplasmic accumulation of viral late mRNAs was reduced to similar levels, although host protein synthesis was already suppressed (e.g., refs. 2, 24, and 25).

Suppression of Cellular Translation Does Not Correlate with Phosphorylation of eIF-2α. Previous studies have provided intriguing but conflicting results concerning a potential role for DAI kinase activity in suppression of cellular protein synthesis. A strain of HeLa cells low in DAI activity was found to be resistant to translation inhibition by wt Ad (3), whereas KB cells, another human cell line low in DAI activity, were not (12). Therefore experiments were performed to investigate the correlation between suppression of host protein synthesis and the activity of DAI kinase and phosphorylation state of eIF-2α.

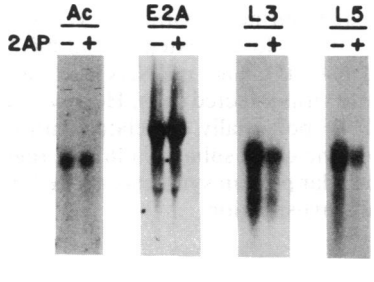

FIG. 3. Effect of 2AP on cytoplasmic accumulation of mRNAs at late times after infection with wt300 virus. Analysis of mRNAs for actin (Ac), Ad early-protein DBP (E2A), and Ad late-proteins hexon (L3) and fiber (L5) are shown. Cytoplasmic poly(A)$^+$ RNA was purified from cells 18 hr after infection, and equal amounts were subjected to Northern analysis. Autoradiograms were quantitated by densitometry. Lanes: −, without 2AP; +, with 2AP.

Table 1. Effect of 2AP on Ad-induced block to transport of cellular RNAs

RNA	% of uninfected control			
	Mock	Mock + 2AP	Ad-inf.	Ad-inf. + 2AP
Cell mRNAs	100	100	28	27
18S rRNA	100	90	5	5

Cells were labeled with [^{3}H]uridine 18 hr after infection, and RNAs were prepared as described. Levels of newly synthesized and transported cytoplasmic 18S rRNA were determined by electrophoresis in formaldehyde/agarose gels followed by fluorography and densitometry of autoradiograms. Cytoplasmic levels of newly synthesized cellular mRNAs were determined by exhaustive hybridization of 10^5 cpm of poly(A)$^+$ RNA to filters containing Ad5 DNA as described (20). All values are expressed as the ratio of test sample to uninfected control. Ad-inf., Ad-infected; Mock, uninfected.

Extracts were prepared from 293 cells 18 hr after infection with wt300 or dl331 Ad, as well as from duplicate plates treated with 2AP. Although both eIF-2α and eIF-2β subunits are substrates for phosphorylation by different kinases, only phosphorylation of eIF-2α is regulated and alters eIF-2 activity (26, 27). As shown previously (3, 5), DAI kinase activity was found to be increased 2- to 3-fold by infection with high multiplicities of wt300 virus and ≈20 fold by infection with mutant dl331, in comparison with uninfected cell extracts (Fig. 4). Curiously, treatment with 2AP was not found to alter the activity of DAI kinase in wt300 virus-infected cell extracts, and only reduced by half the large activation of DAI in cells infected with dl331. The levels of eIF-2α phosphorylation found in infected cells after treatment with 2AP, particularly in those infected with dl331 virus, clearly should result in the global inhibition of protein synthesis and are inconsistent with the ongoing translation of both viral and cellular mRNAs. In reticulocyte lysates, for example, a 3-fold increase in DAI kinase activity generally reduces protein synthesis (28), whereas similar increases were observed in translationally competent wt300 virus-infected cells. Therefore, to further characterize the mechanism for suppression of cellular protein synthesis by Ad, we measured the level of eIF-2α phosphorylation *in vivo*.

Total cell proteins were resolved in isoelectric focusing gels, transferred to nitrocellulose, and immunoblotted with a monoclonal antibody directed against eIF-2α (Fig. 5). The phosphorylated form of eIF-2α is more acidic and migrated slightly ahead of the unmodified polypeptide (22). In uninfected cells, typically only 5–10% of eIF-2α molecules were found to be phosphorylated, in good agreement with other

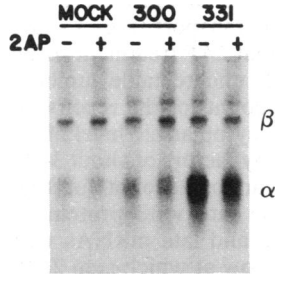

FIG. 4. *In vitro* analysis of eIF-2α phosphorylation in 293 cells infected with wt300 or dl331 Ad without (lanes −) or with (lanes +) 2AP treatment. Extracts were prepared 18 hr after infection, and DAI kinase assays were performed with added eIF-2 and [γ-^{32}P]ATP as described (10). Labeled eIF-2 was immunoprecipitated with a polyclonal mouse antisera, and electrophoresis was carried out as described (10). The α and β subunits were positively identified by silver staining of purified, coelectrophoresed eIF-2 (not shown).

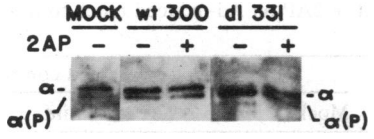

FIG. 5. *In vivo* analysis of eIF-2α phosphorylation in 293 cells infected with wt300 or dl331 Ad without (lanes −) or with (lanes +) 2AP treatment. Whole-cell lysates were prepared, and polypeptides were resolved in isoelectric focusing gels as described (22, 23). Proteins were transferred to nitrocellulose and immunoblotted with a mouse monoclonal antibody to eIF-2α. Coelectrophoresis of purified eIF-2 was used to identify the unphosphorylated (α) and phosphorylated [α(P)] forms of eIF-2α (not shown). The faint second acidic band in the dl331 lanes occurs after excessive phosphorylation at a second site in eIF-2α. Autoradiograms were quantitated by densitometry.

published reports (3, 22, 23). At late times after infection, ≈25% of eIF-2α was phosphorylated in wt300 virus-infected cells and 90% in mutant dl331-infected cells. Treatment with 2AP failed to reduce the level of eIF-2α phosphorylation in wt300 virus-infected cells and only reduced by half the extensive phosphorylation in dl331 virus-infected cells. Thus, these data confirm the results from *in vitro* phosphorylation assays and demonstrate that the activation of DAI kinase and phosphorylation of eIF-2α are not sufficient for the suppression of cellular protein synthesis during Ad infection.

DISCUSSION

We have demonstrated that suppression of cellular protein synthesis by Ad is prevented by treatment with the drug 2AP, but that the role of DAI kinase and phosphorylation of eIF-2α are unclear. Cells normally sensitive to translation shut-off by Ad can be made resistant by treatment with 2AP (Fig. 1); however, the site of action for 2AP in this system must now be determined. Although this drug has been shown to inhibit the activity or activation of DAI kinase *in vitro* (13, 14), it also has been shown to prevent the transcriptional induction of genes normally responsive to α interferon and double-stranded RNA (29–32). Thus, although cells deficient in induction of DAI kinase are resistant to translation shut-off by Ad (3), other genes are also likely to be involved.

Treatment with 2AP had only a modest effect on viral growth (Fig. 2), reducing final virus yields by factors of 2–3. Therefore, we can exclude defects in viral replication as the basis for recovery of host translation mediated by 2AP. Furthermore, 2AP was not noticeably toxic to cells during the exposure period, as observed by others as well (30–33). Finally, treatment with 2AP did not appear to alter the cytoplasmic level of cellular mRNAs or to reverse the normal viral block to their transport (Fig. 3 and Table 1). We think it highly unlikely that 2AP treatment resulted in the renewed transport of a small, undetectable number of cellular mRNAs that then could account for the reactivation of host translation. Although the cytoplasmic level of late Ad mRNAs was generally reduced by factors of about 3–4 during exposure to 2AP, this alone cannot account for the recovery of host translation because late viral protein synthesis was only slightly diminished and late mRNAs were still quite abundant. We do not know at which step 2AP acts to reduce the level of late mRNAs. It is unlikely to affect the viral 55-kDa E1B–34-kDa E4 complex, since we did not observe a failure to block host mRNA transport or the severe reductions in viral replication (100 fold) and late mRNA accumulation associated with defects in this function (34–36).

The demonstration that 2AP acts as an inhibitor of protein kinases *in vitro* (13, 14), rescued protein synthesis in dl331 virus-infected cells, and prevented the suppression of host mRNA translation by wt300 Ad, would suggest that the drug acts by inhibiting activation of DAI kinase. Therefore, it was surprising to find that 2AP had no detectable effect on DAI kinase activity in wt300-infected cells and only reduced by about half the massive phosphorylation of eIF-2α in dl331-infected cells (Figs. 4 and 5). Previous studies have shown that 2AP enhanced translation of plasmid-derived mRNAs (33), which were apparently restricted by activation of DAI kinase (37), although the direct effect of 2AP on eIF-2α phosphorylation was not determined *in vivo*. However, it has recently been shown that in poliovirus-infected cells treatment with 2AP also failed to reduce eIF-2α phosphorylation (23). Consistent with these observations, 2AP was found to act early in the interferon pathway by preventing the induction of interferon but not the subsequent activation of the double-stranded RNA-dependent antiviral enzymes (31).

It is now clear that seemingly inhibitory levels of DAI kinase activity and eIF-2α phosphorylation persist in a number of systems without the global inhibition of protein synthesis, such as in Ad dl331-infected cells treated with 2AP (this report) or coinfected with influenza virus (38), cells infected with high multiplicities of wt Ad (ref. 3; this report), poliovirus-infected cells (23, 39), cell-free translation systems (28), and cells transfected with a variety of plasmids (37). It has been proposed that phosphorylation of eIF-2α may contribute to mRNA specificity in translational control (37). Our observations support this notion and suggest that there may exist a critical "window" or level of eIF-2α phosphorylation required to promote this specificity, which in turn may be regulated in Ad-infected cells by VAI RNA. It may be of critical importance, then, that only partial restoration of translation occurs in dl331-infected cells treated with 2AP, which correlates with a reduction in phosphorylated eIF-2α levels to about 40%. Perhaps this level of eIF-2α phosphorylation is not fully inhibitory in 293 cells. Obviously, the regulation of translation in Ad-infected cells and the role of eIF-2α phosphorylation are not understood. The observation that phosphorylation eIF-2α and its reversing factor GEF could be differentially distributed within cells, possibly bound to 60S ribosomal subunits (40–42), may partially resolve this paradox in late Ad translation.

Finally, these results suggest a potential mechanism by which Ad may suppress cellular protein synthesis. Perhaps Ad infection results in the modification of other translation factors, possibly through a pathway activated by double-stranded RNA or viral gene products. The activity of many initiation factors is regulated by their state of phosphorylation during heat-shock, stress, and other physiologically relevant processes (43). We have previously demonstrated that late Ad mRNAs, which contain a common 5' noncoding region called the tripartite leader, translate without a requirement for initiation factor eIF-4F (16). Nevertheless, the function of the tripartite leader during the viral life cycle is unclear, since its translation effects do not extend to all mRNAs to which it is attached (44, 45), and mRNAs that lack it are still translated in late viral-infected cells. However, given that the activity of eIF-4F potentially correlates with the phosphorylation state of one of its subunits (46), its regulation in the inhibition of cellular protein synthesis at late times during Ad infection needs investigation.

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