A Late Adenovirus Factor Induces eIF-4E Dephosphorylation and Inhibition of Cell Protein Synthesis

YAN ZHANG, DAVID FEIGENBLUM, AND ROBERT J. SCHNEIDER*

Department of Biochemistry and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016

Received 6 June 1994/Accepted 1 August 1994

Adenovirus prevents host cell protein synthesis during its late phase of replication in large part by causing the underphosphorylation of translation initiation factor eIF-4E, a component of initiation factor eIF-4F (cap-binding protein complex). Late adenovirus mRNAs are preferentially translated because they possess a reduced requirement for eIF-4F. This study continues the characterization of the mechanism by which adenovirus inhibits cellular protein synthesis. First it is shown that adenovirus blocks the addition of phosphate to eIF-4E rather than enhancing its removal, establishing that the virus impairs a signalling pathway or protein kinase activity involved in eIF-4E phosphorylation. It is then shown that shutoff of cell protein synthesis and translation of late viral mRNAs are uncoupled, in that shutoff actually occurs a short time (1 to 3 h) after late adenovirus mRNAs are already undergoing translation. Finally, by using a variety of genetic mutants stalled at different stages in the viral life cycle, it was found that dephosphorylation of eIF-4E and inhibition of cell translation are not caused by early adenovirus gene products acting at late times or by events related to viral DNA replication. Instead, it is shown that inhibition of eIF-4E phosphorylation and cell translation are mediated upon activation of the viral major late transcription unit. These and other results presented indicate that the adenovirus signal which induces eIF-4E dephosphorylation and shutoff of cell protein synthesis is linked either to an activity of one or more late viral polypeptides, to double-stranded RNA produced by opposition of the early and late viral transcription units, or to both.

The late phase of adenovirus (Ad) infection is marked by inhibition of host cell protein synthesis and preferential translation of late viral mRNAs. Both suppression of cellular protein synthesis and selective translation of late Ad mRNAs involve virus-mediated inactivation or alteration of translation factor eIF-4F activity (cap-binding protein complex) (31). eIF-4F consists of three components: eIF-4E, a 24-kDa cap-binding protein (24K protein) that specifically binds to mRNA cap structures; eIF-4A, a 50K protein with ATPase and RNA helicase activities (26, 50, 59); and p220, a 220K protein of unknown function (reviewed in reference 24). Upon association of eIF-4F with m⁷GTP caps, eIF-4A and p220 are believed to assemble into the eIF-4F complex, acting as a cap- and ATP-dependent RNA helicase. Presumably eIF-4F unwinds RNA duplexes near the cap, facilitating binding by 43S ribosomal subunits (reviewed in reference 56). The activity of eIF-4F is apparently more complex than first realized, since it displays 5'-to-3' unwinding on capped mRNAs and 3'-to-5' unwinding on uncapped messages (59). Activity of the eIF-4F complex is also thought to confer discriminatory and regulatory activities to the process of translation initiation. For instance, addition of eIF-4F to cell-free translation extracts relieves mRNA competition, indicating that it is a rate-limiting factor (52, 62).

Late Ad mRNAs all contain a common 5' noncoding region called the tripartite leader, which is required for translation of mRNAs at late but not early times after Ad infection (4, 41). The tripartite leader is therefore required for preferential translation of mRNAs during late virus infection. Several studies have explored the property of the tripartite leader responsible for late-specific translation. Translation of late Ad

mRNAs, unlike most others, was found to be resistant to inhibition by superinfecting poliovirus (10, 15), which inactivates eIF-4F and inhibits cell protein synthesis through proteolysis of the p220 protein (reviewed in reference 24). Moreover, the Ad tripartite leader was shown to confer eIF-4F-independent translation to any mRNA to which it is attached and to do so without any requirement for Ad gene products (15). Secondary structure and mutagenesis studies demonstrated that the tripartite leader possesses a relaxed 5' end, which was found to be critical for its ability to promote translation when eIF-4F is limiting (14, 75). In vitro translation studies by Thomas et al. (70) subsequently established that the tripartite leader does not function in the complete absence of eIF-4F activity but probably requires low levels of this factor. These results are supported by studies showing that minimal 5' secondary structure in mRNA correlates with a reduced requirement for eIF-4F during initiation or the ability to efficiently recruit limiting amounts of eIF-4F (21, 38).

Inactivation of eIF-4F by Ad was shown to correlate with dephosphorylation of eIF-4E and inhibition of host cell protein synthesis (31). The mechanism by which eIF-4E regulates translation is not well understood, but many studies indicate that its activity is enhanced by phosphorylation at serine residue 53 (reviewed in references 24 and 56). Reduced phosphorylation of eIF-4E correlates with inhibition of translation during heat shock (17, 36, 48, 73), during mitosis (7), and in Ad- and influenza virus-infected cells (19, 30). Increased phosphorylation of eIF-4E correlates with enhanced translation after activation of cells by mitogens or serum (35, 43, 46) and in cells transformed with the *src* or *ras* oncogene (22). Overexpression of eIF-4E in cells also results in aberrant and enhanced cell growth (66), including transformation (13, 39). Phosphorylation of eIF-4E is required for this effect as well.

It was therefore proposed that in Ad-infected cells, the ability of late viral mRNAs to translate with little if any

* Corresponding author. Phone: (212) 263-6006. Fax: (212) 263-8166.

requirement for eIF-4F, coupled to inactivation of eIF-4F through dephosphorylation of eIF-4E, is largely responsible for inhibition of cell protein synthesis and preferential translation of late viral mRNAs (31; reviewed in reference 65). Since tripartite leader-containing mRNAs probably recruit the remaining small amounts of active eIF-4F efficiently, they maintain high translation rates while cellular protein synthesis is selectively inhibited. Several lines of evidence consistent with this interpretation were established. First, tripartite leader mRNAs were found to translate in metaphase-arrested cells, in which inhibition of cell protein synthesis is linked to dephosphorylation of eIF-4E (7, 31). Second, addition of the kinase inhibitor 2-aminopurine (2AP) was shown to prevent Ad shutoff of cell protein synthesis (30) and the dephosphorylation of eIF-4E (31). Third, Ad cannot induce inhibition of cell translation in certain cell lines, which correlates with an inability to dephosphorylate eIF-4E (31).

The regulation of eIF-4E phosphorylation likely involves modulation of both kinase and phosphatase activities, although the specific proteins have not been definitively identified. Treatment of cells in culture with okadaic acid, an inhibitor of protein phosphatases 1 and 2A, enhances eIF-4E phosphorylation but not when induced by epidermal growth factor (16). Thus, the regulation of eIF-4E phosphorylation by epidermal growth factor in vivo may be mediated by a phosphatase. Rychlik et al. (60) have shown an increased rate of both phosphorylation and dephosphorylation of eIF-4E (i.e., turnover) in B cells stimulated to proliferate with either lipopolysaccharide or phorbol ester, indicating that both regulated kinase and phosphatase activities are involved in eIF-4E control. Enhanced phosphorylation and perhaps an increased rate of phosphate turnover on eIF-4E was also shown in nerve growth factor-stimulated PC12 cells (23), Src-transformed 3T3 cells (22), and Ras-transformed cloned rat embryo fibroblasts (58).

To further understand the molecular mechanisms by which Ad controls the host translational machinery, it was important to determine whether Ad underphosphorylates eIF-4E by enhancing a phosphatase or blocking an unknown eIF-4E kinase and to determine which Ad gene products are involved in these processes. It was previously shown that Ad induces inhibition of host cell protein synthesis only after it enters the late phase of infection (reviewed in reference 44), but this issue has not been systematically studied. In this report, we demonstrate that Ad blocks phosphorylation of eIF-4E by downregulating the addition of phosphate rather than by enhancing its removal. This is consistent with viral inhibition of an eIF-4E kinase or a cell signalling pathway leading to eIF-4E phosphorylation. It is also shown that shutoff of cellular protein synthesis and the efficient translation of late viral mRNAs are uncoupled; shutoff occurs slightly after Ad late mRNAs are already well translated. Through the use of Ad genetic mutants stalled at various points in the viral life cycle, we show that inhibition of cell translation and dephosphorylation of eIF-4E involves the activity of one or more late-acting viral factors that are either not produced or not effective until after a high level of activation of the viral major late promoter (MLP) and synthesis of Ad late mRNAs.

MATERIALS AND METHODS

Viruses and cells. Ad5 *dl309* (H5*dl309*) is a phenotypically wild-type variant of Ad5 that contains a series of altered restriction enzyme cleavage sites (32). Ad5 *ts125* (H5*ts125*), originally isolated by Ensinger and Ginsberg (18), carries a temperature-sensitive mutation in E2A-72K DNA-binding

protein (DBP). Mutant Ad5 *dl338* contains a deletion in the carboxy terminus of the E1B-55K protein which eliminates its activity (51). Mutant Ad5 *dl331* contains a deletion in the intragenic control region of the VAI gene and therefore does not produce VAI RNA (69). Ad5 *ts1* carries a temperature-sensitive point mutation in its L4-100K protein (28). Ad TATA₀ contains several MLP mutations that severely impair late transcription (54). 293 cells are a human embryonic kidney cell line transformed with the E1 region of Ad5 (25). HeLa and 293 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Titers of all virus stocks were determined on 293 cells before use.

Analysis of polypeptides. Cells were labeled with [³⁵S]methionine for 1 h, using 50 μ Ci of *trans*-³⁵S-methionine (ICN) per ml in DMEM without methionine. Equal numbers of cells were used for preparation of extracts. Cells were lysed in 50 mM KCl–10 mM Tris HCl (pH 7.4)–1 mM EDTA at 4°C by sonication and cleared of debris by centrifugation at 10,000 $\times$ g, and equal amounts of soluble S10 protein were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and fluorography. Quantitation of autoradiograms was performed by densitometry.

Analysis of viral mRNAs and genomic DNAs. Cells were infected with viruses and harvested at indicated times. A cytoplasmic RNA fraction was prepared, and poly(A)⁺ RNA was isolated by oligo(dT)–agarose bead selection, using a Qiagen kit according to the manufacturer's instructions. Equal amounts of poly(A)⁺ mRNA were subjected to Northern (RNA) analysis in formaldehyde–agarose gels, transferred to a Nytran membrane, and hybridized to ³²P-labeled probes prepared as described previously (20). Probes were described previously (15). Viral genomic DNAs were prepared by Hirt extraction of whole cell lysates as described previously (61). Equal *A*₂₆₀ amounts of nucleic acid were digested with *Hind*III and resolved by electrophoresis in 1% agarose gels.

Characterization of eIF-4E. For in vivo labeling of eIF-4E with ³²PO₄, cells were washed in phosphate-free DMEM and incubated for 2 h at 37°C in 1 ml of phosphate-free DMEM containing 250 μ Ci of carrier-free ³²PO₄ (New England Nuclear) per ml. Preparation of ³²P-labeled cell extracts for affinity chromatography on m⁷GTP–Sephacrose was carried out as described previously (31). Briefly, equal numbers of cells were resuspended in solubilization buffer (100 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 0.2 mM EDTA, 10% [wt/vol] glycerol, 1 mM dithiothreitol, 0.5% Triton X-100), nuclei and mitochondria were removed by centrifugation at 10,000 $\times$ g at 4°C, and the S10 supernatant was collected. Equal amounts of protein supernatant was then incubated at 4°C for 1 h with a 300- μ l slurry of m⁷GTP–Sephacrose in microcentrifuge tubes. Sepharose was washed with solubilization buffer followed by 1 ml of 1.0 mM GTP and then eluted with 75 μ M m⁷GTP. Eluted protein was recovered by precipitation with 6 volumes of acetone at –20°C and prepared for SDS–polyacrylamide gel electrophoresis.

Kinetics of eIF-4E phosphorylation. To measure the rate of addition of ³²P on eIF-4E, 6-cm-diameter plates of cells were pulse-labeled with 250 μ Ci of ³²PO₄ per ml in 1 ml of phosphate-free DMEM for increasing times as indicated in the text. For determining the half-life of ³²P removal from eIF-4E, cells were labeled with 80 μ Ci of ³²PO₄ per ml in phosphate-free DMEM for 3 h and then subjected to a chase with standard DMEM medium without ³²PO₄ for the indicated times. Cell extracts were prepared and the level of eIF-4E phosphorylation was analyzed as described above.

RESULTS

Ad blocks phosphorylation rather than enhances dephosphorylation of eIF-4E. To determine whether the reduced phosphorylation of eIF-4E results from an Ad-induced phosphatase activity, the relative half-life of phosphate on eIF-4E was examined. If the turnover time of ^{32}P is unchanged on eIF-4E in uninfected and late Ad-infected cells, this would provide compelling evidence that Ad does not enhance the activity of an eIF-4E phosphatase. Cells were labeled with $^{32}\text{PO}_4$ for several hours until the pool of eIF-4E protein was saturated and were then chased for increasing periods of time in medium containing no isotope. The levels of labeled eIF-4E protein were then examined by performing cap affinity chromatography, SDS-polyacrylamide gel electrophoresis, and autoradiography, using equal amounts of soluble cytoplasmic protein (Fig. 1). It was shown previously that the phosphorylation state of eIF-4E does not influence cap-binding affinity (7, 17, 31). The intensity of protein bands corresponding to ^{32}P -labeled eIF-4E decreased as the chase time increased, as expected. Autoradiograms were quantitated by densitometry, and the results were plotted as a function of time (Fig. 1A). Similar half-lives for ^{32}P on eIF-4E were calculated in both uninfected and late Ad-infected cells (32 to 36 min), assuming that turnover proceeds as a pseudo-first-order reaction controlled by a biological decay constant. By plotting these data on semilog plots, two almost parallel exponential decay curves, corresponding to uninfected and infected samples, were also obtained (Fig. 1C). These results demonstrate that loss of ^{32}P from eIF-4E occurs at similar rates in uninfected and infected cells. Calculation of half-lives for phosphate turnover on eIF-4E from respective slopes yields similar rates in uninfected cells (half-life of 32 ± 5 min) and Ad-infected cells (half-life of 36 ± 5 min). Thus, Ad does not cause underphosphorylation of eIF-4E by enhancing an eIF-4E phosphatase.

To determine whether Ad blocks phosphorylation of eIF-4E, cells were pulse-labeled with $^{32}\text{PO}_4$ for increasing periods of time. The rate of phosphate addition to eIF-4E was determined in both uninfected and late Ad-infected cells after purification of eIF-4E from equal amounts of protein lysate by cap affinity chromatography and SDS-polyacrylamide gel electrophoresis as described above. The levels of ^{32}P incorporated into eIF-4E increased incrementally with time (Fig. 2B). Quantitation of autoradiograms by densitometry demonstrated that the rate of addition of ^{32}P to eIF-4E was on average 13 to 14 times slower in late Ad-infected cells than in uninfected cells, as revealed by the apparent difference in the slopes for each curve. In addition, the final accumulation of phosphorylated eIF-4E was ~ 14 -fold lower in late Ad-infected cells. Rates were calculated from the later time point data during linear labeling of eIF-4E because early in labeling, nonlinear uptake, probably resulting from initial limitations in the pool of $^{32}\text{PO}_4$, is apparent. Ad therefore blocks one or more steps leading to phosphorylation of eIF-4E, which is catalyzed by an unidentified kinase. Inhibition of eIF-4E phosphorylation therefore fully accounts for the underphosphorylation of eIF-4E during late Ad infection. In addition, these data and others (31) indicate that metabolic labeling of eIF-4E with $^{32}\text{PO}_4$ accurately measures the state of its phosphorylation in these experiments.

It would seem that the ability to measure the block in phosphorylation of eIF-4E might serve as a good biochemical assay by which to identify the eIF-4E kinase and its Ad regulators by using cell fractionation techniques. However, all attempts failed to produce an *in vitro* extract from late Ad-infected cells that could quantitatively mimic the block in

FIG. 1. Kinetic analysis of eIF-4E dephosphorylation in uninfected and late Ad-infected 293 cells. At late times after infection (20 h), cells were labeled with $^{32}\text{PO}_4$ until the accumulation in eIF-4E plateaued (3 h), and then label was chased by incubating cells with cold medium for 0, 30, 60, and 120 min in medium without isotope. eIF-4E was purified from equal amounts of protein extract by m⁷GTP-Sepharose affinity chromatography, resolved by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. (A) Plot of labeled eIF-4E phosphorylation quantitated at each time point by densitometry of autoradiograms. The effective half-life ($t_{1/2}$) of ^{32}P -labeled eIF-4E was calculated as shown. (B) SDS-polyacrylamide gel electrophoresis analysis and autoradiogram of pulse-chase-labeled eIF-4E. (C) Semi-log plot of the log of relative intensity of ^{32}P -labeled eIF-4E versus time after chase.

eIF-4E kinase activity observed *in vivo*. Instead of a biochemical approach, we therefore used a genetic analysis to characterize the Ad gene products involved in late translation shutoff and dephosphorylation of eIF-4E. Different Ad mutants with genetic defects that stall the virus at well-described stages of its life cycle were exploited to track the viral functions involved in shutoff of host protein synthesis.

Shutoff of host protein synthesis occurs after efficient translation of Ad late mRNAs is underway. We first examined

FIG. 2. Kinetic rate analysis of eIF-4E phosphorylation in 293 cells at late times after Ad infection. At late times (20 h) after Ad infection, 293 cells were labeled with 250 μCi of $^{32}\text{PO}_4$ per ml for 15, 30, 60, and 120 min. Extracts obtained from equal numbers of cells were subjected to m⁷GTP-Sepharose affinity chromatography, and eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. (A) Plot of ^{32}P -labeled eIF-4E quantitated at each time point by densitometry of autoradiograms. The slope of the linear range of the curve was used to calculate the relative rate for accumulation of ^{32}P in eIF-4E. (B) Electrophoretic analysis and autoradiogram of ^{32}P -labeled eIF-4E that was quantitated by densitometry.

precisely when host protein shutoff occurs during the viral life cycle to better map the point at which viral intervention of cell translation takes place. Several previous studies showed that under certain conditions, Ad-infected cells could simultaneously support late viral and cellular protein synthesis and that translation of late viral mRNAs does not require suppression of cell translation (30, 36, 48, 76), suggesting that cell and Ad late translation may overlap. Polypeptide synthesis was therefore monitored at 2-h intervals during the viral life cycle by pulse-labeling infected cells for a short duration with [^{35}S]methionine. Polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. By 10 h after infection, Ad had already entered late phase, evidenced by viral DNA replication and transcription from the MLP (data not shown). Synthesis of late viral proteins was already apparent by 10 h (Fig. 3) and plateaued by 12 to 14 h postinfection. In contrast, shutoff of host translation was not evident until 16 h postinfection and was marked by a gradual decrease in background cell protein bands over a period of several hours. Between 10 and 14 h after infection, both late viral and cellular mRNAs were efficiently cotranslated. These results support the studies described earlier which indicated that suppression of host translation enhances but is not essential for late viral protein synthesis. The kinetics of virus replication and shutoff of host cell protein synthesis vary among different cell lines analyzed, occurring in some cases more rapidly and in other cases more slowly than observed here. Regardless, overlapping translation of late Ad and cell mRNAs was also observed in HeLa, KB, Chang liver, and other 293 cells lines and in all cases was evident for 1 to 3 h

FIG. 3. Kinetics for Ad inhibition of cell protein synthesis and dephosphorylation of eIF-4E. 293 cells were infected with wt Ad and labeled at the indicated times with [^{35}S]methionine (A) or $^{32}\text{PO}_4$ (B). [^{35}S]methionine-labeled protein obtained from equal numbers of cells was resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. Preparation and analysis of ^{32}P -labeled eIF-4E were carried out as described in Materials and Methods.

before shutoff occurred (data not shown). Therefore, inhibition of cell translation actually occurs after viral entrance into the late phase of replication rather than concurrently with it.

Sustained expression of early Ad gene products and viral DNA replication are not sufficient to inhibit cell translation and phosphorylation of eIF-4E. The Ad genome is temporally organized into early and late transcription units that are activated before or with, respectively, the onset of viral DNA replication. The six early transcription units encode a variety of polypeptides involved in a number of functions, including transcriptional activation, viral DNA replication, and regulation of nuclear-to-cytoplasmic transport of cellular and viral mRNAs (3, 27). Early viral gene products modulate the activity of a number of cellular factors, suggesting that they might mediate a cellular pathway leading to inhibition of eIF-4E phosphorylation during the late phase of infection. To examine this possibility, Ad5 mutant *ts125* was first used. Ad5 *ts125* contains a temperature-sensitive lesion in the viral DBP (18) and is unable to replicate its DNA or enter the late phase of infection at the restrictive temperature of 39.5°C. Protein synthesis was monitored by labeling cells with [^{35}S]methionine and then performing SDS-polyacrylamide gel electrophoresis. As expected from previous reports, cells infected with Ad5 *ts125* at 39.5°C failed to enter late phase and did not inhibit host protein synthesis (Fig. 4A). Levels of eIF-4E phosphorylation were examined by in vivo labeling of cells with $^{32}\text{PO}_4$. Factor eIF-4E was purified by cap affinity chromatography and detected by SDS-polyacrylamide gel electrophoresis and autoradiography. As expected, phosphorylation of eIF-4E decreased significantly during the late phase of wild-type (wt) Ad infection (39.5°C), which correlated with the shutoff of host protein synthesis (Fig. 4B). In cells infected with Ad5 *ts125*, however, there was no dephosphorylation of eIF-4E, which was actually somewhat elevated (~2-fold). From this experiment and others using additional Ad mutants (see below), it was concluded that early Ad gene products acting at late times during infection are not sufficient to induce a block in eIF-4E phosphorylation and prevent host protein synthesis.

Ad DNA replication marks viral entrance into the late phase

FIG. 4. Analysis of eIF-4E phosphorylation in 293 cells after infection with Ad5 mutant *ts125*. Uninfected, wt300-infected, and *ts125*-infected cells were labeled at 36 h (32°C) or 18 h (39.5°C) postinfection, and extracts were prepared. (A) SDS-polyacrylamide gel electrophoretic analysis and autoradiogram of polypeptides obtained from equal numbers of cells labeled with [³⁵S]methionine. (B) Electrophoretic analysis and autoradiogram of ³²P-labeled eIF-4E.

of the infectious cycle and induces activation of the MLP transcription unit. To determine whether replication of viral DNA is related in some manner to reduced phosphorylation of eIF-4E, Ad5 mutant *dl338* was used. Ad5 *dl338* fails to synthesize the E1B-55K protein (51) and displays a complicated phenotype. Although it expresses all early genes except E1B-55K and replicates genomic viral DNA to near normal levels, it fails to synthesize late mRNAs to high levels and cannot block the transport of cellular mRNAs at late times after infection. Thus, Ad5 *dl338* possesses a defect later in the viral life cycle than Ad5 *ts125*. Additionally, *dl338* cannot inhibit cellular protein synthesis at late times after infection, a defect also observed with other E1B-55K and E4 mutants that fail to synthesize or transport late Ad mRNAs to normal levels (3, 8, 27, 51, 72). If events related to Ad DNA replication induce dephosphorylation of eIF-4E, such an effect should occur during prolonged infection with *dl338*. HeLa cells were used to study the effect of Ad5 *dl338* on phosphorylation of eIF-4E because 293 cells express complementing regions E1A and E1B. HeLa cell protein synthesis was inhibited by wt Ad at late times in infection (Fig. 5A), but not by Ad5 mutant *dl338* as expected. As observed for Ad5 *ts125*, wt Ad caused dephosphorylation of eIF-4E but Ad5 *dl338* did not, instead slightly elevating its level. Thus infection of both 293 and HeLa cells with two different Ad mutants that are stalled in the early phase of viral infection resulted in slightly enhanced phosphorylation of eIF-4E. Analysis of eIF-4E by two-dimensional isoelectric focusing (2-D IEF) gels and Western immunoblotting showed a single phosphorylated isoelectric form of eIF-4E in Ad5 *dl338*-infected cells (data not shown), suggesting that it was not phosphorylated at multiple sites. Although the increased phosphorylation of eIF-4E by Ad early mutants is not

FIG. 5. Analysis of eIF-4E phosphorylation in HeLa cells at late times after infection with wt Ad and mutant *dl338*. Uninfected, wt300-infected, and *dl338*-infected cells were labeled at late times (24 h) after infection, and extracts were prepared as described in Materials and Methods. (A) SDS-polyacrylamide gel electrophoretic analysis and autoradiogram of polypeptides obtained from equal number of cells labeled with [³⁵S]methionine. (B) Electrophoretic analysis and autoradiogram of ³²P-labeled eIF-4E.

understood, it can be concluded that sustained early gene expression, or the mere replication of Ad genomes, is not sufficient to induce the dephosphorylation of eIF-4E or the shutoff of host translation.

Shutoff of cell translation and dephosphorylation of eIF-4E are induced by a late Ad function. Since sustained expression of early Ad gene products and viral DNA replication are not sufficient to prevent eIF-4E phosphorylation or shutoff cell protein synthesis, it was possible that one or more late-acting Ad factors were involved. Several different effects can be envisioned for the control of host translation by Ad late gene products. For example, double-stranded RNA (dsRNA) is produced from the opposition of the Ad MLP to many early transcription units, which only occurs at significant levels during late infection when extended transcription from the MLP occurs (42). In fact, it was suggested that Ad dsRNA might activate a cell stress or interferon response (42; reviewed in reference 65), although this issue has not been directly addressed. It is possible, then, that dephosphorylation of eIF-4E and inhibition of cell protein synthesis are associated with a dsRNA-induced response triggered by entrance into late infection. Ad VAI RNA, which is synthesized in large amounts only after the replication of the Ad genome is under way, functions to inhibit activation of the dsRNA-activated interferon-induced (DAI) kinase and thereby ensure the synthesis of viral proteins (48, 55, 64, 69; reviewed in reference 44). VAI RNA counters a host antiviral response that otherwise inhibits both viral and cellular protein synthesis as the late phase of replication commences (reviewed in reference 44). One can envision a role for VAI RNA in regulating cell translation in

which it antagonizes the activity of an eIF-4E kinase, perhaps by directly binding and preventing its activation in a manner analogous to its inhibition of DAI kinase activity. Finally, the five families of Ad late mRNAs encode a large number of structural and nonstructural polypeptides. Apart from clear roles as structural proteins, the functions of most of these proteins have not yet been well defined. It is therefore possible that one or more late Ad polypeptides function to inhibit eIF-4E phosphorylation.

Additional Ad mutants were used to distinguish between the different possible mechanisms outlined above for dephosphorylation of eIF-4E. Ad5 *dl331* is unable to express VAI RNA but replicates its DNA and synthesizes normal levels of late viral mRNAs. However, cells infected with Ad5 *dl331* undergo a global shutoff of both cellular and viral protein synthesis due to elevated phosphorylation of translation factor eIF-2 α (55, 63, 69). Thus, *dl331*-infected cells possess late viral mRNAs and possibly dsRNA produced from the action of the major late promoter but do not synthesize either VAI RNA or late viral polypeptides. As expected, protein synthesis was globally inhibited in *dl331*-infected cells, as demonstrated by SDS-polyacrylamide gel electrophoresis of [³⁵S]methionine-labeled polypeptides (Fig. 6A). The level of eIF-4E phosphorylation was probed in *dl331*-infected cells by *in vivo* labeling with ³²PO₄ as described above. Unexpectedly, eIF-4E was not dephosphorylated at late times after infection with Ad5 *dl331* but in fact was phosphorylated ~2-fold more strongly than in uninfected cells (Fig. 6B), which was confirmed by 2-D IEF immunoblot analysis (Fig. 6C). It was extremely unlikely that VAI RNA itself could block eIF-4E phosphorylation, since its expression in transfected cells actually stimulates translation (67). These results therefore suggest that there is a requirement for synthesis of either host or viral polypeptides in dephosphorylation of eIF-4E as the virus enters late phase.

To more precisely address the requirement for late-acting viral factors in shutoff of host protein synthesis, Ad mutant Ad5 *ts1* was used. The Ad5 *ts1* mutation was characterized by Hayes et al., who showed that it occurs in the late Ad 100K protein (28). The 100K protein is a nonstructural polypeptide involved in morphogenesis of viral particles, but it also binds viral and cellular poly(A)⁺ RNAs *in vivo* as strongly as a ribonucleoprotein particle and therefore likely possesses a second unknown function (1). The Ad5 *ts1* mutant was shown to replicate viral genomic DNA normally, to synthesize normal levels of VAI RNA and late viral messages, but to be largely defective in the preferential translation of these late viral mRNAs (28). In our hands, translation of both host and viral mRNAs are significantly reduced in Ad5 *ts1*-infected cells, which is thought to result from a decreased ability of mutant 100K protein to strongly bind mRNAs (57). Protein synthesis in cells infected at late times with Ad5 *ts1* indicated that translation of both viral and cell mRNAs was inhibited >15- to 20-fold at the restrictive temperature but clearly not complete, except in the case of hexon and fiber proteins, syntheses of which were less impaired (Fig. 7A). Examination of eIF-4E phosphorylation by using equal amounts of protein extract from Ad5 *ts1*-infected cells at the restrictive temperature showed that it was only partially dephosphorylated, to levels about threefold lower than that of uninfected cells (Fig. 7B). Similar results were obtained by 2-D IEF-immunoblot analysis (Fig. 7C). Because normal levels of VAI RNA are present in Ad5 *ts1*-infected cells, it can be excluded that VAI RNA itself functions as a viral mediator of eIF-4E kinase inhibition or eIF-4E dephosphorylation. Instead, these results are consistent with the notion that translation shutoff and dephosphorylation

FIG. 6. Phosphorylation status of eIF-4E in 293 cells infected with Ad5 mutant *dl331*. Uninfected, wt Ad-infected, and *dl331*-infected cells were labeled at late times (18 h) after infection as described in Materials and Methods. (A) SDS-polyacrylamide gel electrophoresis and autoradiogram of [³⁵S]methionine-labeled polypeptides. (B) Electrophoresis of ³²P-labeled eIF-4E. (C) 2-D IEF gel and immunoblot analysis of eIF-4E. Focusing gel analysis was carried out as described previously (19, 31). a and b refer to phosphorylated (acidic) and nonphosphorylated (basic) forms, respectively, of eIF-4E. Establishment of eIF-4E species was based on isoelectric point and molecular weight correlations.

of eIF-4E are induced by a high level of activation of the viral MLP and synthesis of late mRNAs and/or proteins.

Translation shutoff and dephosphorylation of eIF-4E are linked to activation of the MLP. The role of viral late mRNA transcription and translation in host cell shutoff was addressed

FIG. 7. Analysis of eIF-4E phosphorylation in 293 cells at late times after infection with wt Ad or mutant Ad5 τ s1. Uninfected, wt300-infected, and Ad5 τ s1-infected cells were labeled at late times after infection (18 h) with [35 S]methionine or 32 PO $_4$ at the restrictive temperature for the τ s1 mutation (39.5°C), and extracts were prepared and analyzed as described in Materials and Methods. (A) SDS-polyacrylamide gel electrophoretic analysis and autoradiogram of [35 S]methionine-labeled polypeptides. (B) Electrophoretic analysis and autoradiogram of 32 P-labeled eIF-4E. (C) 2-D IEF gel and immunoblot analysis of eIF-4E. a and b refer to phosphorylated (acidic) and nonphosphorylated (basic) forms, respectively, of eIF-4E.

by using an Ad mutant containing a defect in the MLP. Reach et al. constructed several mutants that are impaired in MLP activation and synthesis of late Ad mRNAs (53, 54). HeLa cells were infected with the wt parental virus Ad-MLP-wt or MLP-

FIG. 8. Analysis of eIF-4E phosphorylation and host cell translation shutoff in cells infected with Ad mutant TATA₀. (A) Uninfected, wt Ad-infected, and mutant TATA₀-infected HeLa cells were labeled at late times (50 h) after infection with [³⁵S]methionine (A) or ³²PO₄ (B), and proteins were analyzed as described in Materials and Methods. Viral proteins are identified. Ac, actin. HeLa cells were used because the TATA₀ virus displayed only a partial loss of function phenotype in 293 cells.

defective virus Ad TATA₀. These cells do not complement for the loss of function mutation in the MLP. As reported previously (53), the Ad TATA₀ virus was found to replicate its genomic DNA to wt levels and displayed a >20-fold decrease in synthesis of late mRNAs, as shown by Northern blot analysis of selected poly(A)⁺ mRNAs extracted from cells at late times (50 h) after infection (data not shown). Duplicate plates of cells were examined for inhibition of cell protein synthesis by metabolic labeling with [³⁵S]methionine (Fig. 8A) or dephosphorylation of eIF-4E by labeling with ³²PO₄ (Fig. 8B). It was evident that even at very late times after infection, the MLP-defective virus Ad TATA₀ failed to shut off cellular protein synthesis, noticeable by the prominent accumulation of actin and background host polypeptides. Ad TATA₀-infected cells accumulated very low levels of many but not all late Ad polypeptides, averaging a 10- to 20-fold reduction. However, hexon (protein II), 100K protein, and protein pVII accumulated to significant levels, reduced by only two- to threefold in Ad TATA₀-infected cells. The reason for efficient accumulation of these proteins is under investigation. As observed in several previous reports, the failure to inhibit cell translation also correlated with dramatically enhanced levels of Ad early and intermediate polypeptides that are also present at late times (15, 30, 31, 76), which is evident in Fig. 8 for Ad protein pIX and the 72K DBP. The Ad TATA₀ virus also failed to induce severe dephosphorylation of eIF-4E (Fig. 8B). Levels of phosphorylated eIF-4E in Ad TATA₀-infected cells were only slightly lower than in uninfected controls, whereas in wt Ad-infected cells eIF-4E phosphorylation was reduced ~10- to 15-fold. The inhibition of cell protein synthesis and eIF-4E phosphorylation is therefore induced following strong activation of the Ad late transcription unit and/or accumulation of certain late polypeptides.

TABLE 1. Phenotypic and genotypic summary of Ad mutant analysis

Virus ^a	Mutation	Late gene expression				eIF-4E dephosphorylation
		DNA	VAI RNA	mRNA	Protein	
Ad5 <i>ts125</i>	E2A-DBP	—	—	—	—	—
Ad5 <i>dl338</i>	E1B-55K	+	+/-	—	—	—
Ad5 <i>dl331</i>	VAI RNA	+	—	+	—	—
Ad5 <i>ts1</i>	L4-100K	+	+	+	-/+	-/+
Ad TATA ₀	MLP	+	+	- ^b	—	—

^a All viruses exhibited early gene expression.^b MLP activity decreased >20-fold.

DISCUSSION

Reversible serine phosphorylation is a primary means for adjusting translation initiation factor activity to effect changes in translation rates, particularly for eIF-4E and eIF-2 α (reviewed in references 24 and 29). In late Ad-infected cells, shutoff of cellular translation is associated with the dephosphorylation of eIF-4E (reviewed in reference 65) and in some circumstances perhaps enhanced phosphorylation of eIF-2 α (reviewed in reference 45). Ad infection therefore serves as a good model system with which to study translation regulation mediated by changes in eIF-4E phosphorylation.

Previous studies found that the turnover of phosphate on eIF-4E can be regulated by its rate of addition and removal (22, 23, 58, 60, 71). It has been suggested that the rate of phosphate cycling on eIF-4E may regulate translation initiation such that rapid phosphorylation and dephosphorylation may accelerate initiation events without altering the percentage of eIF-4E which is phosphorylated (60), indicating that both eIF-4E kinase and phosphatase activities are subject to regulation. In our study, it was found that Ad underphosphorylates eIF-4E by blocking phosphate addition rather than by enhancing phosphate removal (Fig. 1 and 2). Dephosphorylation of eIF-4E in late Ad-infected cells occurred quickly ($t_{1/2}$ of ~36 min) when phosphorylation was blocked, apparently because it is mediated by a potent phosphatase activity. In this study, we therefore concentrated on characterizing the Ad gene activities that block eIF-4E phosphorylation, since they are also involved in inhibition of cell protein synthesis.

Table 1 summarizes the genetic analysis of Ad mutants presented in this study which were stalled at various points in the viral life cycle. It is clear that sustained expression of Ad early gene products and replication of viral genomic DNA were not sufficient to mediate the viral block in eIF-4E phosphorylation and the shutoff of host protein synthesis. It is also evident that sustained expression of early viral genes in the absence of Ad DNA replication (Ad5 *ts125*) or in its presence (Ad5 *dl338*) did not induce inhibition of cell protein synthesis or eIF-4E dephosphorylation. Moreover, Ad DNA replication in the absence of MLP expression (Ad TATA₀) was not sufficient to induce inhibition of cell translation or dephosphorylation of eIF-4E. These results indicate that host cell translation shutoff is mediated either by the synthesis of late Ad transcripts, perhaps acting as a dsRNA inducer (42), or by one or more protein products translated from late mRNAs, or both. The Ad5 *dl331* virus induces rapid inhibition of both viral and cell protein synthesis as Ad enters into late phase and does not block phosphorylation of eIF-4E. The Ad5 *ts1* virus behaves in a similar but less severe fashion in our hands and accordingly causes a partial dephosphorylation of eIF-4E. We believe that the most reasonable interpretation of these two effects is that a period of ongoing viral and/or cellular protein

synthesis is required for Ad shutoff of cell translation as it proceeds into the late phase of replication. If host cell shutoff is triggered by viral dsRNA produced upon activation of the Ad MLP, these data suggest that it would require new protein synthesis for at least a short period of time to mediate this effect.

Previous studies that investigated the failure of a variety of E1B and E4 mutants to shut off cellular protein synthesis are consistent with the results reported here. These studies also support the contention that cytoplasmic accumulation and/or translation of late Ad mRNAs is required for inhibition of host cell protein synthesis. For instance, Ad5 E1B-55K mutant *dl338* (51) and E4 mutant Ad5 *dl1007* (8) replicate viral DNA to near normal levels, display a 30- to 100-fold impairment in cytoplasmic accumulation of late Ad mRNAs, and do not inhibit cell translation. In contrast, E4 mutant Ad5 *dl1004* is less impaired in late mRNA accumulation (~10-fold) and showed a greater but incomplete ability to shut off cell protein synthesis (8). It was concluded that cytoplasmic accumulation of late Ad mRNAs is related to viral inhibition of cell protein synthesis (8).

We have demonstrated a requirement for late-acting Ad factors (RNA and/or polypeptides) in eIF-4E dephosphorylation and host cell translation shutoff, which now begins the process of identifying the individual factors involved. A number of possibilities by which late-acting viral factors block eIF-4E phosphorylation can be entertained. For example, one or more late viral polypeptides might bind and sequester the eIF-4E kinase, proteolytically degrade it, or possibly act as a competitive inhibitor for the eIF-4E phosphorylation site. Ad synthesizes one known protease (L3-23K) at late times after infection that is involved in maturation of the viral particle (5, 73) and destabilization of cell structural integrity (11, 76) leading to cell lysis (76). However, the possibility that this proteinase proteolytically degrades the eIF-4E kinase can be excluded, since Ad mutant Ad2 *ts1*, which carries a lesion in the proteinase, inhibits host translation at the restrictive temperature as efficiently as wt Ad (76). On the other hand, Ad could activate a cellular protease that degrades the eIF-4E kinase in a manner analogous to poliovirus-induced proteolysis of DAI kinase (6). There is also precedence for cellular and viral polypeptides directly modulating the activities of certain translation factors. Vaccinia virus encodes two polypeptides that inhibit translation factor eIF-2 α phosphorylation; one (pK3r) mimics eIF-2 α and binds DAI kinase (9, 12), whereas the other (E3L) scavenges dsRNA produced during infection, preventing activation of DAI kinase (12). DAI kinase is also regulated by binding to a 58K cellular inhibitor which has been recently cloned (40) and to other cellular inhibitors that are not as well identified (34, 47). Although analogous sequestration of eIF-4F components has not been reported to date, it remains a formal possibility. Finally, Ad might interfere with the signal transduction pathway(s) that maintains phosphorylation of eIF-4E and thereby prevents cellular mRNA translation. Little is known about the signalling events or their control that lead to eIF-4E phosphorylation, other than the fact that they are inducible by growth factors and activated Ras or Src (reviewed in reference 24). In addition, we showed that the ability of Ad to dephosphorylate eIF-4E is blocked by the kinase inhibitor 2AP (30, 31). 2AP is reported to inhibit DAI kinase *in vitro*, and possibly *in vivo* in some cells, and to impair transmission of certain interferon-stimulated gene-signalling responses (reviewed in reference 65). These data suggest that Ad might utilize one or more late proteins to induce shutoff of cell translation by interfering with a signalling cascade that maintains eIF-4E phosphorylation. Alternatively, a late viral

polypeptide or potential production of dsRNA from the Ad genome might activate a cellular pathway sensitive to 2AP that induces eIF-4E dephosphorylation. Both possibilities are currently being explored.

In this study, we also addressed and confirmed a recent report from our laboratory regarding the biological significance of Ad-induced inhibition of cell translation. In previous studies, inhibition of host cell protein synthesis was found to enhance but was not essential for translation of late viral mRNAs (30, 31, 76). We recently showed that Ad-induced shutoff of host translation facilitates lysis of infected cells and release of mature viral particles by preventing repair of the cytokeratin network which is proteolysed by the late Ad L3-23K proteinase (76). Thus, in cultured cells, Ad-induced host translation shutoff enhances cytopathic effects and virus release in addition to boosting late viral mRNA translation during the late phase of infection. These observations are underscored in this report by Fig. 3, in which late Ad translation proceeds at reasonably high levels without host cell translation shutoff. It is not surprising that inhibition of cell protein synthesis increased translation of late Ad mRNAs by only ~3- to 5-fold. Similar results were reported for heat shock mRNAs (33), avian myeloblastosis virus type 4 mRNAs (2), and internal ribosome entry site-containing mRNAs (68), which also translate with a reduced requirement for eIF-4F. These studies all indicate that elimination or reduction of eIF-4F dependence does not vastly increase the translation frequency of an mRNA but instead ensures its translation when the amount of this factor is limiting. With respect to late Ad translation, depletion of eIF-4F impairs cell mRNA translation and simultaneously provides selective translation of late viral mRNAs, not because these transcripts represent strong mRNAs but because they possess a reduced requirement for eIF-4F.

In conclusion, this study has shown that shutoff of host cell translation and the block in eIF-4E phosphorylation are induced by a late Ad function that occurs subsequent to strong expression of the viral late transcription unit. It was also shown that shutoff of cell protein synthesis is not coupled to efficient translation of late Ad mRNAs. These results will now permit the independent investigation of two complicated molecular mechanisms: the preferential translation of late Ad tripartite leader mRNAs and the viral block to eIF-4E phosphorylation. This study has therefore narrowed the identification of viral factors that contribute to inhibition of cell translation and preferential translation of late Ad mRNAs.

ACKNOWLEDGMENTS

We thank Latika Khatri for excellent technical assistance with parts of this work, and we thank Tom Shenk and Hamish Young for certain virus stocks.

This work was supported by Public Health Service grant CA-42357 to R.J.S.

REFERENCES

- Adam, S. A., and G. Dreyfuss. 1987. Adenovirus proteins associated with mRNA and hnRNA in infected HeLa cells. *J. Virol.* **61**:3276-3283.
- Altmann, M., N. Sonenberg, and H. Trachsel. 1989. Translation in *Saccharomyces cerevisiae*: initiation factor 4E-dependent cell-free system. *Mol. Cell. Biol.* **9**:4467-4471.
- Babiss, L. E., and H. S. Ginsberg. 1984. Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. *J. Virol.* **50**:202-212.
- Berkner, K. E., and P. A. Sharp. 1985. Effect of tripartite leader on synthesis of a non-viral protein in an adenovirus 5' recombinant. *Nucleic Acids Res.* **13**:841-857.
- Bhatti, A. R., and J. Weber. 1979. Protease of adenovirus type 2: partial characterization. *Virology* **96**:478-485.
- Black, T. L., G. N. Barber, and M. G. Katze. 1993. Degradation of the interferon-induced 68,000- M_r protein kinase by poliovirus requires RNA. *J. Virol.* **67**:791-800.
- Bonneau, A. M., and N. Sonenberg. 1987. Involvement of the 24kd cap-binding protein in regulation of protein synthesis in mitosis. *J. Biol. Chem.* **262**:11134-11139.
- Bridge, E., and G. Ketner. 1989. Redundant control of adenovirus late gene expression by early region 4. *J. Virol.* **63**:631-638.
- Carroll, K., O. Elroy-Stein, B. Moss, and R. Jagus. 1993. Recombinant vaccinia virus K3L gene product prevents activation of double-stranded RNA-dependent, initiation factor 2 alpha-specific protein kinase. *J. Biol. Chem.* **268**:12837-12842.
- Castrillo, J. L., and L. Carrasco. 1987. Adenovirus late protein synthesis is resistant to the inhibition of translation induced by poliovirus. *J. Biol. Chem.* **262**:7328-7334.
- Chen, P. H., D. A. Ornelles, and T. Shenk. 1993. The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. *J. Virol.* **67**:3507-3514.
- Davies, M. V., H. W. Chang, B. L. Jacobs, and R. J. Kaufman. 1993. The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double-stranded RNA-dependent protein kinase by different mechanisms. *J. Virol.* **67**:1688-1692.
- DeBenedetti, A., and R. E. Rhoads. 1990. Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. *Proc. Natl. Acad. Sci. USA* **87**:8212-8216.
- Dolph, P. J., J. Huang, and R. J. Schneider. 1990. Translation by the adenovirus tripartite leader: elements which determine independence from cap-binding protein complex. *J. Virol.* **64**:2669-2677.
- Dolph, P. J., V. Racaniello, A. Villamarin, F. Palladino, and R. J. Schneider. 1988. The adenovirus tripartite leader eliminates the requirement for cap-binding protein during translation initiation. *J. Virol.* **62**:2059-2066.
- Donaldson, R. W., C. H. Hagedorn, and S. Cohen. 1991. Epidermal growth factor or okadaic acid stimulates phosphorylation of eukaryotic initiation factor 4F. *J. Biol. Chem.* **266**:3162-3166.
- Duncan, R., S. C. Milburn, and J. W. B. Hershey. 1987. Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. *J. Biol. Chem.* **262**:380-388.
- Ensinger, M., and H. Ginsberg. 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. *J. Virol.* **10**:328-339.
- Feigenblum, D., and R. J. Schneider. 1993. Modification of eukaryotic initiation factor 4F during infection by influenza virus. *J. Virol.* **67**:3027-3035.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
- Fletcher, L., S. D. Corbin, K. G. Browning, and J. M. Ravel. 1990. The absence of a m7G cap on beta-globin mRNA and alfalfa mosaic virus 4 increases the amounts of initiation factor 4F required for translation. *J. Biol. Chem.* **265**:19582-19587.
- Frederickson, R. M., K. S. Montine, and N. Sonenberg. 1991. Phosphorylation of eucaryotic translation initiation factor 4E is increased in Src-transformed cell lines. *Mol. Cell. Biol.* **11**:2896-2900.
- Frederickson, R. M., W. E. Mushynski, and N. Sonenberg. 1992. Phosphorylation of translation initiation factor eIF-4E is induced in a *ras*-dependent manner during nerve growth factor-mediated PC12 cell differentiation. *Mol. Cell. Biol.* **12**:1239-1247.
- Frederickson, R. M., and N. Sonenberg. 1993. eIF-4E phosphorylation and the regulation of protein synthesis, p. 143-162. In J. Ilan (ed.), *Translational regulation of gene expression*, vol. 2. Plenum Press, New York.
- Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59-72.

26. Grifo, J. A., S. M. Tahara, M. A. Morgan, A. J. Shatkin, and W. C. Merrick. 1983. New initiator activity required for globin mRNA translation. *J. Biol. Chem.* **258**:5804–5810.
27. Halbert, D. N., J. R. Cutt, and T. Shenk. 1984. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J. Virol.* **56**:250–257.
28. Hayes, B. W., G. C. Telling, M. M. Myat, J. F. Williams, and S. J. Flint. 1990. The adenovirus L4 100-kilodalton protein is necessary for efficient translation of viral late mRNA species. *J. Virol.* **64**:2732–2742.
29. Hershey, J. W. B. 1991. Translational control in mammalian cells. *Annu. Rev. Biochem.* **60**:717–755.
30. Huang, J., and R. J. Schneider. 1990. Adenovirus inhibition of cellular protein synthesis is prevented by the drug 2-aminopurine. *Proc. Natl. Acad. Sci. USA* **87**:7115–7119.
31. Huang, J., and R. J. Schneider. 1991. Adenovirus inhibition of cellular protein synthesis involves inactivation of cap binding protein. *Cell* **65**:271–280.
32. Jones, N., and T. Shenk. 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* **17**:683–689.
33. Joshi-Barve, S., A. DeBenedetti, and R. E. Rhoads. 1992. Preferential translation of heat shock mRNAs in HeLa cells deficient in protein synthesis initiation factors eIF-4E and eIF- γ . *J. Biol. Chem.* **267**:21038–21043.
34. Judware, R., and R. Petryshyn. 1992. Mechanism of action of a cellular inhibitor of the dsRNA-dependent protein kinase from 3T3-F442A cells. *J. Biol. Chem.* **267**:21685–21690.
35. Kaspar, R., W. Rychlik, M. W. White, R. E. Rhoads, and D. R. Morris. 1990. Simultaneous cytoplasmic redistribution of ribosomal protein L32 mRNA and phosphorylation of eukaryotic initiation factor 4E after mitogenic stimulation of Swiss 3T3 cells. *J. Biol. Chem.* **265**:3619–3622.
36. Kitajewski, J., R. J. Schneider, B. Safer, and T. Shenk. 1986. An adenovirus mutant unable to express VA1 RNA displays different growth responses and sensitivity to interferon in various host cell lines. *Mol. Cell. Biol.* **6**:4493–4498.
37. Lamphear, B. J., and R. Panniers. 1990. Cap binding protein complex that restores protein synthesis in heat shocked Ehrlich cell lysates contains highly phosphorylated eIF-4E. *J. Biol. Chem.* **265**:5333–5336.
38. Lawson, T. G., B. K. Ray, J. T. Dodds, J. A. Grifo, R. D. Abramson, W. C. Merrick, D. F. Betsch, H. L. Weith, and R. E. Thach. 1986. Influence of 5' proximal secondary structure on the translational efficiency of eukaryotic mRNAs and on their interaction with initiation factors. *J. Biol. Chem.* **261**:13979–13989.
39. Lazaris-Karatzas, A., K. S. Montine, and N. Sonenberg. 1990. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature (London)* **345**:544–547.
40. Lee, T. G., N. Tang, S. Thompson, J. Miller, and M. G. Katze. 1994. The 58,000-dalton cellular inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR) is a member of the tetratricopeptide repeat family of proteins. *Mol. Cell. Biol.* **14**:2331–2342.
41. Logan, J., and T. Shenk. 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. *Proc. Natl. Acad. Sci. USA* **81**:3655–3659.
42. Maran, A., and M. B. Mathews. 1988. Characterization of the double-stranded RNA implicated in inhibition of protein synthesis in cells infected with a mutant adenovirus defective for VA RNA I. *Virology* **164**:106–113.
43. Marino, M. W., L. M. Pfeffer, P. T. Guidon, and D. B. Donner. 1989. Tumor necrosis factor induces phosphorylation of a 28kd mRNA cap-binding protein in human cervical carcinoma cells. *Proc. Natl. Acad. Sci. USA* **86**:8417–8421.
44. Mathews, M. B. 1990. Control of translation in adenovirus-infected cells. *Enzyme* **44**:250–264.
45. Mathews, M. B., and T. Shenk. 1991. Adenovirus virus-associated RNA and translational control. *J. Virol.* **65**:5657–5662.
46. Morley, S. J., and J. A. Traugh. 1989. Phorbol esters stimulate phosphorylation of eukaryotic initiation factors 3, 4B and 4F. *J. Biol. Chem.* **264**:2401–2404.
47. Mundschauf, J. L., and D. V. Faller. 1992. Oncogenic ras induces an inhibitor of double-stranded RNA-dependent eukaryotic initiation factor 2 alpha-kinase activation. *J. Biol. Chem.* **267**:23092–23098.
48. O'Malley, R. P., R. F. Duncan, J. W. B. Hershey, and M. B. Mathews. 1989. Modification of protein synthesis initiation factors and the shut-off of host protein synthesis in adenovirus infected cells. *Virology* **168**:112–118.
49. Panniers, R., E. B. Stewart, W. C. Merrick, and E. C. Henshaw. 1985. Mechanism of inhibition of polypeptide chain initiation in heat shocked Ehrlich cells involves reduction of eukaryotic initiation factor 4F activity. *J. Biol. Chem.* **260**:9648–9653.
50. Pause, A., and N. Sonenberg. 1992. Mutational analysis of a DEAD RNA helicase: the translation initiation factor eIF-4A. *EMBO J.* **11**:2643–2654.
51. Pilder, S., J. Logan, and T. Shenk. 1986. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.* **6**:470–476.
52. Ray, B. K., T. G. Brendler, S. Adya, S. D. McQueen, J. K. Miller, J. W. B. Hershey, J. A. Grifo, W. C. Merrick, and R. E. Thach. 1983. Role of mRNA competition in regulating translation: further characterization of mRNA discriminatory initiation factors. *Proc. Natl. Acad. Sci. USA* **80**:663–667.
53. Reach, M., L. E. Babiss, and C. S. H. Young. 1990. The upstream factor-binding site is not essential for activation of transcription from the adenovirus major late promoter. *J. Virol.* **64**:5851–5860.
54. Reach, M., L.-X. Xu, and C. S. H. Young. 1991. Transcription from the adenovirus major late promoter uses redundant activating elements. *EMBO J.* **10**:3439–3446.
55. Reichel, P. A., W. C. Merrick, J. Siekierka, and M. B. Mathews. 1985. Regulation of a protein initiation factor by adenovirus associated RNA I. *Nature (London)* **313**:196–200.
56. Rhoads, R. E. 1993. Regulation of eukaryotic protein synthesis by initiation factors. *J. Biol. Chem.* **268**:3017–3020.
57. Riley, D., and S. J. Flint. 1993. RNA-binding properties of a translational activator, the adenovirus L4 100-kilodalton protein. *J. Virol.* **67**:3586–3595.
58. Rinker-Schaeffer, C. W., V. Austin, S. Zimmer, and R. E. Rhoads. 1992. *ras*-transformation of cloned rat embryo fibroblasts results in increased rates of protein synthesis and phosphorylation of eukaryotic initiation factor 4E. *J. Biol. Chem.* **267**:10659–10664.
59. Rozen, F., I. Edery, K. Meerovitch, T. E. Dever, W. C. Merrick, and N. Sonenberg. 1990. Bidirectional RNA helicase activity of eukaryotic translation initiation factors 4A and 4F. *Mol. Cell. Biol.* **10**:1134–1144.
60. Rychlik, W., J. S. Rush, R. E. Rhoads, and C. J. Waechter. 1990. Increased rate of phosphorylation/dephosphorylation of the translation initiation factor eIF-4E correlates with the induction of protein and glycoprotein biosynthesis in activated B-lymphocytes. *J. Biol. Chem.* **265**:19467–19471.
61. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
62. Sarkar, G., I. Edery, R. Gallo, and N. Sonenberg. 1984. Preferential stimulation of rabbit α globin mRNA translation by cap binding protein complex. *Biochim. Biophys. Acta* **783**:122–127.
63. Schneider, R. J., B. Safer, S. Munemitsu, C. E. Samuel, and T. Shenk. 1985. Adenovirus VA1 RNA prevents phosphorylation of the eukaryotic initiation factor 2 alpha subunit subsequent to infection. *Proc. Natl. Acad. Sci. USA* **82**:4321–4325.
64. Schneider, R. J., C. Weinberger, and T. Shenk. 1984. Adenovirus VA1 RNA facilitates the initiation of translation in virus infected cells. *Cell* **37**:291–298.
65. Schneider, R. J., and Y. Zhang. 1993. Translational regulation in adenovirus infected cells, p. 227–250. *In* J. I. I. (ed.), *Translational regulation of gene expression*, vol. 2. Plenum Press, New York.
66. Smith, M. R., M. Jaramillo, Y.-I. Liu, T. E. Dever, W. C. Merrick, H. F. Kung, and N. Sonenberg. 1990. Translation initiation factors induce DNA synthesis and transform NIH 3T3 cells. *New Biol.* **2**:648–654.
67. Svensson, C., and G. Akusjarvi. 1985. Adenovirus VAI RNA mediates a translational stimulation which is not restricted to the viral mRNAs. *EMBO J.* **4**:957–964.
68. Tahara, S. M., T. A. Dietlin, T. E. Dever, W. C. Merrick, and L. M.

- Worrlow. 1991. Effect of eukaryotic initiation factor 4F on AUG selection in a bicistronic mRNA. *J. Biol. Chem.* **266**:3594–3601.
69. Thimmapaya, B., C. Weinberger, R. J. Schneider, and T. Shenk. 1982. Adenovirus VA1 RNA is required for efficient translation of viral mRNA at late times after infection. *Cell* **31**:543–551.
70. Thomas, A. M., G. C. Scheper, M. Kleijn, M. DeBoer, and H. O. Voorma. 1992. Dependence of the adenovirus tripartite leader on the p220 subunit of eukaryotic initiation factor 4F during in vitro translation. *Eur. J. Biochem.* **207**:471–477.
71. Tuazon, P. T., S. J. Morley, T. E. Dever, W. C. Merrick, R. E. Rhoads, and J. A. Traugh. 1990. Association of initiation factor eIF-4E in a cap binding protein complex (eIF-4F) is critical for and enhances phosphorylation by protein kinase C. *J. Biol. Chem.* **265**:10617–10621.
72. Weinberg, D. H., and G. Ketner. 1983. A cell line that supports the growth of a defective early region 4 deletion mutant adenovirus type 2. *Proc. Natl. Acad. Sci. USA* **80**:5383–5386.
73. Yeh-Kai, L., G. Akusjarvi, P. Alestrom, U. Pettersson, M. Tremblay, and J. Weber. 1983. Genetic identification of an endoproteinase encoded by the adenovirus genome. *J. Mol. Biol.* **167**:217–222.
74. Zapata, J. M., F. G. Maroto, and J. M. Sierra. 1991. Inactivation of mRNA cap-binding protein complex in *Drosophila melanogaster* embryos under heat shock. *J. Biol. Chem.* **266**:16007–16014.
75. Zhang, Y., P. J. Dolph, and R. J. Schneider. 1989. Secondary structure analysis of adenovirus tripartite leader. *J. Biol. Chem.* **264**:10679–10684.
76. Zhang, Y., and R. J. Schneider. 1994. Adenovirus inhibition of cell translation facilitates release of virus particles and enhances degradation of the cytokeratin network. *J. Virol.* **68**:2544–2555.