Complementation of adenovirus virus-associated RNA I gene deletion by expression of a mutant eukaryotic translation initiation factor

(translational control/phosphorylation/eukaryotic initiation factor 2/double-stranded RNA protein kinase)

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ABSTRACT Adenovirus VA RNAs (virus-associated RNAs) are small polymerase III transcripts that are required for efficient initiation of mRNA translation late in adenovirus infection. VAI RNA prevents double-stranded RNA (dsRNA) activation of the interferon-induced protein kinase (DAI kinase). Activation of this kinase results in phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2 α) and correlates with inhibition of translation initiation. In this report we show growth complementation of adenoviruses harboring deletions in the VAI gene in cell lines expressing a serine-to-alanine mutant of eIF-2 α . This serine-to-alanine mutant is resistant to phosphorylation by DAI kinase. These results directly show that the primary function of VAI RNA in the lytic adenovirus infection is the inhibition of eIF-2 α phosphorylation by DAI kinase and identify eIF-2 α as the target that mediates the effects of DAI kinase activation. Cells that express a mutant eIF-2 α will enable the isolation of specific host-range mutants for other types of viruses that are defective in the ability to inhibit DAI kinase.

Polypeptide chain synthesis initiates when the ternary complex of eukaryotic initiation factor 2 (eIF-2), GTP, and initiator Met-tRNA binds the 40S ribosomal subunit to generate a 43S preinitiation complex. Subsequently, mRNA binds, and the 60S ribosomal subunit joins to form the 80S initiation complex with the concomitant hydrolysis of GTP to GDP. For eIF-2 to promote another round of initiation, GDP bound to eIF-2 must be exchanged for GTP, a reaction catalyzed by the guanine nucleotide exchange factor [eIF-2B or GEF (1, 2)]. Inhibition of protein synthesis occurs as a result of phosphorylation of eIF-2 α , which stabilizes the eIF-2-GDP-eIF-2B complex and consequently prevents GTP exchange and eIF-2 recycling (3–5). Induction and activation of double-stranded RNA (dsRNA)-activated protein kinase (DAI kinase), which phosphorylates eIF-2 α , are part of the interferon-induced antiviral response. Many interferon-resistant viruses have evolved mechanisms to prevent DAI kinase activation (6).

As the lytic cycle of adenovirus proceeds from the early to the late phase, cells accumulate large amounts of mRNAs derived from the adenovirus major late promoter as well as large quantities of the adenovirus virus-associated RNAs I and II (VAI and VAII RNAs) transcribed by RNA polymerase III (7). An adenovirus VAI RNA gene deletion mutant (dl331) produces functional viral mRNAs that are not translated *in vivo* (8, 9). Translation extracts prepared from dl331-infected cells are blocked at the level of initiation, and addition of either eIF-2 or eIF-2B can restore translation (41).

In dl331-infected cells, activation of DAI kinase (10, 11) correlates with eIF-2 α phosphorylation (12, 13) and inhibition of protein synthesis. Since VA RNA can bind and inhibit the activation of DAI kinase (14), it has been proposed that VA RNA functions to circumvent the host antiviral response (15, 16).

In a similar manner, translation of plasmid-derived mRNA may be inefficient as a consequence of DAI kinase activation in transfected cells (17, 18). Expression of an eIF-2 α mutant harboring a serine-to-alanine change at the site of DAI kinase-mediated phosphorylation (residue 51) corrects the translation defect in transfected cells (19). Curiously, a serine-to-alanine mutation at residue 48 also corrects the translation defect, although this mutant is phosphorylated by DAI kinase (19). These findings show that the effects of DAI kinase activation can be mediated through eIF-2 α phosphorylation.

The importance of VA RNA in adenovirus infection as an inhibitor of DAI kinase activation and subsequent eIF-2 α phosphorylation was studied by asking whether expression of nonphosphorylatable eIF-2 α mutants can complement the growth of adenovirus VA RNA gene deletion mutants. For this study, serine-to-alanine mutants at either amino acid residue 48 or 51 were expressed in human adenovirus-transformed 293 cells. Expression of these mutant subunits of eIF-2 α can correct the defect in protein synthesis and virus production for adenovirus VA RNA deletion mutants.

MATERIALS AND METHODS

Plasmid Constructions. p48A VA[−] and p51A VA[−], which encode the alanine-48 and alanine-51 mutants of eIF-2 α have been described (19). In these vectors the eIF-2 α cDNA is transcribed from the adenovirus major late promoter and simian virus 40 (SV40) enhancer. The transcript contains most of the adenovirus tripartite leader and a small intron within the 5' untranslated region. The 3' untranslated region of the mRNA contains a dihydrofolate reductase (DHFR) coding region and the SV40 early polyadenylation signal. The DHFR expression plasmid pD61 (19) and pSV2-neo (20) have been described.

Cell Culture. The parental 293 cell line (21) used in this study was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units of penicillin per ml, 100 μ g of streptomycin per ml, 2 mM glutamine, and 10%

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Abbreviations: eIF-2, eukaryotic initiation factor 2; VA RNA, virus-associated RNA; DAI kinase, dsRNA-activated protein kinase; GEF, guanine nucleotide exchange factor (eIF-2B); pfu, plaque-forming units.

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(vol/vol) heat-inactivated fetal calf serum. eIF-2 α -expressing cell lines were obtained by calcium phosphate coprecipitation (22) of the eIF-2 α expression plasmid (20 μ g) mixed with pSV2-neo (1 μ g) and pD61 (2 μ g). Transformants were selected by growth in medium containing 1 mg of G418 per ml. Two clones derived from p48A VA⁻ transfection were F148 and 48S2. One clone, 51P, derived from p51A VA⁻ transfection, was subsequently selected for its increased resistance to methotrexate (resistant to a maximum of 5.0 μ M methotrexate) in order to amplify the transfected DNA.

Adenovirus Infection. Wild-type adenovirus serotype 5 and adenovirus 5 VA RNA gene deletion mutant lines dl704 and dl720 have been described (23, 24). dl704 harbors a deletion of the VAI RNA gene only and has a wild-type phenotype (23). dl720 is deleted in both VAI and VAI RNA genes and is severely defective for growth in 293 cells (24). These viruses were used to infect 293 cells at a multiplicity of infection of 10 plaque-forming units (pfu) per cell. Twenty hours after infection, cells were labeled with [³⁵S]methionine (100 μ Ci/ml; 1000 Ci/mmol, Amersham; 1 Ci = 37 GBq) for 30 min, and cell extracts were prepared by lysis in Nonidet P-40 (NP-40)/sodium dodecyl sulfate (SDS) lysis buffer (0.15 M NaCl/0.05 M Tris-HCl, pH 8.0/0.05% SDS/1% NP-40/0.1 mM phenylmethylsulfonyl fluoride) for analysis by electrophoresis on SDS/20% polyacrylamide gels (24). Growth kinetics were studied by infecting the various cell lines at a multiplicity of infection of 3 pfu per cell, and the virus yield was determined at the indicated times after infection by plaque assay on HeLa cells (23).

Analysis of eIF-2 α Expression. For measure of eIF-2 α synthesis, subconfluent cells were pulse-labeled for 30 min with [³⁵S]methionine (200 μ Ci/ml; 1000 Ci/mmol, 5 mCi/0.33 ml; Amersham) in methionine-free medium. Cell extracts were prepared by lysis in NP-40/SDS lysis buffer and immunoprecipitated by using a rabbit anti-human eIF-2 α antiserum and protein A-Sepharose for immunoadsorption. Immunoprecipitates were analyzed by electrophoresis on SDS/12% polyacrylamide gels (25). For analysis of steady-state levels of eIF-2 α , cell extracts were prepared, and 20 μ g of each lysate was electrophoresed on a SDS/12% polyacrylamide gel and transferred to nitrocellulose. The filters were incubated with rabbit anti-human eIF-2 α and developed with ¹²⁵I-labeled protein A (Amersham). For analysis of eIF-2 α mRNA expression, total cellular RNA was isolated from cells by guanidine thiocyanate extraction, subjected to electrophoresis on an agarose gel, transferred to nitrocellulose, and hybridized to a ³²P-labeled cDNA encoding eIF-2 α prepared by nick-translation (19). The band intensities on autoradiograms were quantitated by using digital imaging technology (Visage 2000; BioImage, Ann Arbor, MI).

DAI Kinase Activity Determination. Wild-type 293 and eIF-2 α -expressing cell lines were mock-treated or infected with either dl704 or dl720 mutant adenovirus. Twenty hours after infection, cell lysates were prepared, and DAI kinase activity was determined by the agarose-poly(I)-poly(C) binding method (15).

RESULTS AND DISCUSSION

Expression of eIF-2 α Mutants in Human 293 Cells. Adenovirus VAI RNA gene deletion mutants grow poorly in 293 cells (26). Therefore, we chose this cell line to study the effect of expression of mutant eIF-2 α on infection by adenovirus mutant lines that do not express VAI RNA. Two mutant eIF-2 α molecules were previously shown to enhance translation by transient expression assays in COS-1 monkey cells (19). A serine-to-alanine mutation at residue 51, the site of phosphorylation (27, 28), renders the eIF-2 α molecule resistant to phosphorylation by activated DAI kinase and thus resistant to inactivation by phosphorylation. A serine-

to-alanine mutation at residue 48 served as a substrate for DAI kinase; however, its phosphorylation did not result in inactivation of eIF-2 α (19). The two eIF-2 α expression plasmids containing serine-to-alanine mutations at amino acid positions 48 or 51 (19) were stably introduced into 293 cells by cotransfection with a G418-resistance expression plasmid and a dihydrofolate reductase expression plasmid as described. Transformants were selected by growth in medium containing G418. Two cell lines expressing the alanine-48 mutant [Ala⁴⁸]eIF-2 α , were called F148 and 48S2. One cell line, 51P, expressing the mutant [Ala⁵¹]eIF-2 α was subsequently selected because of its increased resistance to methotrexate (up to 5.0 μ M methotrexate) in order to amplify the transfected DNA.

Blot-hybridization (Northern) analysis of RNA using an eIF-2 α -specific probe detected the primary endogenous eIF-2 α species migrating at 1.6 kb and a 4-kb eIF-2 α cross-hybridizing species in the original 293 cells (Fig. 1A, lanes 1 and 4). In the eIF-2 α -transfected cells an additional 2.4-kb species was detected (Fig. 1A, arrow), which is the expected size of the eIF-2 α transcript derived from the expression vector. Expression of the mutant [Ala⁴⁸]eIF-2 α in F148 and 48S2, yielded 3- to 4-fold lower (lane 2) and 6-fold greater (lane 3) levels of mutant eIF-2 α mRNA relative to the endogenous eIF-2 α mRNA, respectively. The G418- and methotrexate-resistant transformant, 51P, obtained after transfection with the alanine-51 mutant virus expressed 10-fold greater mutant [Ala⁵¹]eIF-2 α mRNA compared with endogenous eIF-2 α mRNA (lane 5). At these high levels of mutant eIF-2 α expression, there was no alteration in the level of eIF-2 α mRNA derived from the endogenous gene.

eIF-2 α synthesis was monitored by [³⁵S]methionine pulse-labeling, immunoprecipitation with eIF-2 α -specific antiserum, and SDS/polyacrylamide gel electrophoresis of the immunoprecipitates. No increase over endogenous eIF-2 α synthesis was detected in clone F148 (Fig. 1B, compare lanes 6 and 8), consistent with the low level of mRNA in this clone. For clones 48S2 and 51P, eIF-2 α synthesis was increased

FIG. 1. Expression of eIF-2 α mutants in 293 cells. (A) Total cell RNA was isolated from the indicated clones, and equal amounts (10 μ g) were analyzed by electrophoresis on an agarose gel and Northern blot hybridization to a ³²P-labeled cDNA encoding eIF-2 α . Lanes 1 and 2 were overexposed 5-fold to identify the mutant eIF-2 α mRNA. The arrow shows migration of the mutant eIF-2 α mRNA. The asterisks identify the endogenous eIF-2 α mRNA species (lower arrow) and a cross-hybridizing species (upper arrow). The cross-hybridizing species was not detected after more stringent washing of the Northern blot. (B) eIF-2 α synthesis was monitored by pulse-labeling growing cells with [³⁵S]methionine for 30 min. Cell extracts were prepared and analyzed by immunoprecipitation with a rabbit anti-human eIF-2 α antiserum as described. The arrow shows migration of eIF-2 α . (C) The steady-state level of eIF-2 α was monitored by immunoblotting (Western) procedures. Extracts were prepared from the indicated clones for SDS/polyacrylamide gel electrophoresis, transfer to nitrocellulose, and incubation with rabbit anti-human eIF-2 α as described. The arrow identifies eIF-2 α . The band of slower mobility represents an immuno-cross-reacting polypeptide.

approximately 5- and 7-fold, respectively (Fig. 1B, lanes 7 and 9). The steady-state level of eIF-2 α in the transfected cells was monitored by immunoblot analysis. All cell lines, irrespective of their level of eIF-2 α synthesis, exhibited similar levels of eIF-2 α protein by this analysis (Fig. 1C, lanes 10–13, arrow). These observations suggest that much of the overexpressed eIF-2 α is not stable in these cells, possibly because it is not incorporated into the eIF-2 trimer complex. Overexpression of the α subunit of eIF-2 likely does not result in overproduction of the β and γ subunits of the eIF-2 heterotrimer. We have recently demonstrated the exchange of overexpressed wild-type and mutant eIF-2 α into the eIF-2 complex in transiently transfected COS-1 cells (Sang-Yum Choi, J. Schnier, R.J.K., and J.W.B.H., unpublished observations). We expect that increased synthesis of the eIF-2 α mutant results in a greater proportion of eIF-2 trimers that contain the eIF-2 α mutant. To date we have not observed any obvious changes in the phenotype or growth properties of the 293 cells that overexpress either [Ala⁴⁸]eIF-2 α or [Ala⁵¹]eIF-2 α .

Expression of a Mutant eIF-2 α Complements Growth of a VAI RNA Gene Deletion Mutant Adenovirus. The ability for mutant eIF-2 α expression to complement growth of an adenovirus VAI gene deletion mutant virus was studied by infecting 293 cells with a phenotypically wild-type adenovirus VAI RNA gene deletion mutant virus dl704 (23) and a VA⁻ negative mutant virus dl720, which lacks both VAI and VAI genes. Mutant dl720 is severely defective for translation at late times after infection, and infectious virus production is low (24). Polypeptide synthesis was monitored 20 hr after infection by [³⁵S]methionine pulse-labeling and SDS/polyacrylamide gel electrophoresis of prepared cell extracts. Infection of the original 293 cells with either wild-type (data not shown) or the dl704 adenovirus yielded the expected pattern of adenovirus polypeptide synthesis (Fig. 2A, lane 2). Infection with dl720 resulted in very low levels of adenovirus-specific polypeptide synthesis (Fig. 2A, lane 3). Infection of 293 cells that were transfected and selected in G418-containing medium to express the transfected wild-type eIF-2 α cDNA with dl704 and dl720 yielded results similar to

results of infection of parental 293 cells (data not shown). By contrast, similar levels of adenovirus polypeptide synthesis were observed for the dl704 and dl720 adenovirus mutants upon infection of cells that express low or high levels of [Ala⁴⁸]eIF-2 α (Fig. 2A, compare lane 5 with 6 and lane 8 with 9) and in cells that express [Ala⁵¹]eIF-2 α (Fig. 2A, lanes 11 and 12). Thus, expression of phosphorylation-resistant eIF-2 α resulted in late adenovirus protein synthesis in the absence of VA RNA expression.

The ability for the mutant eIF-2 α to complement infectious virus production of the VA RNA-deficient adenovirus was studied by monitoring the kinetics of infectious virus production. Infection of 293 cells with wild-type or dl704 adenovirus yielded infectious virus titers of 5×10^8 pfu/ml 5 days after infection (Fig. 2B). The virus titer obtained from dl720 infection of 293 cells was reduced by a factor of 200. dl720 infection of the F148 cells, which express low levels of mutant [Ala⁴⁸]eIF-2 α , yielded 2.5×10^8 pfu/ml, whereas dl720 infection of 48S2 or 51P cells, which express high levels of [Ala⁴⁸]- and [Ala⁵¹]eIF-2 α mutants, respectively, yielded infectious virus at 6.5×10^8 pfu/ml, similar to levels obtained by infection with wild-type or dl704 adenovirus. These results show that expression of the eIF-2 α mutant can complement the growth of the VA RNA-gene-deleted adenovirus to produce virus at levels obtained by wild-type adenovirus infection. The ability for low levels of mutant eIF-2 α to complement virus production is consistent with the findings that small changes in the phosphorylation state of eIF-2 α can dramatically affect protein synthesis (reviewed in ref. 4).

One possible explanation for the complementation observed may be that the mutant eIF-2 α can act directly as a DAI kinase inhibitor. To assess this possibility, we assayed DAI kinase levels in lysates from adenovirus-infected cells by phosphorylation of exogenously added eIF-2 α . DAI kinase is activated by dl720 infection in the original 293 cell line (Fig. 3, compare lanes 1 and 2 to lane 3) as described (10, 14–16). Infection of the cell lines that express the [Ala⁴⁸]- and [Ala⁵¹]eIF-2 α mutants also results in high levels of DAI kinase activation (Fig. 3, lanes 6, 9, and 12). Thus, DAI kinase is activated in these infected cells. However, expres-

FIG. 2. Expression of mutant eIF-2 α complements the growth of adenovirus VA gene deletion mutant lines. (A) Wild-type, dl704, or dl720 adenoviruses were used to infect (multiplicity of infection = 10 pfu per cell) F148, 48S2, 51P, and control 293 cells. Twenty hr after infection, cells were labeled with [³⁵S]methionine for 30 min, and cell extracts were prepared for analysis by electrophoresis on 20% polyacrylamide gels (24). (B) The growth kinetics of wild-type and mutant viruses was compared in the F148, 48S2, 51P, and original 293 cells. Cells were infected at a multiplicity of 3 pfu per cell, and the virus yield at the indicated times after infection was measured by plaque assay on HeLa cells (23). (Left) Comparison of growth in the original 293 line and 51P cell line. (Right) Comparison of growth in lines expressing high (48S2) and low (F148) levels of mutant [Ala⁴⁸]eIF-2 α .

FIG. 3. DAI kinase activity in wild-type and eIF-2 α mutant-expressing 293 cells subsequent to adenovirus infection. F148, 48S2, 51P, and wild-type 293 cells were mock-treated or infected with either dl704 or dl720 adenovirus mutants. Twenty hours after infection, cell lysates were prepared, and eIF-2 α kinase activity was determined by the agarose-poly(I)-poly(C) binding method (15). α denote the position of phosphorylated α subunit of eIF-2.

sion of the mutant eIF-2 α can complement the defect in translation of adenovirus mRNAs. This shows that the phosphorylation of eIF-2 α by DAI kinase is responsible for the translation defect in dl720-infected cells.

Our results substantiate the importance of eIF-2 α phosphorylation mediated by DAI kinase activation as a means to inhibit host protein synthesis in virally infected cells. A mechanism for control of translation initiation mediated by eIF-2 α phosphorylation has been proposed from *in vitro* studies (3–5, 29). Phosphorylated eIF-2 retains its ability to bind GEF but it cannot promote GTP exchange (1, 30, 31). In the absence of GTP exchange, GEF cannot dissociate and recycle. Since inhibition of polypeptide chain initiation can occur with low levels of eIF-2 α phosphorylation, it was proposed that phosphorylated eIF-2 blocks recycling of eIF-2 by sequestering limiting amounts of GEF. Our results show that low levels of mutant eIF-2 α expression can act in a dominant way to prevent inhibition of translation and appear to contradict the sequestering hypothesis. However, a couple alternatives may account for the discrepancy. First, if the percent incorporation of the mutant eIF-2 α into the eIF-2 complex is high, the amount of wild-type eIF-2 that is available for phosphorylation and subsequent sequestering of GEF may be very low. We are presently quantitating the phosphorylation state of eIF-2 α in these mutant eIF-2 α -expressing cells. Alternatively, the mutant forms of eIF-2 may have a reduced requirement for GEF to catalyze GTP exchange. The data with the mutant forms of eIF-2 α presented here, as well as elsewhere (19), suggest that control of translation by eIF-2 α phosphorylation is more complicated than previously proposed and that new hypotheses may be needed to reconcile all of the experimental data. With the ability to produce and isolate mutant forms of eIF-2, we will be able to approach the mechanism by which the mutant eIF-2 can potentiate translation.

These experiments show that the primary function for adenovirus VAI RNA is prevention of eIF-2 α phosphorylation. Since cells that express the eIF-2 α mutant can complement the growth of adenoviruses lacking the VA genes, they will provide a useful host for the efficient propagation of VA RNA gene-deleted adenoviruses. In addition, since a variety of different viruses, including vaccinia virus (32, 33), influenza virus (13), reovirus (34, 35), poliovirus (36, 37), the herpes viruses (38), and possibly human immunodeficiency

virus (39, 40) have evolved mechanisms to prevent DAI kinase activation, the eIF-2 α mutant cells will provide a convenient host to isolate and propagate viral mutants defective in the ability to prevent eIF-2 α phosphorylation. These cells should be of general use in the development of attenuated virus strains for vaccine production.

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