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Suppression of the Translation Defect Phenotype Specific for a Virus-Associated RNA-Deficient Adenovirus Mutant in Monkey Cells by Simian Virus 40

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Human cells infected with adenovirus type 2 (Ad2) or Ad5 require VAI RNA for efficient translation of viral mRNAs at late times after infection. The Ad5 mutant *dl-sub*720 synthesized neither virus-associated I (VAI) nor VAII RNAs, and infection of human cells with this mutant resulted in reduced virion polypeptide synthesis. Infection of monkey cells with this mutant also resulted in drastic reduction of polypeptide synthesis compared with wild-type (WT) adenovirus infections. Steady-state levels of hexon-specific mRNA were found to be comparable in WT- and mutant-infected monkey cells. The in vitro translation experiments showed that double-mutant- and WT-infected cells contained comparable levels of translatable hexon mRNA (and other adenovirus late mRNAs), suggesting that the severe inhibition of hexon protein synthesis in the VA mutant involves a translation block. Preinfection of monkey cells with simian virus 40 fully restored the efficient translation of this mRNA in the VA mutant infections to the level observed in WT-infected cultures. These results raise the possibility that simian virus 40 may encode or induce factors that suppress the translation block that occurs during adenovirus infections in the absence of the VA RNAs.

Human cells infected with adenovirus synthesize large amounts of two low-molecular-weight RNAs designated virus-associated (VA) I and II RNAs (32, 41). The genes coding for these two RNAs map at about 29.0 map units (m.u.) on the adenovirus physical map and are transcribed by RNA polymerase III (32, 46). The VA RNAs are about 157 to 162 nucleotides long, and they can fold to form compact stem-loop structures (1). Portions of VA RNAs are present in the cytoplasm as ribonucleoprotein particles complexed with a host-coded 45,000-dalton phosphoprotein (La antigen) (14, 24, 25).

The major species, VAI RNA, has been shown to be required for the efficient initiation of translation of viral mRNAs at late times in the productive cycle (38, 43). The mechanism by which VAI RNA enhances translation appears to be the blocking of activity of a kinase which phosphorylates protein synthesis initiation factor eIF-2 (P1/eIF-2α kinase) (21, 28, 33, 37, 39). Because phosphorylated eIF-2 is unable to recycle, inhibition or reversal of eIF-2 phosphorylation is necessary for continued protein synthesis.

The adenovirus type 5 (Ad5) mutant *dl-sub*720 (double mutant) synthesizes neither VAI nor VAII RNAs (5, 7), and human cells infected with this mutant synthesize viral polypeptides at drastically reduced levels at late times after infection (8). We have observed that an African green monkey kidney cell line, CV-1p, infected with mutant *dl-sub*720 also shows the phenotype characteristic of human cells. However, if CV-1p cells are preinfected with wild-type (WT) simian virus 40 (SV40), the phenotype is suppressed. In this paper, we present the results of these experiments and discuss the possible explanations for this intriguing observation.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Cell lines 293 (an Ad5-transformed human embryo cell line), HeLa, CV-1p (African green monkey kidney), and COS-1 (an SV40-transformed monkey kidney cell line) (15) were maintained in Dulbecco modified Eagle minimal essential medium containing 10% calf serum. The WT SV40 is a plaque-purified derivative of strain SVS (42). Mutant *dl*704 is a phenotypically WT variant of Ad5 and lacks functional VAII RNA gene due to a 17-base-pair (bp) deletion within its coding sequences (6). Mutant *dl-sub*720 is VAI$^-$/VAII$^-$ (double mutant). Construction of mutants *dl*704 and *dl-sub*720 was described in earlier reports (5–7). pA5-130 is a plasmid in which DNA sequences between 30.0 m.u. (*Eco*RI site) and 59.5 m.u. (*Bam*HI site) of Ad5 variant *dl*704 (7) were cloned between the *Bam*HI and *Eco*RI sites of pBR322.

Polypeptide analysis. To analyze viral polypeptides, cell lines were infected at a multiplicity of infection (MOI) of 5 PFU/cell, except where noted, and at indicated times were labeled with [^{35}S]methionine (50 μCi/ml, specific activity, >800 Ci/mmol; Amersham Corp.) for 1 h. The cells were washed twice with phosphate-buffered saline (pH 7.4) and lysed in cold RIPA buffer (0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 1.0% sodium deoxycholate, 1.0% Triton X-100, 1 mM EDTA, 20 mM Tris, pH 7.4) at a concentration of 2.0×10^6 cells per ml as described by Cepko and Sharp (9). Cell extracts derived from equal numbers of cells were diluted into SDS gel sample buffer and loaded directly onto 20% SDS-polyacrylamide gels (acrylamide-bisacrylamide, 20.0:0.1) to analyze late polypeptides.

Hexon polypeptide was immunoprecipitated with a mono-

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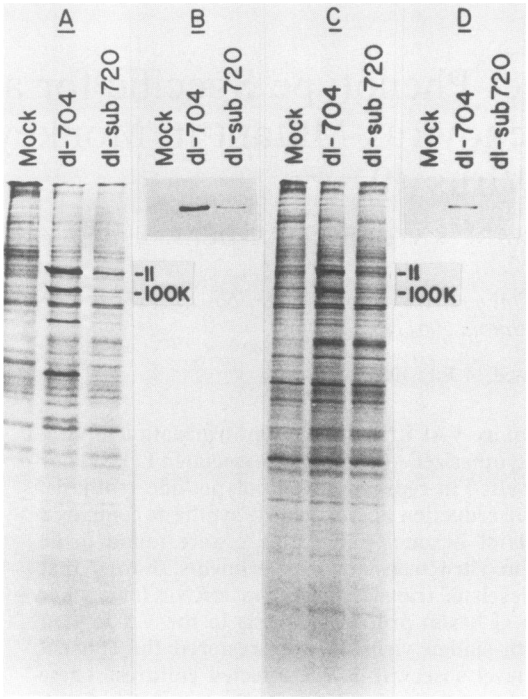

FIG. 1. Electrophoretic analysis of viral proteins synthesized in human 293 cells or CV-1p cells with *dl*704 or the double mutant. Cells were infected with Ad5 variants at 5 PFU/cell for 19 h and labeled with [^{35}S]methionine for 1 h, and the viral polypeptides were analyzed as described in Materials and Methods. Equal quantities of extracts were loaded on a 20% polyacrylamide gel containing 0.1% SDS and electrophoresed for 15 h at 17 mA. (A) Viral polypeptides synthesized in 293 cells. (B) Immunoprecipitation of hexon polypeptide synthesized in 293 cells. To ensure quantitative immunoprecipitation, the supernatant after the first immunoprecipitation was reimmunoprecipitated. Negligible amounts of hexon protein were observed. (C) Viral polypeptides synthesized in CV-1p cells. (D) Immunoprecipitation of hexon polypeptide synthesized in CV-1p cells. The positions of hexons (II) and 100,000-M_r proteins (100K) are shown.

clonal antibody against the Ad2 hexon protein (9) (kind gift of Constance Cepko and Phillip Sharp, Massachusetts Institute of Technology).

The in vitro translation of polyadenylated [poly(A)$^+$] RNA isolated from CV-1p cells infected with adenovirus variants was carried out in cell-free rabbit reticulocyte lysates (29) with a translation kit (catalog no. 001; New England Nuclear Corp.) (see the legend to Fig. 4).

Northern blot analysis of hexon mRNAs. Northern-type analysis of cytoplasmic RNA or the poly(A)$^+$ selected fraction was performed with nitrocellulose filters for RNA transfer. Cells were infected with Ad5 mutants (5 PFU/cell) or SV40 (10 PFU/cell) for the duration indicated in the legend to Fig. 3. Total cytoplasmic RNA or poly(A)$^+$ RNA fractions were subjected to 1.2% agarose–formaldehyde gels (31) and transferred to nitrocellulose filters, and the filters were hybridized with a nick-translated 3.3-kilobase (kb) DNA fragment specific for the hexon mRNA (see the legend to Fig. 3).

RESULTS

Viral polypeptide synthesis in human and monkey cells infected with Ad5 mutants. Mutant *dl*704 is VAI$^+$/VAII$^-$ and shows no growth defects in HeLa or human 293 cells (6, 8). This virus was used as a WT control in all these experiments. Mutant *dl-sub*720 synthesizes neither VAI nor VAII RNA owing to mutations which affect their transcription (7) (see Materials and Methods). Infection of human 293 cells with this mutant resulted in drastic reduction of viral polypeptide synthesis at late times compared with *dl*704 infections (8). Human 293 cells were infected with the Ad5 variants *dl*704 and *dl-sub*720 at 5 PFU/cell for 19 h and then labeled for 1 h with [^{35}S]methionine. The viral polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described under Materials and Methods. Figure 1A shows that the double mutant synthesized viral polypeptides at drastically reduced levels compared with *dl*704, consistent with our earlier report (8). The phenotype exhibited by *dl-sub*720 was similar to that of mutant *dl*331, a VAI$^-$ mutant reported earlier (43), except that *dl-sub*720 was much more defective for translation than *dl*331 (8). To quantitate more accurately the decrease in viral polypeptide synthesis in these experiments, we immunoprecipitated and quantitated the hexon polypeptide with a monoclonal antibody specific for hexon protein (9) (Fig. 1B). Densitometer scanning of the autoradiogram of the gel (Fig. 1B) indicated that *dl-sub*720 synthesized hexon polypeptide at approximately eightfold-lower levels than *dl*704.

To examine whether the double mutant showed a similar phenotype in African green monkey kidney cells, CV-1p cells were infected with Ad5 variants at an MOI of 5, and at 19 h after infection the cells were labeled for 1 h with [^{35}S]methionine. Viral polypeptides were analyzed as described before. Figure 1C shows that the WT virus (*dl*704) synthesized viral polypeptides at considerably reduced levels. This result is in agreement with several earlier reports and typical of abortive infection of monkey cells with WT adenovirus (4, 10, 16, 17, 26). In contrast, viral polypeptide synthesis was nearly undetectable in cells infected with *dl-sub*720. This was further confirmed by analysis of the hexon polypeptide by immunoprecipitation (Fig. 1D).

Suppression of the translation defect phenotype by SV40. SV40 is known to enhance the expression of adenovirus genes in monkey cells due to the helper function (4, 10, 16, 17, 26), which is associated with the large T antigen (11, 13, 22). Therefore, we sought to determine the effect of SV40-encoded products on the translation block developed during adenovirus infection of monkey cells in the absence of VA RNAs. We reasoned that if any of the SV40-encoded gene products are somehow capable of preventing the translation block developed during adenovirus infection, preinfection of monkey cells with SV40 should prevent the development of such a translation block. Accordingly, CV-1p cells were preinfected with SV40 for 24 h and superinfected with adenovirus variants *dl*704 or the double mutant. At 19 h after superinfection, cells were labeled for 1 h with [^{35}S]methionine and viral polypeptides were analyzed by SDS-PAGE as described in Materials and Methods. Figure 2A shows that the double mutant synthesized viral polypeptides at a level comparable to that of our WT control. Immunoprecipitation of hexon polypeptide from these cell lysates showed that the double mutant synthesized hexon protein at a level comparable to that of the WT (Fig. 2B). These results suggest that SV40 is capable of suppressing the adenovirus VA RNA-specific translation defect in monkey cells by an unknown mechanism.

Determination of steady-state levels of hexon-specific mRNA in infected monkey cells. The process of adenovirus infection in monkey cells is rather complex, and the enhancement of

adenovirus gene expression in monkey cells by SV40 occurs at various levels (3, 13, 19, 22). While the VA defect in human cells involves translational efficiency, it was also possible that the transcription or stability of viral mRNAs was altered in the double-mutant infections of monkey cells. We therefore determined the steady-state levels of hexon-specific mRNA in CV-1p cells infected with *dl*704 or the double mutant and also in cells preinfected with WT SV40 before adenovirus infection.

Cytoplasmic RNAs were isolated from cells infected with the double mutant and WT controls and subjected to denaturing agarose-formaldehyde gels both with and without selection for the poly(A)$^+$ RNA fraction. The RNAs were then transferred to nitrocellulose filters as described previously (31). The filters were probed with a nick-translated 3.3-kb DNA fragment specific for the hexon mRNA (50.1 to 59.5 m.u.; *Hin*dIII and *Bam*HI sites, respectively) (Fig. 3). The level of hexon-specific mRNA found in double-mutant-infected cells was comparable to that of WT controls when analyzed as total cytoplasmic RNA or poly(A)$^+$ selected fraction, even though much less hexon polypeptide was produced in the mutant infections (Fig. 1B). Similarly, the level of the hexon-specific mRNA in CV-1p cells infected with Ad5 variants and preinfected with SV40 was found to be comparable (Fig. 3A and B). Note that when the poly(A)$^+$ fraction was analyzed, the RNA was resolved into two species; the slower-migrating band probably corresponds to pVI protein (44). SV40 is known to increase the levels of adenovirus-specific mRNAs in monkey cells due to a helper function provided by SV40 (4, 10, 16, 17, 26). Interestingly, these experiments did not detect an increase in the level of hexon-specific mRNAs in cells preinfected with SV40. However, in agreement with the published results (3, 13, 19, 22), we did see a two- to threefold increase in the levels of hexon mRNA when the cells were coinfected with SV40 and *dl*704 (Fig. 3C).

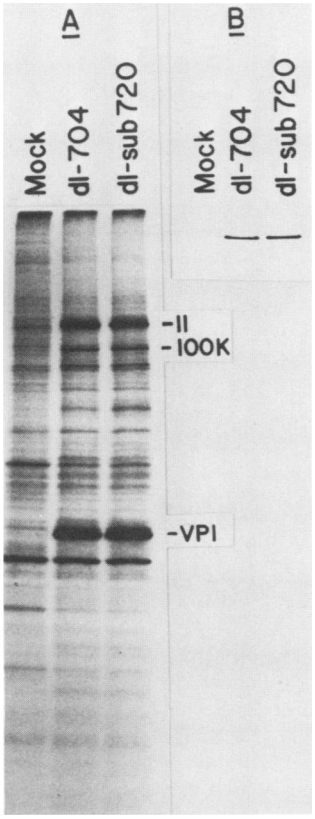

FIG. 2. Analysis of viral proteins synthesized by *dl*704 and the double mutant in CV-1p cells after preinfection with SV40. CV-1p cells were preinfected with SV40 at 10 PFU/cell for 24 h, at which time they were superinfected with *dl*704 or the double mutant for 19 h and labeled for 1 h, and the viral polypeptides were analyzed as described in the text. (A) Total viral proteins synthesized in CV-1p cells. (B) Immunoprecipitation of hexon polypeptide.

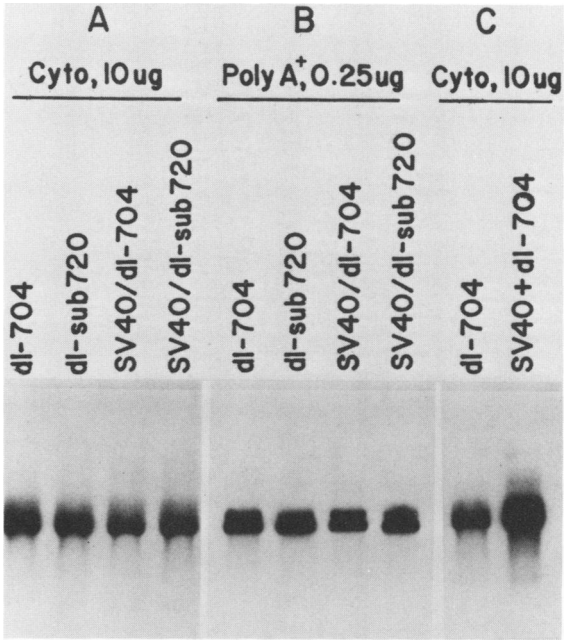

FIG. 3. Northern blot analysis of hexon-specific mRNA synthesized by the Ad5 variants in CV-1p cells. The cells were infected with *dl*704 or the double mutant at 5 PFU/cell for 20 h, and the cytoplasmic (Cyto) and poly(A)$^+$ RNAs were prepared as described earlier (43). In the preinfection experiments, the cells were infected with SV40 for 24 h before adenovirus infection. For coinfection, cells were infected with SV40 and Ad5 variants at the same time at 10 and 5 PFU/cell, respectively, for 20 h. The RNA samples were analyzed on 1.2% agarose–formaldehyde gels and blot-transferred to nitrocellulose filters as described by Rave et al. (31). The blots were hybridized to a nick-translated 3.3-kb DNA fragment specific to the hexon mRNA (50.1 to 59.5 m.u. [*Hin*dIII and *Bam*HI sites, respectively]) of pA5-130. Amounts of RNA [cytoplasmic or poly(A)$^+$] used for analysis are indicated at the top. (A) Levels of hexon mRNA analyzed in total cytoplasmic RNA after Ad5 variant infection of CV-1p cells with or without preinfection with SV40. (B) Levels of hexon mRNA analyzed in poly(A)$^+$ RNA after Ad5 variant infection of CV-1p cells with and without preinfection with SV40. (C) Levels of hexon mRNA in total cytoplasmic RNA with and without coinfection with SV40.

Efficient translation of double-mutant-specific mRNAs isolated from CV-1p cells in cell-free reticulocyte lysates. To confirm that the monkey cells infected with *dl-sub*720 contained normal levels of intact mRNAs, we examined the translatability of these mRNAs in cell-free reticulocyte lysates. CV-1p cells were infected for 20 h with the double mutant or *dl*704 with and without preinfection with SV40. Poly(A)$^+$ RNAs were prepared from these cells and translated in cell-free rabbit reticulocyte lysates as described by Pelham and Jackson (29). The response of *dl*704- and *dl-sub*720-specific mRNAs with and without SV40 preinfection was essentially identical (Fig. 4) and was dependent on the concentration of added RNA (data not shown).

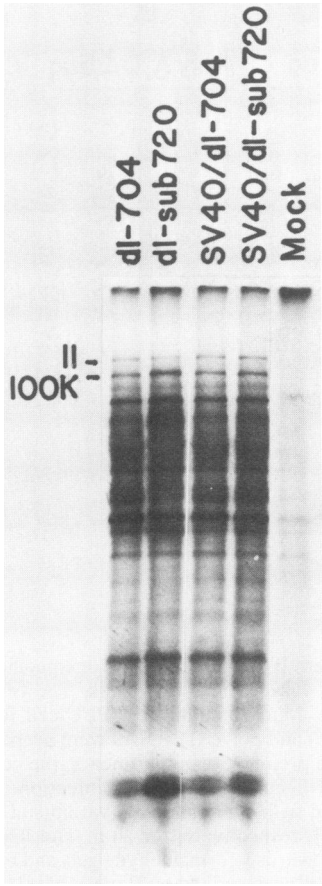

FIG. 4. Electrophoresis analysis of polypeptides synthesized in cell-free rabbit reticulocytes programmed with poly(A)$^+$ RNAs. CV-1p cells were infected with Ad5 variants at 5 PFU/cell and cytoplasmic poly(A)$^+$ RNA was isolated at 20 h after infection. In the preinfection experiments, cells were infected with SV40 at 10 PFU/cell for 24 h before Ad5 infection. A total of 200 ng of poly(A)$^+$ RNA was translated in mRNA-dependent reticulocyte lysates (29) with an in vitro translation kit. Under the conditions described by the supplier (New England Nuclear). The in vitro ^{35}S-labeled polypeptides were analyzed in a 20% SDS–polyacrylamide gel as described in the legend to Fig. 1.

The results of Northern blot hybridization (Fig. 3) and in vitro translation experiment (Fig. 4) together suggest that the monkey cells infected with the double mutant contained translatable mRNAs at levels comparable to those of WT controls. This suggests that the reduced polypeptide synthesis in the double-mutant infections reflected primarily a translation defect.

Absence of double-mutant-specific phenotype in a monkey cell line transformed with SV40. We then asked whether monkey cells synthesizing SV40 large T antigen would allow the translation of adenovirus polypeptides at levels comparable to that in WT-infected cells. If the phenotypic suppression is mediated by SV40 large-T, COS-1 cells, which synthesize SV40 large-T constitutively (15), should be capable of translating adenovirus-specific mRNAs at normal levels without VA RNAs. COS-1 cells were infected with *dl*704 or *dl-sub*720 and at 19 h postinfection were labeled with [^{35}S]methinine for 1 h. The labeled cell lysates were analyzed on polyacrylamide gels as described above. The *dl-sub*720 mRNAs were translated as efficiently as those of the WT controls in COS-1 cells (Fig. 5).

DISCUSSION

Studies of the VA RNA genes in human cells have shown that efficient translation of viral mRNAs at late times requires the VAI RNAs (43). It has been reported that VAI RNA prevents the phosphorylation of eIF-2, which causes the block to translation initiation (21, 28, 33, 37, 39). As in human cells, the VA RNAs appear to affect the efficiency of translation of viral mRNAs in monkey cells. CV-1p cells infected with either WT adenovirus (*dl*704) or the double mutant (*dl-sub*720) contained comparable levels of the hexon mRNA. However, the levels of hexon protein were substantially reduced in the mutant-infected cells compared with WT-infected cells. This suggests that the efficient translation of the hexon mRNA in monkey cells requires the VA RNA function. Surprisingly, preinfection of cells wtih SV40 fully restored the translation of hexon mRNA in the absence of the VA RNAs. Levels of hexon protein observed in SV40-preinfected cultures were the same in both WT- and VA mutant-infected cells. No differences were found in the levels of hexon mRNA in WT- and mutant-infected cultures (Fig. 3), and the mRNAs isolated from mutant-infected cultures were efficiently translated in cell-free reticulocyte

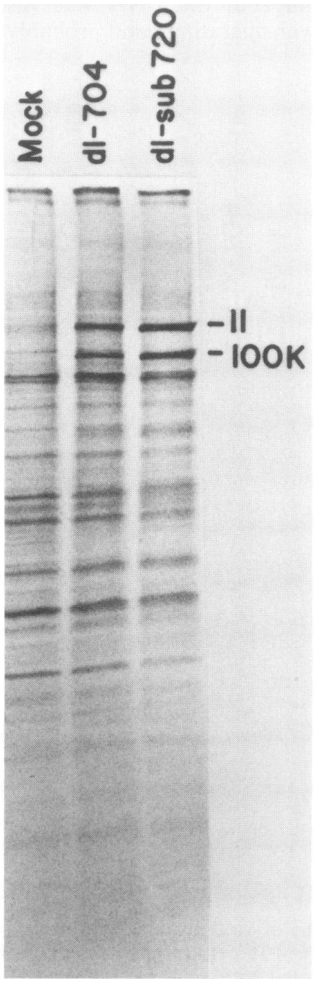

FIG. 5. Analysis of viral proteins synthesized in COS-1 cells. COS-1 cells were infected with AD5 variants at 5 PFU/cell for 19 h, and viral polypeptides were labeled, extracted, and analyzed as described in the legend to Fig. 1.

lysates (Fig. 4). These results clearly indicate that the restoration of viral protein synthesis by SV40 in mutant-infected cultures occurs at the level of translation.

The mechanism by which SV40 overcomes the double-mutant-specific translation defect in monkey cells is not clear. SV40 provides a helper function for productive adenovirus infection in monkey cells, and this helper function is associated with large-T (11, 13, 22). The biochemistry of this helper function is rather complex and believed to be at least partly due to an increased rate of transcription, reduced premature transcription termination (19), and proper mRNA processing (22). No difference was found in the stability of adenoviral mRNAs during abortive and productive infection of monkey cells (19). In the experiments described in this report, WT- and double-mutant-infected cells contained similar amounts of hexon mRNA in both productive and abortive infections. Thus, in abortive infections the VA RNA genes play no apparent function in the production of stable mRNAs. Nonetheless, much less hexon protein was synthesized in the absence of both VA RNA genes.

The observation that SV40 infection of monkey cells 24 h prior to adenovirus infection did not increase the level of hexon mRNA is somewhat surprising. It is conceivable that common cellular factors are used by SV40 and adenovirus for gene expression, and these factors may be present in limiting amounts in monkey cells. These factors are utilized efficiently by SV40 for its gene expression during preinfection and thus are not present at optimum levels for adenovirus gene expression when the cells are superinfected after 24 h with adenovirus.

Recent data suggest that VAI RNA enhances translation by blocking the activation of a kinase (P1/eIF-2α kinase), which phosphorylates the initiation factor eIF-2. The P1/eIF-2α kinase is induced by interferon (21, 34, 36, 48) and activated by double-stranded RNA (18, 27). The function of VA RNAs appears to be to inhibit the activation of interferon-induced P1/eIF-2α kinase during adenovirus infection (21, 28). Therefore, the VA RNAs may serve as a defense against interferon action. The phenotype displayed by the double mutant in monkey cells is probably due to the activation of this kinase. Which of the SV40-encoded products is responsible for the suppression of the double-mutant-specific phenotype in monkey cells is not clear at present. Although SV40 does not have genes coding for polymerase III-directed small RNAs, the virus does express a small RNA (SAS-RNA) which is a cleavage product of viral late mRNAs (2). However, it seems unlikely that the SAS-RNA has an obligate role in efficient translation of SV40 late mRNAs because SV40 mutants which prevent the production of SAS-RNA are fully viable (45). It seems equally unlikely that SAS-RNA is involved in complementation of the adenovirus VA-deficient mutants because complementation can occur in COS cells, in which late transcription equivalent to a productive SV40 infection does not occur.

SV40 large-T is known to induce transcription of cellular RNA polymerase III genes (40), and therefore infection of monkey cells with SV40 may lead to enhanced synthesis of one or more cellular small RNAs which may mimic the VA RNA function. Alternatively, the SV40 large-T antigen is a multifunctional protein and may function directly to prevent phosphorylation of eIF-2 and promote efficient translation or may enhance translation by a mechanism independent of the kinase which is yet to be defined. Several large-T deletion mutants which vary in their biologic properties are available and may be useful in distinguishing between these possibilities. A recently described deletion mutant of large-T (*dl*2459) may be of particular interest in these studies. This mutant has a deletion near the 3′ end of large-T and was shown to be replication competent but essential for the productive infection of CV-1p cells (45). The block in this case appears to be after the onset of viral DNA replication, and reduced levels of SV40 late proteins are observed in the mutant infections (45). It will be interesting to determine whether this function is associated with translation control of viral mRNAs late after infection.

The data presented in this paper are not sufficient to prove that the phenotypic suppression of the double mutant is due to blocking of the activity of the P1/eIF-2α kinase. However, if this mechanism were operating, then SV40 may have developed its own mechanism to prevent the activation of the P1/eIF-2α kinase, thus serving as a defense mechanism against the action of interferon. If it is true, it is similar to the systems that adenovirus and a few other known viruses have developed to counteract the activation or function of interferon-induced P1/eIF-2α kinase. For example, influenza virus mRNAs are translated efficiently in cells infected with a VAI$^-$ mutant (20). The P1/eIF-2α kinase activation is prevented by a factor in cells infected with vaccinia virus (47). Mengovirus RNA is reported to inhibit the activation of the kinase (35). Finally, Epstein-Barr virus encodes two low-molecular-weight RNAs which appear to be functionally similar to the VA RNAs (6, 8).

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