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Adenovirus VAI RNA prevents phosphorylation of the eukaryotic initiation factor 2 α subunit subsequent to infection

(translation control/interferon/double-stranded RNA)

ROBERT J. SCHNEIDER*, BRIAN SAFER†, SUSAN M. MUNEMITSU‡, CHARLES E. SAMUEL‡, AND THOMAS SHENK*§

*Department of Molecular Biology, Princeton University, Princeton, NJ 08544; †Section on Protein Biosynthesis, Laboratory of Molecular Hematology, National Heart, Lung and Blood Institute, Bethesda, MD 20205; and ‡Section of Biochemistry and Molecular Biology, Department of Biological Sciences, University of California, Santa Barbara, CA 93106

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ABSTRACT **The virus-associated VAI RNA of adenovirus is a small, RNA polymerase III-transcribed species required for efficient translation of mRNAs late after infection. Deletion mutant dl331 fails to produce this RNA and, as a result, grows poorly. Three lines of evidence suggest that VAI RNA facilitates translation by preventing inactivation of the function of eukaryotic initiation factor 2 (eIF-2). First, the mutant's translational defect can be relieved by addition of eIF-2 or eIF-2B (GTP recycling factor). Second, extracts of mutant-infected cells exhibit enhanced protein P_1/eIF-2α subunit kinase activity. Third, dl331 can grow with nearly normal kinetics in cells that do not express the kinase.**

The virus-associated (VA) RNAs of adenovirus are small RNAs (about 160 nucleotides) synthesized in large amounts late after infection (1, 2). These RNAs are encoded by two genes, termed *VAI* and *VAII*, located at about 30 map units on the viral genome (3, 4), and their nucleotide sequence has been delineated (5–7). The *VA* genes are transcribed by RNA polymerase III (8–10). VAI RNA is made in much greater quantities late after infection than is the VAII species.

To probe the functions of these RNAs, we previously constructed two adenovirus type 5 (Ad5) variants, each of which fails to synthesize one of the VA species (11, 12). The mutant that fails to produce the minor VAII RNA grows normally, while the variant that cannot synthesize the major VAI species grows more poorly than its parent. Analysis of the mutant's growth defect indicated that VAI RNA is required for efficient translation late after infection, and the translational defect was localized to an early event in the initiation process.

We now report that VAI RNA facilitates translation by preventing activation of the protein kinase that phosphorylates protein P_1 and the α subunit of eukaryotic initiation factor 2 (eIF-2α) (P_1/eIF-2α kinase). This kinase is induced by interferon, and its activity is dependent on double-stranded RNA (13–17). Phosphorylation of eIF-2α by this kinase results in a dramatic decrease in initiation events, presumably by sequestering the GTP recycling factor (eIF-2B) in an inactive complex (reviewed in ref. 18).

MATERIALS AND METHODS

Viruses and Cells. Wild-type adenovirus type 5 (strain H5wt300) is a plaque-purified derivative of a stock originally obtained from H. Ginsberg. H5dl309 is a phenotypically wild-type virus that carries a series of altered restriction endonuclease cleavage sites (19). H5dl331 carries a 29-base-pair (bp) deletion within the intragenic control region of the *VAI* gene, and, as a result, fails to encode VAI RNA (11). The 293 cell line (a human embryonic kidney cell line transformed with a DNA fragment carrying the left 11% of the adenovirus type 5 genome) has been described (20). GM2767A cells are a human fibroblast cell line in which P_1/eIF-2α kinase activity is not detectably induced by interferon (21). 293 and GM2767A cells were propagated in medium containing 10% calf serum and 20% fetal calf serum, respectively.

Cell-Free Translation. Cell-free translation extracts were prepared from 293 cells 24 hr after infection at a multiplicity of 20 plaque-forming units (pfu) per cell. Extracts were prepared by the procedure of Brown *et al.* (22) except that various GTP levels were used. eIF-2 and eIF-2B were purified from rabbit reticulocyte lysates as described by Konieczny and Safer (23). eIF-2 and eIF-2B preparations were not significantly cross-contaminated as judged by electrophoretic analysis of their subunit compositions. Initiation complexes and viral polypeptides produced in cell-free extracts were analyzed as described (12). Immunoprecipitation of polypeptide IX utilized a monoclonal antibody (24) kindly provided by C. Cepko and P. Sharp.

Polypeptide Phosphorylation. Extracts were prepared from 293 cells at various times after infection at 20 pfu per cell. Procedures for *in vitro* phosphorylation assays have been described in detail (25, 26). Briefly, the reaction mixture (25 μl) contained 20 mM Hepes (pH 7.5), 48 mM KCl, 2 mM dithiothreitol, 4 mM $Mg(OAc)_2$, 100 μM ATP containing 10 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP, and 10 μl of S-10 extract containing about 20 μg of protein. When utilized, reovirus double-stranded RNA and eIF-2 were included, respectively, at 1 and 20 μg/ml. Samples were processed for electrophoresis after incubation at 30°C for 10 min. Immunoprecipitations utilized sheep polyclonal antiserum raised against purified eIF-2 (unpublished data).

RESULTS

Purified eIF-2 and eIF-2B Can Restore Translational Activity to Cell-Free Extracts Derived from dl331-Infected Cells. We have previously demonstrated that cell-free extracts derived from dl331-infected cells maintain the *in vivo* phenotype of the VAI^- virus (12). Using inhibitors that block initiation and cause intermediates to accumulate, we localized the defect

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Abbreviations: VA, virus-associated; eIF, eukaryotic initiation factor; bp, base pair(s); P_1/eIF-2α kinase, protein kinase phosphorylating protein P_1 and the α subunit of eIF-2; pfu, plaque-forming unit(s).

§To whom reprint requests should be addressed.

Table 1. Stimulation of translation by eIF-2 and eIF-2B

Virus	GTP, mM	Incorporated [^{35}S]Met, cpm × 10^{-3} (fold increase)		
		No addition	With eIF-2	With eIF-2B
dl309	0.02	26.3	31.1 (1.2)	47.9 (1.8)
	0.10	28.1	32.7 (1.1)	58.6 (2.0)
	1.00	44.2	44.2 (1.0)	43.9 (1.0)
dl331	0.02	2.7	8.0 (3.0)	15.4 (5.7)
	0.10	2.7	10.8 (4.0)	29.5 (11.0)
	1.00	4.0	9.0 (2.2)	10.5 (2.6)

Protein-synthesizing extracts were prepared at 24 hr after infection with dl309 or dl331 at 20 pfu per cell. Extracts were held on ice while either eIF-2 (120 μg/ml) or eIF-2B (5 μg/ml) preparations were added. The mixtures were incubated at 30°C for 15 min in the presence of [^{35}S]methionine (1100 Ci/mmol; 200 μCi/ml), and then acid-precipitable radioactivity was determined. The levels of initiation factor preparations used in this experiment were previously determined to provide optimal enhancements of mutant extract activity in dose–response experiments. Edeine-resistant cpm (≈30% of total) have been subtracted from the values presented in this table. The "fold-increase" value shown in parentheses is the increase relative to a comparable extract that received no added initiation factor.

responsible for reduced translational activity to an early step in the initiation process.

Inhibition early during initiation has been described in several systems and shown to result from loss of eIF-2 activity (reviewed in ref. 27). This factor functions in a complex with GTP and Met-tRNA$_i$ (ternary complex) to bind the initiator tRNA (tRNA$_i$) to a 40S ribosomal subunit. GTP is hydrolyzed during this process, so eIF-2·GDP must be reconverted to eIF-2·GTP if it is to function catalytically. This is accomplished by a second factor termed eIF-2B or the GTP recycling factor. About 20–30% of the eIF-2α subunits are phosphorylated in extracts that have lost eIF-2 activity. The substantial reduction in initiation presumably results from sequestration of the limited quantities of eIF-2B in complexes containing phosphorylated eIF-2α. Thus, GTP is no longer recycled and eIF-2 cannot function catalytically (reviewed in ref. 18).

The phosphorylation-induced block can be overcome by addition of either eIF-2 or eIF-2B to defective extracts. Accordingly, factors purified from rabbit reticulocyte lysates were tested for their ability to restore activity to extracts prepared from dl331-infected cells. Mutant extracts were about 1/10th as active as those derived from wild-type virus (dl309)-infected cells (Table 1). Both eIF-2 and eIF-2B substantially enhanced translation in mutant extracts that contained low levels of GTP (0.02 or 0.1 mM). Incorporation of [^{35}S]methionine into the acid-precipitable product could be increased as much as 11-fold by addition of eIF-2B, increasing the level of translation in the mutant extract to within 50% of the comparable wild-type extract. The stimulation is not a general or nonspecific effect because little or no increase in activity was observed upon addition of purified factors to

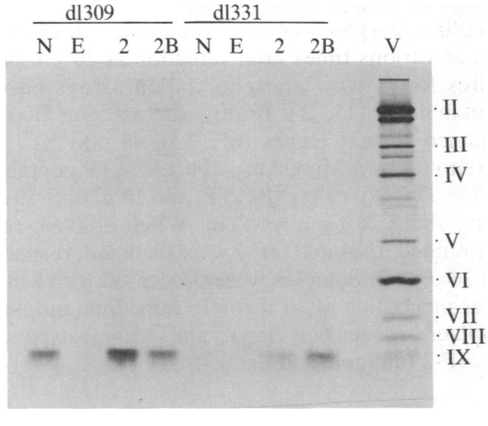

FIG. 1. Electrophoretic analysis of immunoprecipitated polypeptide IX synthesized in cell-free extracts. Protein-synthesizing extracts were prepared at 24 hr after infection with dl309 or dl331 at 20 pfu/cell. Extracts were held on ice while either edeine (10 μM), eIF-2 (120 μg/ml), or eIF-2B (5 μg/ml) preparations were added. The mixtures were then incubated for 15 min at 30°C in the presence of [^{35}S]methionine (1100 Ci/mmol; 200 μCi/ml). Polypeptide IX was immunoprecipitated from the extracts by using a specific monoclonal antibody, and the precipitates were subjected to electrophoresis for 18 hr at 15 mA in a 12.5% polyacrylamide gel containing 1% NaDodSO$_4$. Radioactive polypeptides were visualized by fluorography. Virion polypeptides were included as markers (lane V), and their identities are indicated by Roman numerals. Lanes: N, no addition; E, edeine; 2, eIF-2; 2B, eIF-2B.

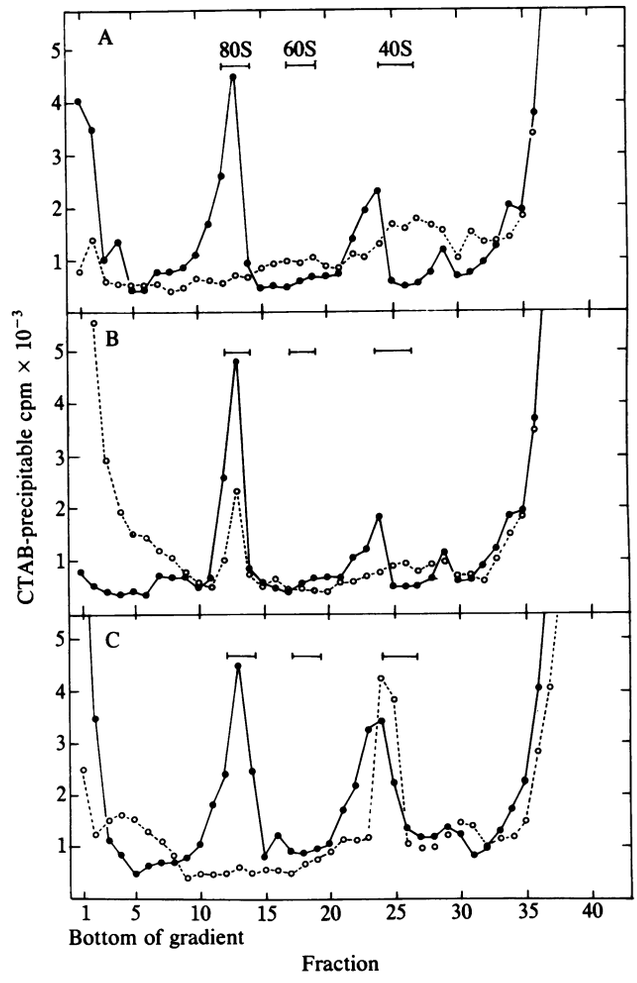

FIG. 2. Anisomycin-induced accumulation of 80S initiation complexes in cell-free extracts. Protein-synthesizing extracts were prepared at 24 hr after infection with dl309 (●) or dl331 (○). Extracts were held on ice while anisomycin (10 μM) with 0.1 mM GTP (*A*) or 1.0 mM GTP (*C*) or 0.1 mM GTP and eIF-2 preparation (120 μg/ml) (*B*) were added. The mixtures were incubated for 5 min at 30°C, [^{35}S]methionine (1100 Ci/mmol; 200 μCi/ml) was added, and incubation was continued for another 10 min; then extracts were analyzed by centrifugation in a 10–30% (wt/vol) sucrose gradient at 36,000 rpm for 4 hr at 4°C in a Beckman SW-41 rotor. Gradients were fractionated, and hexadecyltrimethylammonium bromide (CTAB) was added to aliquots.

wild-type extracts. Mutant extracts were somewhat more active in the presence of relatively high GTP levels (1.0 mM) and were less responsive to added eIF-2 or eIF-2B, consistent with the ability of GTP to partially compensate for sequestered eIF-2B (23, 28). Examination of the synthesis of a specific viral polypeptide by immunoprecipitation confirmed the ability of eIF-2 and eIF-2B preparations to stimulate translation in extracts derived from dl331-infected cells (Fig. 1).

As a final test of the ability of eIF-2 to stimulate translation in mutant extracts, accumulation of 80S initiation complexes was monitored. This experiment utilized anisomycin, which inhibits translocation (29, 30). In the presence of this drug, initiation can proceed through the formation of an 80S ribosome–mRNA complex, but the ribosome cannot move from the initiation codon. Fig. 2 depicts an experiment in which extracts were treated with anisomycin, incubated in the presence of [^{35}S]methionine, and then subjected to centrifugation in sucrose gradients. Without added eIF-2 (Fig. 2*A*), wild-type but not mutant extracts accumulated 80S complexes. Addition of eIF-2 allowed accumulation of 80S complexes in mutant extracts (Fig. 2*B*). As we observed previously (12), high GTP levels (1.0 mM) in the absence of added eIF-2 leads to accumulation of a 43S-like species (*bona fide* 43S preinitiation complex contains ternary complex, the 40S ribosomal subunit, and additional initiation factors) but no 80S particle (Fig. 2*C*). The nature of this 43S species is not clear, but it may be similar to complexes described in hemin-deficient reticulocyte lysates subsequent to phosphorylation of eIF-2α (31–33).

Our experiments indicate that addition of exogenous eIF-2 or eIF-2B can overcome the translational defect in extracts of dl331-infected cells. It is conceivable that a factor other than eIF-2 or eIF-2B that contaminates the initiation factor preparations relieves the translational block. However, this appears extremely unlikely, given the evidence that follows for inactivation of resident eIF-2 in mutant extracts.

The P_1/eIF-2α Kinase Is Activated in dl331-Infected but Not Wild-Type Virus-Infected Cells. As mentioned above, eIF-2-related translational defects have been shown to result from phosphorylation of the eIF-2α subunit. In reticulocyte lysates this phosphorylation can be activated by either hemin deficiency or double-stranded RNA (e.g., ref. 34). In cultured cells, a ribosome-associated, double-stranded RNA-dependent protein kinase (P_1/eIF-2α kinase) mediates phosphorylation of the ribosome-associated protein P_1 and the eIF-2α subunit (13–17, 26, 35, 36).

To test for activation of the kinase, extracts were prepared at various times after infection of 293 cells and assayed for phosphorylation of P_1 and eIF-2α polypeptides. Phosphorylation of P_1 was significantly elevated in extracts prepared from VAI$^-$ mutant dl331-infected cells as compared to uninfected or wild-type virus dl309-infected cell extracts (Fig. 3*A*). Increased P_1 phosphorylation was first evident at

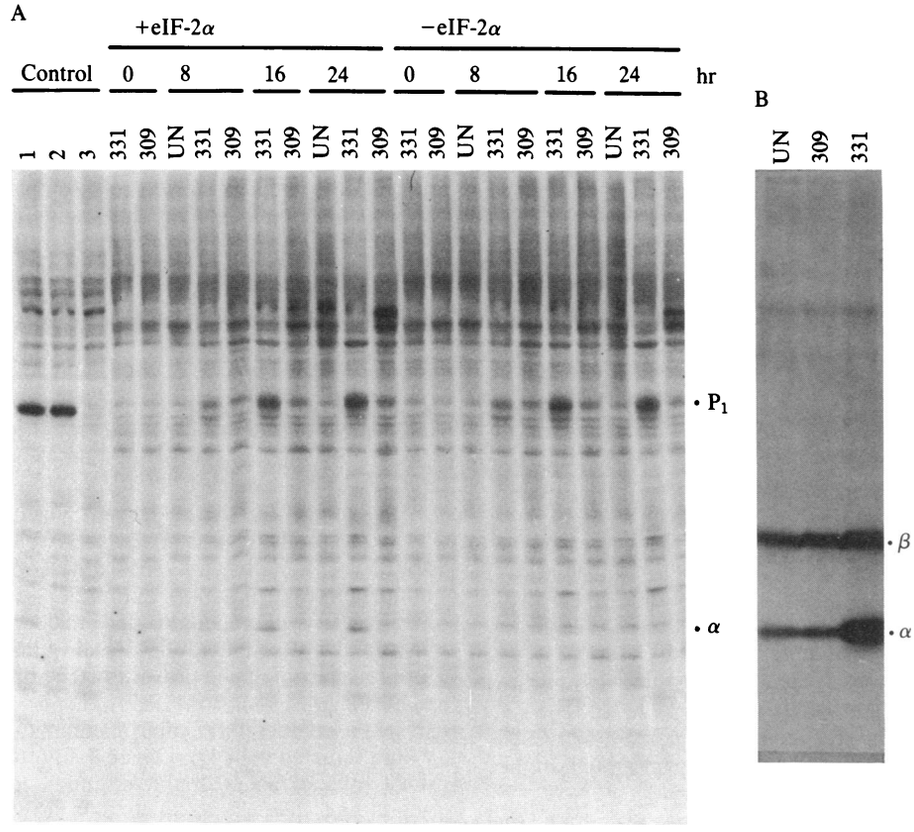

FIG. 3. Phosphorylation of P_1 and eIF-2α polypeptides in cell-free extracts prepared from uninfected cells (lanes UN) or cells infected with either dl309 (lanes 309) or dl331 (lanes 331). (*A*) Electrophoretic analysis of polypeptides phosphorylated by extracts of 293 cells prepared at 0, 8, 16, and 24 hr after infection at 20 pfu per cell. Where indicated, mixtures contained exogenously added eIF-2 (20 μg/ml) to provide additional substrate for the kinase. Extracts were prepared from uninfected human U cells (25) to serve as a positive control. Lanes: Control 1, U cell extract plus double-stranded reovirus RNA plus eIF-2; Control 2, U cell extract plus double-stranded RNA; Control 3, U cell extract. Samples were subjected to electrophoresis for 3 hr at 20 mA in a 10% polyacrylamide gel containing 1% $NaDodSO_4$. Radioactive polypeptides were visualized by autoradiography. The positions of P_1 and eIF-2α are indicated. (*B*) Electrophoretic analysis of immunoprecipitated eIF-2 subunits that have been phosphorylated by 293 cell extracts. Extracts were prepared from either uninfected cells (lane UN) or at 24 hr after infection with dl309 (lane 309) or dl331 (lane 331). All reaction mixtures were supplemented with purified eIF-2. Extracts were subjected to immunoprecipitation with a polyclonal sheep antiserum to eIF-2 subunits and electrophoresis was as described above. Purified eIF-2 was immunoprecipitated and its subunits were visualized by silver stain to provide positive identification of α and β subunits, which are labeled.

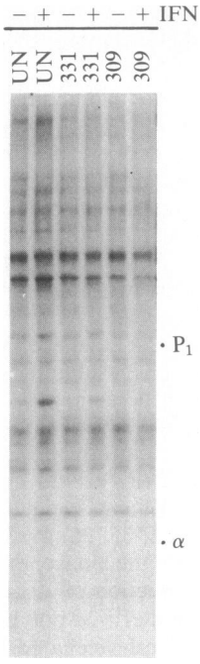

FIG. 4. Electrophoretic analysis of polypeptides phosphorylated by extracts of GM2767A cells. Cells were either not treated (lanes −) or treated (lanes +) with 1000 units/ml of leukocyte interferon (IFN) for 24 hr prior to infection with dl331 (lanes 331) or dl309 (lanes 309) at 10 pfu per cell. Sample analysis was as described in the legend to Fig. 3. The U cell extracts depicted in Fig. 3*A* served as positive controls for this experiment. UN, uninfected.

8 hr after infection. Densitometric scanning of autoradiograms indicated that, at 24 hr after infection, P_1 phosphorylation in mutant extracts was increased about 4-fold compared to uninfected extracts and 3-fold compared to extracts from wild-type virus-infected cells. Phosphorylation of endogenous eIF-2α was difficult to detect in these extracts. It became evident, however, when extracts were supplemented with purified eIF-2 (Fig. 3*A*). To quantitate the level of eIF-2α phosphorylation, extracts were subjected to immunoprecipitations with antiserum specific for the eIF-2 (Fig. 3*B*). The level of eIF-2α phosphorylation in mutant extracts was about 5-fold greater than that observed in extracts of uninfected or wild-type virus-infected cells. Addition of double-stranded RNA to the extracts did not substantially increase kinase activity (data not shown), indicating that there was sufficient endogenous double-stranded RNA (perhaps generated by opposing viral transcription units) in the extracts to fully activate the double-stranded RNA-dependent kinase. The eIF-2β subunit also was phosphorylated in this experiment; α and β subunits are phosphorylated by different kinases, and β-subunit phosphorylation is not known to affect eIF-2 activity (37, 38).

Clearly, extracts of dl331-infected cells contain increased levels of P_1/eIF-2α kinase activity as compared to wild-type virus-infected cell extracts.

Mutant dl331 Grows Nearly As Well As Wild-Type Virus in Cells That Do Not Express P_1/eIF-2α Kinase Activity. One might predict that dl331 could grow with near wild-type kinetics on a cell line such as GM2767A, which does not express detectable P_1/eIF-2α kinase (21). Extracts from both untreated and interferon-treated GM2767A cells infected with dl331 failed to exhibit enhanced P_1/eIF-2α phosphorylation (Fig. 4). Whereas dl331 grew poorly in 293 cells, which express the kinase activity, the mutant virus replicated with near wild-type kinetics to produce an almost normal yield in GM2767A cells (Fig. 5). This result is consistent with the

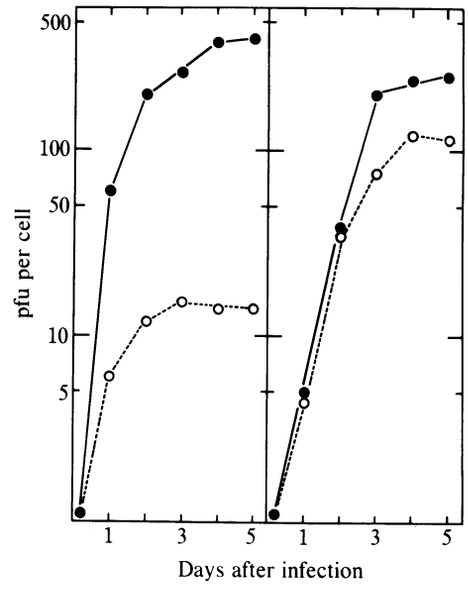

FIG. 5. Growth kinetics of dl309 (●) and dl331 (○) in 293 cells (*Left*) as compared to GM2767A cells (*Right*). Cells were infected at 1 pfu per cell, and virus yield at the indicated times after infection was monitored by plaque assay on 293 cells.

notion that the VAI⁻ virus (dl331) growth defect in 293 cells results from activation of the P_1/eIF-2α kinase.

DISCUSSION

VAI RNA facilitates translation late after Ad infection by preventing inactivation of eIF-2 function. Three lines of evidence support this assertion. First, the translational block in extracts of dl331-infected cells can be relieved by addition of either eIF-2 or eIF-2B (Table 1; Figs. 1 and 2). Added eIF-2 presumably acts in a stoichiometric fashion to stimulate translation; eIF-2B likely reactivates the catalytic recycling of resident eIF-2 that has not yet been phosphorylated. Reichel *et al.* (39) have noted recently a similar effect of eIF-2 and eIF-2B on dl331-infected cell extracts. Second, extracts of mutant-infected cells exhibit enhanced P_1/eIF-2α kinase activity as compared to extracts of wild-type cells (Fig. 3). Third, dl331 grows nearly as well as the wild-type virus in GM2767A cells, which do not express the P_1/eIF-2α kinase activity (Figs. 4 and 5).

How does VAI RNA prevent the inactivation of eIF-2 observed in dl331-infected cells? As yet, the mechanism is not clear. One attractive possibility is that VAI RNA functions directly to inhibit expression of P_1/eIF-2α kinase activity. It is interesting to note that double-stranded RNA paradoxically activates the protein kinase when present at low concentration and prevents activation at high concentrations (34, 40, 41). VAI RNA displays extensive double-stranded character (7), and accumulates in large amounts within infected cells (1). Conceivably, then, VA RNA functions by increasing double-stranded RNA concentrations within infected cells to levels that prevent activation of the kinase. This proposal would argue that, beyond the ability to form a double-stranded structure, primary sequence may not be terribly important to VA RNA function.

Alternatively, VAI RNA might function by interacting with eIF-2 to alter activity of the protein phosphatase (42) that catalyzes dephosphorylation of eIF-2α-*P*. However, the fact that dephosphorylation of eIF-2α-*P* occurs at comparable rates in the presence or absence of exogenously added reovirus double-stranded RNA (43) argues against a direct effect of VAI RNA on the activity of the phosphatase. Finally, it is conceivable that VAI RNA exerts its effect on

eIF-2 indirectly by influencing another parameter of the virus-infected cell. For example, the VAI-minus mutant (dl331) might produce increased levels of double-stranded RNA subsequent to infection, which could, in turn, activate the P_1/eIF-2α kinase. We have no evidence to support this notion, but, as yet, it cannot be entirely ruled out.

The site of VAI RNA action fits well with the observation that translation of all viral and host cell mRNAs is dependent on VAI RNA late after infection (11, 12). Svensson and Akusjarvi (44) and Kaufman (45) have reported that VAI RNA can enhance translation of mRNAs encoded by recombinant plasmids subsequent to transfection of cultured cells. Given our present results, it seems likely that the P_1/eIF-2α kinase is activated in transfected cells and that VAI RNA protects against activation when present.

Interferon treatment of many animal cell types can induce the P_1/eIF-2α kinase, and activity of this kinase is dependent on double-stranded RNA. Adenoviruses likely deal with both interferon and double-stranded RNA during their normal life cycle. Animal hosts could mount interferon responses to infections and opposing transcription units offer the potential to generate double-stranded RNAs. Nevertheless, adenoviruses are not particularly sensitive to the antiviral effects of interferon (46). Quite possibly this relative insensitivity is due, at least in part, to the VA RNAs that prevent activation of the P_1/eIF-2α kinase. These small RNAs may play a central role in the ability of adenoviruses to maintain inapparent infections in animals for months or even years after a primary infection.

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