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A Novel Translational Regulation Function for the Simian Virus 40 Large-T Antigen Gene

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Cells use the interferon-induced, double-stranded-RNA-dependent protein kinase PKR as a defense against virus infections. Upon activation, PKR phosphorylates and thereby inactivates the protein synthesis initiation factor eIF-2, resulting in the cessation of protein synthesis. Viruses have evolved various strategies to counteract this cellular defense. In this paper, we show that simian virus 40 (SV40) large-T antigen can antagonize the translational inhibitory effect resulting from the activation of PKR in virus-infected cells. Unlike the situation with other virus-host cell interactions, SV40 large-T antigen does not block the activation of PKR, suggesting that SV40 counteracts the cellular antiviral response mediated by PKR at a step downstream of PKR activation. Mutational analysis of large-T antigen indicates that a domain located between amino acids 400 and 600 of large-T antigen is responsible for this function. These results define a novel translational regulatory function for the SV40 large-T antigen.

Interferon-induced, double-stranded-RNA-activated protein kinase PKR (also referred to as p68 kinase, double-stranded-RNA-activated inhibitor [DAI], and eIF-2α kinase) is an enzyme that cells use as a defense against virus infections. PKR, upon activation, phosphorylates and thereby inactivates the vital protein synthesis initiation factor eIF-2 (reviewed in references 22, 23, 36, 44, and 47). The initiation factor eIF-2 is required at one of the earliest steps in translational initiation. During polypeptide chain initiation, eIF-2 forms a ternary complex with GTP and $tRNA^{Met}$. In the subsequent step of the initiation pathway, the GTP moiety bound to eIF-2 is hydrolyzed to GDP, which is then exchanged for GTP before the eIF-2 can function in a new round of initiation. This exchange reaction is carried out by another factor, termed eIF-2B. PKR acts by phosphorylating eIF-2, which then forms a tight complex with the limiting amounts of eIF-2B, preventing the recycling of eIF-2 and thereby reinitiation of protein synthesis (42).

Viruses must overcome this cellular antiviral response for successful infection (reviewed in references 23, 36, and 44). Different viruses have evolved different strategies to combat the translation inhibitory effects resulting from PKR activation. The best studied is the strategy used by adenoviruses (Ad), which use an RNA polymerase III-directed small RNA, which binds to and inactivates PKR (reviewed in references 37, 47, and 54). In cells infected with Ad mutants lacking the VAI RNA gene, protein synthesis is dramatically reduced as a result of PKR activation. We previously showed that simian virus (SV40) can rescue the translational defect in monkey cells that results from infection of an Ad mutant lacking the VA RNA genes (51). We now report that this complementation function is encoded by the large-T gene and that the complementation occurs at the level of the protein. Surprisingly, unlike what occurs with other viruses, large-T antigen of SV40 does not prevent the activation of PKR and the complementation appears to occur at a step downstream of PKR activation. Using mutational analysis, we have identified a domain in the C-terminal region of large-T antigen that is responsible for the complementation. These results define a novel translational regulation function for the SV40 large-T antigen.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Human 293, A549 (a human lung carcinoma cell line), CV-1p, and COS cells (CV-1 cells stably transformed with the SV40 early region) were maintained in Dulbecco's modified Eagle medium (DME) containing 10% calf serum (CV-1p and A549) or fetal calf serum (293 and COS cells). The Ad5 mutants used in this study are mutant *dl*704, which is a phenotypically wild-type (WT) variant which does not code for the minor VAII RNA species; mutant *sub*722, which does not code for the major VAI RNA and grows to a 20-fold-reduced titer; and mutant *sub*720, which lacks both the VAI and VAII RNA genes and grows to a 100-fold-reduced titer in comparison with the WT virus (5–7, 17). All of the Ad stocks were propagated in A549 cells.

Following are the details of the SV40 mutants used in this study. Mutant *dl*888 does not synthesize the small-t protein because of a 100-bp deletion which overlaps the splice donor site of the message that codes for small-t antigen (41a, 49). Mutants *dl*1263 and *dl*1265 contain deletions in the C-terminal region of the large-T gene; they are defective for the Ad helper function of SV40, each retaining about 33 and 8% of the activity, respectively (14, 21). *dl*1263 has a 33-bp in-frame deletion that extends from map units 0.195 to 0.201 and causes the large-T antigen to shorten by 11 amino acids. *dl*1265 has a 39-bp deletion between map units 0.172 and 0.179 and lacks the last 9 amino acids of the large-T protein, although it has 4 new amino acids not normally present in the protein (57).

The WT and mutant plasmids containing the large-T gene used for assaying the large-T translational complementation phenotype of the VA-negative Ad mutants have been described previously (53, 56, 57). These recombinant plasmids contain the entire SV40 genome in a pBR322 background, and the viral large-T gene is expressed from the SV40 early promoter. The positions of the mutations in the large-T gene of these plasmids are shown in Fig. 7. Both the WT and mutants (except mutants 401/450, 451/500, and 601/650) are replication defective because of a small deletion in the replication origin. Mutants 401/450, 451/500, and 601/650 contain only the SV40 early region and promoter-enhancer in an ori^+ background (30). Plasmid pR137 was constructed so that the initiator AUG codon of the large-T gene was mutated into a chain terminator codon by

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using the PCR approach. Plasmid pR108 expresses only the SV40 small-t protein (39).

Preparation of labeled, infected cell extracts. Cells in 35-mm-diameter dishes were infected with the appropriate virus (SV40 or Ad) for 19 h at 10 PFU per cell. Metabolic labeling with 100 μCi of [^{35}S]methionine per ml was then performed for 1 h in methionine-free DME. After two rinses in ice-cold phosphate-buffered saline (PBS), the cells were lysed in 500 μl of ice-cold 1× radioimmunoprecipitation assay buffer (0.01 M Tris-HCl [pH 7.2], 0.15 M sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 5 mM EDTA, 0.2% azide, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Lysates were spun at 30,000 × *g* for 30 min at 4°C, and the supernate was stored at −20°C until further use. Twenty microliters of the lysate was denatured in SDS sample-loading buffer and analyzed by SDS–20% polyacrylamide gel electrophoresis (PAGE), when the whole-cell lysate was used to evaluate the phenotype (17). Routinely, 150 to 200 μl of the lysate was used for the various immunoprecipitations described below.

Immunoprecipitation of hexon, fiber, DBP, and large-T antigen. Lysates corresponding to 1.5 × 10^5 CV-1p cells were used for each immunoprecipitation, and the number of cells was kept constant for comparison of the phenotypes. All immunoprecipitation steps were carried out at 4°C. The lysates were incubated with the appropriate dilution of the respective antibody for 45 min and then treated with 40 μl of a 10% suspension of *Staphylococcus aureus* cells for 15 min. The immune complexes were then washed twice with solution I (100 mM Tris-HCl [pH 8.8], containing 500 mM lithium chloride) and once with solution II (fivefold dilution of solution I). Complexes were then denatured by boiling in SDS sample buffer and analyzed on SDS–20% PAGE gels (17). Antibodies used were 2Hx-2 for hexon (American Type Culture Collection), PAb419 for large-T antigen (a kind gift of M. K. Rundell, Northwestern University), an anti-72-kDa protein monoclonal antibody for single-stranded-DNA-binding protein (DBP), and a polyclonal antifiber antibody for fiber (the last two were kind gifts of A. Levine, Princeton University).

Transfection protocol. CV-1p cells were plated in 35-mm-diameter plates at an appropriate density so that they were approximately 80% confluent about 24 h later. Ten micrograms of plasmid DNAs containing WT or mutant large-T genes was ethanol precipitated and resuspended in 250 μl of H_2O and then mixed with 250 μl of 2× transfection buffer which contained 250 mM $CaCl_2$; 50 mM BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), pH 7.0; 280 mM NaCl; and 1.5 mM Na_2HPO_4. The samples were incubated at room temperature for 20 to 30 min so that efficient precipitation could occur. The DNA-calcium phosphate precipitate was gently applied to the cells. The cells were incubated with the DNA for 30 min at 37°C in a 3% CO_2 incubator and then fed with medium that contained 10% fetal calf serum, 6.25 mM $CaCl_2$, 1.25 mM BES, 0.0375 mM Na_2HPO_4, and 7 mM NaCl and maintained at 3% CO_2 for 18 to 20 h. The cells were then treated with 20% glycerol for 3 min, washed twice with prewarmed DME, refed with DME containing 10% fetal calf serum, and incubated for an additional 40 h at 8% CO_2. The cells were then either lysed or infected prior to being labeled with [^{35}S]methionine, as indicated in the figure legends. Because the stabilities of the mutant large-T proteins varied considerably, the quantitation of steady-state levels of the protein in the cells was considered more relevant to our assay than was the determination of transfection efficiency. Large-T antigen synthesized by various large-T plasmids was quantitated by Western blots (immunoblots).

Complementation assays. CV-1p cells, in 35-mm-diameter dishes, were transfected with plasmid DNAs containing WT or mutant large-T genes as described above and then infected with *sub*720 at 10 PFU per cell for 19 h; the cells were then labeled with [^{35}S]methionine for 1 h. The cell lysates were prepared, and synthesis of hexon was quantitated by immunoprecipitation with a monoclonal antibody specific for hexon protein. The ability of the WT or mutant large-T genes to complement the translational defect resulting from *sub*720 infections was assessed by comparing the amounts of hexon synthesized in *sub*720-infected cells in the presence and absence of large-T antigen.

Large-T Western blots. To determine the amounts of large-T antigen synthesized from the transfected gene, large-T protein was quantitated by Western blot analysis by using a portion of the cell lysates used for the immunoprecipitation of hexon. Large-T antigen was immunoprecipitated from equal amounts of extracts with the large-T-specific antibodies PAb901 and PAb902 (56). Antibody PAb902 recognizes all the large-T mutant proteins shown in Fig. 7, whereas antibody PAb901 recognizes all but *dl*2459 and *dl*1265. The immunoprecipitated proteins were separated on SDS–7.5% PAGE gels; the proteins were then transferred to nitrocellulose filters, and the filters were blocked with 5% nonfat milk powder. The filters were then probed with the antibodies PAb901 and PAb902. The immunocomplexes were detected by hybridizing the filters with ^{125}I-labeled anti-mouse immunoglobulin G. After three washes in PBS–0.5% Tween, the filters were autoradiographed.

Determination of p68 kinase activation levels. CV-1p cells were infected with the various viruses for 20 h. Cells were rinsed once with phosphate-free DME and labeled with 200 μCi of $^{32}P_i$ per ml for the last 8 h of the infection. All further operations were performed at 4°C. After being rinsed with PBS, the cells were lysed in a buffer containing 1% Triton X-100, 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 mM $MgCl_2$, 0.2 mM PMSF, and 100 μg of aprotinin per ml as described elsewhere (29). Extracts were clarified by centrifugation at 30,000 × *g* for 30 min at 4°C. The extracts were then diluted with 500 μl of buffer I (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 0.4 M NaCl, 1% Triton X-100, 1 mM EDTA, 10 μg of aprotinin per ml, 1 mM dithiothreitol, 0.2 mM PMSF, and 20% glycerol) and incubated for 1 h with 3 μl of a polyclonal antibody raised in rabbits against baculovirus-expressed human PKR, which is capable of recognizing monkey and mouse PKR as well (4). The lysates were then incubated for an additional 1 h with 75 μl of a 10% suspension of protein A-agarose (Boehringer Mannheim). The immune complexes were pelleted and washed four times in buffer I and three times in buffer II (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 10 μg of aprotinin per ml, and 20% glycerol). The immunoprecipitated polypeptides were analyzed by SDS-PAGE and processed for autoradiography.

Primer extension analysis. CV-1p cells were transfected as described above, total cellular RNA was prepared by the guanidine isothiocyanate method, and poly(A)$^+$ RNA was then separated on oligo(dT) columns as described elsewhere (43). Equal quantities of RNA (approximately 1 μg) were used for the large-T primer extension; approximately 0.1 μg was used for the β-actin primer extension to normalize the mRNAs. Primer extensions were performed as described by Ausubel et al. (3), except for the following details. Primers were end labeled at the 5′ end, 10^5 cpm was used per extension reaction, and the annealing was carried out overnight at 30°C. After precipitation of the nucleic acids, the annealed primer was extended for 3 h with 40 U of avian myeloblastosis virus reverse transcriptase (Promega) and the extended products were analyzed on 6% DNA-sequencing gels. The large-T primer extends from positions SV4526 to SV4557 (9), and the β-actin primer used for quantitative normalization of the RNA is complementary to positions +78 to +105 of the human β-actin gene (numbering is in relation to the cap site of actin mRNA).

OA experiments. CV-1p or COS cells were infected with the respective viruses at 10 PFU per cell. After incubation for 1 h to allow virus adsorption, the cells were fed with DME containing 2% calf serum and the appropriate amount of okadaic acid (OA) (a kind gift of Mark Johnson, Northwestern University). The infection was allowed to proceed for 20 h. The cells were labeled during the last 1 h of infection and lysed, as described above. Hexon immunoprecipitations were then performed on the lysates, as described above.

RESULTS

SV40 large-T protein is responsible for the rescue of the translational defect resulting from infection with an Ad mutant lacking the VAI RNA gene. Ad mutants with the VAI RNA gene deleted show a dramatic reduction in viral polypeptide synthesis at late times after infection (46, 55). We previously reported that monkey cells infected with VAI-negative mutants also show this phenotype and that this translational defect can be rescued by coinfecting cells with SV40. Our studies have indicated that this complementation occurs at the level of translation (51).

In order to determine whether the translational defect manifested by monkey cells infected with VA-negative Ad mutants is similar to that of human cells, we analyzed by quantitative immunoprecipitation the levels of three Ad proteins, hexon, fiber, and DBP, in CV-1p cells infected with *dl*704 or *sub*720 (Fig. 1). Hexon and fiber are synthesized abundantly at late times postinfection from the major late promoter. DBP is transcribed from the E2 promoter at both early and late times. The supernatants from the first round of DBP immunoprecipitations were reimmunoprecipitated (see Fig. 1 legend) to enable a more precise quantitation of the translational defect (Fig. 1, lanes 13 to 16). Synthesis of all three viral proteins tested was reduced in the *sub*720-infected cells (Fig. 1, lanes 3, 7, and 15) compared with the WT Ad-infected cells (lanes 2, 6, and 14). Thus, our studies show that the translational defect in CV-1p cells that results from infection of a VA-negative Ad mutant is extended to both early and late viral proteins at late times after infection, indicating that the translational defect caused by the deletion of the VAI gene in CV-1p cells is identical to that observed to occur in human cells. Further, coinfection with SV40 restores the translation of all three Ad proteins to WT levels (Fig. 1, compare lanes 4, 8, and 16 with lanes 2, 6, and 14, respectively). However, the identity of the SV40 gene product responsible for this complementation and the mechanism by which this complementation occurs have not been established.

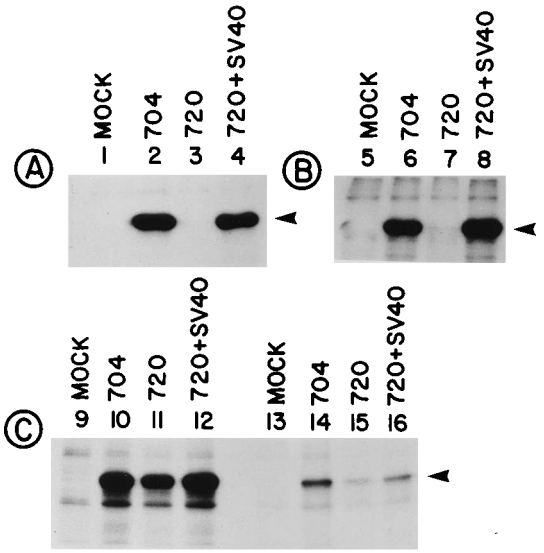

FIG. 1. Synthesis of both early and late viral polypeptides is reduced in CV-1p cells infected with the VA-negative Ad mutant *sub*720. CV-1p cells were infected with the indicated viruses (Ad variants *dl*704 and *sub*720 are phenotypically WT and VAI$^-$ VAII$^-$, respectively) at 10 PFU per cell for 20 h. The cells were labeled with [^{35}S]methionine during the last hour of infection, harvested, and lysed. The labeled viral polypeptides were immunoprecipitated and analyzed on SDS–20% PAGE gels. The antibodies used were 2Hx-2 for hexon (A), an anti-72-KDa protein monoclonal antibody for DBP (C), and a polyclonal antibody for fiber (B) (the last two were kindly donated by A. Levine, Princeton University). The arrowheads indicate the positions of the immunoprecipitated proteins. Because the bands in lanes 10 to 12 in panel C were too intense for quantitative comparison, the supernatants from the first round of DBP immunoprecipitation were reimmunoprecipitated (lanes 13 to 16). DBP bands in lanes 14 to 16 were quantitated by laser densitometry. Radioactive bands for panels A and B were compared by visual inspection.

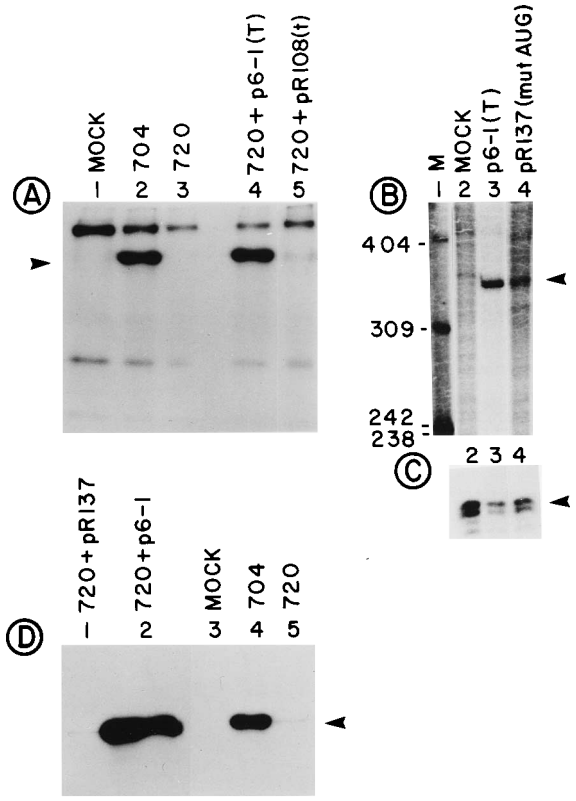

FIG. 2. The large-T protein of SV40 is responsible for the complementation of the Ad VA RNA-negative mutant phenotype. (A) Transfection of the WT SV40 large-T gene complements the *sub*720 (VAI$^-$ VAII$^-$) phenotype. CV-1p cells were either mock infected (lane 1) or infected with a phenotypically WT Ad (*dl*704; lane 2) or *sub*720 (lane 3) at 10 PFU per cell for 20 h. Cells were labeled with [^{35}S]methionine for the last hour of infection, and the amount of hexon polypeptide was quantitated by immunoprecipitation. In the experiments shown in lanes 4 and 5, cells were transfected with 10 μg of p6-1, which contains the WT large-T gene (lane 4), or pR108, which contains the small-t gene (lane 5), for 60 h and then infected with *sub*720. Cells were labeled with [^{35}S]methionine, and hexon polypeptide (arrowhead) was quantitated as previously described. The identity of the band migrating at a position above the hexon polypeptide is not known. (B) Quantitation of the large-T-specific mRNAs produced in CV-1p cells transfected with p6-1, which contains a WT large-T gene, or pR137, which contains a mutant large-T gene with a mutation in the AUG codon (mut AUG). CV-1p cells (100-mm-diameter dishes) were transfected with 10 μg of p6-1 or pR137, and poly(A)$^+$ RNA was isolated and quantitated by a primer extension approach as described in Materials and Methods. The positions of DNA markers are indicated on the left. The arrowhead shows the primer-extended product. The nucleotide sequence of the oligonucleotide used for priming the PCR synthesis in the construction of pR137 is 5′-GCA AAA AGC TTT GCA AAG AGC GGA TAA AGT TT-3′. The nucleotides underlined represent mutations with one substitution (G) and one insertion (C). The WT sequence in this region is 5′-GCA AAA AGC TTT GCA AAG ATG GAT AAA GTT T-3′. The double-underlined sequence is the initiator codon of the large-T gene. M, markers. (C) Quantitation of the β-actin mRNA. β-Actin mRNA was quantitated from RNA samples used in the experiment shown in panel B under conditions the same as those used for panel B with a primer which spans β-actin mRNA sequences from positions +78 to +105 (relative to the mRNA cap site). The arrowhead shows the primer-extended product. (D) Large-T mRNA alone does not complement the VA RNA-negative phenotype of *sub*720. Cells were transfected with either pR137, in which the AUG codon is mutated, or p6-1, containing the WT large-T gene, for 60 h and then infected with *sub*720 for 20 h. Hexon immunoprecipitations were then performed to quantitate the extent of complementation by the two plasmids. Lanes 3, 4, and 5 represent virus infection experiments similar to those shown in lanes 1, 2, and 3 of panel A. The arrowhead shows the hexon protein.

At early times after infection, SV40 encodes three gene products. These are large-T antigen, small-t antigen, and the recently identified 17kT (58), which arise from a single primary transcript as a result of differential splicing. Three capsid proteins and agnoprotein are encoded by the late region. To determine the SV40 gene products responsible for the complementation, we have developed a combined transient transfection and virus infection assay. In this assay, CV-1p cells were first transfected with either a plasmid containing the entire replication-defective SV40 genome or a plasmid expressing only the SV40 small-t antigen. After 60 h, the cells were infected with the translationally defective Ad mutant *sub*720, which had the VAI and VAII RNA genes deleted (6, 17). Rescue of the translational defect resulting from *sub*720 infection by SV40 genes was assayed by quantitative immunoprecipitation of hexon, a major coat protein of Ad. Mock transfection followed by WT Ad or *sub*720 infection were also carried out in parallel as controls. As expected, in control experiments in which cells were infected with phenotypically WT Ad variant *dl*704, large amounts of hexon protein were detectable (Fig. 2A, lane 2), whereas in cells infected with the defective mutant *sub*720, hexon protein was undetectable (lane 3). Plasmid p6-1, which contains the entire SV40 chromosome, was able to rescue the decreased protein synthesis of *sub*720 to WT levels (Fig. 2A, compare lane 4 with lane 2). It is noteworthy that in terms of the levels of hexon protein detected, the transfection experiment (Fig. 2A, lane 4) parallels the infection experiment (lane 2). Considering that only 20 to 30% of the cells are transfected, perhaps the introduction of a large number of plasmid copies into each cell of the transfected population may account for the WT (Fig. 2A, lane 4) or more-than-WT levels (Fig. 2D, lane 2) of hexon detected. A plasmid which expressed only the small-t gene (pR108) did not complement the VAI RNA function (Fig. 2A, compare lane 5 with lanes 2 and 4).

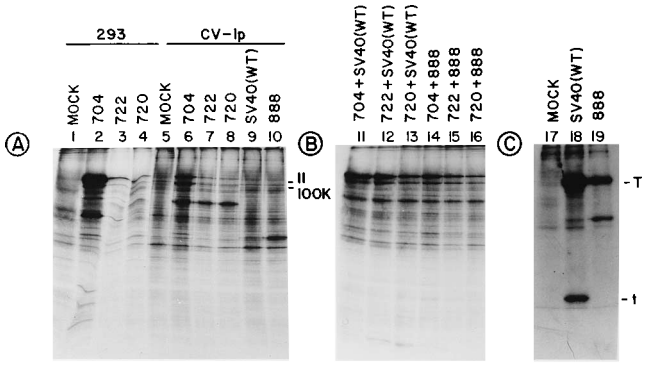

FIG. 3. The SV40 small-t gene is not required for the manifestation of the complementation phenotype. (A) 293 and CV-1p cells were either mock infected or infected with either WT Ad (*dl*704) or the VA-negative mutants (*sub*722 [VAI$^-$ VAII$^+$] and *sub*720 [VAI$^-$ VAII$^-$]) at 10 PFU per cell. Total proteins synthesized at 20 h postinfection were analyzed on denaturing gels as described in Materials and Methods. Lanes 9 and 10 show proteins synthesized during a single infection with WT SV40 and *dl*888 (a small-t-negative mutant), respectively. II and 100K represent the two major late Ad proteins hexon and the 100-kDa protein, respectively. (B) Complementation of the VA-negative phenotype of Ad by coinfection with WT SV40 or *dl*888. CV-1p cells were coinfected with the indicated Ad and SV40 mutants at 10 PFU per cell each for 20 h, and protein profiles were analyzed as described in Materials and Methods. (C) Quantitation of the amount of large-T antigen synthesized in WT SV40 and *dl*888 infections. CV-1p cells were infected with WT SV40 or *dl*888 at 10 PFU per cell each for 20 h, labeled with [^{35}S]methionine during the last hour of infection, harvested, and lysed. Large-T (T) and small-t (t) antigens were immunoprecipitated with a polyclonal antiserum raised against the SV40 tumor antigens in hamsters. Immunoprecipitations were performed in the same lysates that were used for panel A, lanes 9 and 10.

Additional evidence that the small-t antigen did not play a role in causing this phenotype came from virus infection experiments, as shown in Fig. 3, using the SV40 mutant *dl*888, which does not produce small-t mRNA. CV-1p cells were coinfected with SV40 and Ad viruses, and levels of Ad protein synthesized were visualized by radiolabeling in vivo followed by analysis on denaturing gels. As expected, the monkey cells infected with either *sub*722 (lacking the VAI gene) or *sub*720 (lacking both the VA genes) displayed the translational defect characteristic of the VA-negative mutants (Fig. 3, compare lanes 7 and 8 with lane 6). This phenotype is identical to that seen with human 293 cells (Fig. 3, lanes 1 to 4). WT SV40 restored protein synthesis in CV-1p cells infected with either *sub*722 or *sub*720 (compare lanes 12 and 13 of Fig. 3B with lanes 7 and 8 of Fig. 3A). Consistent with the results of the transfection experiments described above, *dl*888 also complemented the translational defect (compare lanes 15 and 16 of Fig. 3B with lanes 7 and 8 of Fig. 3A), albeit slightly less effectively than did WT SV40 (Fig. 3B, compare lanes 15 and 16 with lanes 12 and 13). However, *dl*888 synthesizes lesser amounts of large-T antigen than does WT SV40 (Fig. 3C), presumably because of lower replication rates. This may account for the slightly reduced complementation ability of this mutant. Thus, we conclude that *dl*888 can correct the VA-negative translational defect in the absence of small-t antigen. The role of the recently described 17kT protein, which is derived from the N-terminal region of large-T antigen as a result of alternative splicing (58), has not been evaluated in this study. However, analysis of large-T mutants in this study (see below) suggests that the 17kT protein by itself cannot rescue the translational defect. In addition, we have also found that the large-T gene cloned in a cytomegalovirus expression vector is capable of complementing the VA-negative phenotype in transient assays, ruling out the involvement of late gene products of SV40 in this process (data not shown). Together, these results indicate that the complementation function of SV40 is encoded in the large-T antigen gene.

Because VA RNAs directly affect translation by binding to and inactivating the double-stranded-RNA-activated protein kinase PKR (18, 29), it seemed possible that the rescue of the translational defect by the SV40 large-T antigen might be mediated by the mRNA that encodes the large-T antigen, rather than by the protein. By using the PCR approach, a mutant large-T plasmid (pR137) was constructed in which a single base was substituted at SV5166 and one base was added after SV5166 (numbering is based on the SV40 numbering system [9]). As a result, the translation initiator codon of the gene was mutated, resulting in the frameshift of the reading frame of the large-T protein by one base (see Fig. 2 legend for the nucleotide sequence of the mutation). This mutant is not expected to synthesize the large-T protein. CV-1p cells were transfected with pR137, and the capacity for phenotypic complementation by the mutant large-T gene was quantitated as described above. As shown in Fig. 2D, the mutant large-T gene of pR137 did not complement the VA RNA-specific defect in complementation assays (Fig. 2D, compare lane 1 with lane 2). To ensure that cells transfected with pR137 contained large-T mRNAs comparable to those of the WT control, total RNAs were isolated from one-half of the cells used in the experiments shown in Fig. 2D and the large-T mRNAs were quantitated by a primer extension approach. Poly(A)$^+$ RNAs were prepared from the transfected cells and annealed to a 5′-end-labeled primer spanning positions SV4526 to SV4557. The primers were then extended with reverse transcriptase, and the extended products were analyzed on 6% DNA sequencing gels. Actin mRNAs were also quantitated by using appropriate primers (Fig. 2C). Approximately equal amounts of a cDNA product (~360 nucleotides long) were detected from cells transfected with the WT and pR137 plasmids, indicating that the levels of mRNAs synthesized from the WT and the mutant large-T genes were comparable in CV-1p cells at 60 h posttransfection (Fig. 2B, compare lanes 3 and 4). As expected, we could not detect any large-T protein synthesized from this mutant gene (data not shown). Thus, we conclude that the large-T mRNA by itself does not contribute to the complementation of the translational defect of the Ad mutant lacking the VA RNA genes.

SV40 large-T antigen does not block the activation of PKR in monkey cells infected with VA-negative Ad mutant. Previous studies have shown that when human cells are infected with a VA-negative Ad mutant, PKR is highly phosphorylated (and thereby activated) but the levels of this protein remain unaltered (29). The activation of PKR results in the phosphorylation of the α subunit of eIF-2. Because the activation of PKR is known to play a pivotal role in the manifestation of the VA RNA-negative phenotype of Ad, it seemed possible that the SV40 large-T protein may rescue the translational defect that ensues after infection with *sub*720 by blocking the activation of PKR, a mechanism similar to that of VA RNA (29, 46, 50). Therefore, we compared the levels of phosphorylated PKR in *sub*720-infected CV-1p cells in the absence (Fig. 4A, 720 lane) and presence (720+SV40 lane) of large-T antigen. Parallel control experiments in which levels of phosphorylated PKR were estimated for CV-1p cells infected with SV40 or the phenotypically WT Ad variant *dl*704 were also carried out. To compare these results with those obtained previously with human cells (29), the levels of phosphorylated PKR were also determined for human 293 cells infected with either *dl*704 or *sub*720. Cells were infected with various viruses and labeled with $^{32}P_i$ for the last 8 h of the 20-h infection, and PKR was

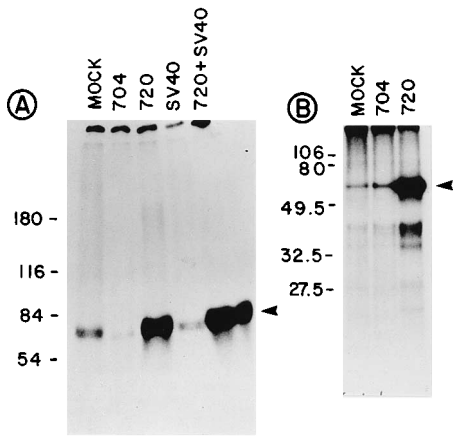

FIG. 4. In vivo phosphorylation of PKR in infected monkey (A) and human (B) cells. CV-1p and 293 cells were infected with the indicated viruses at 25 PFU per cell for 20 h and labeled with $^{32}P_i$ for the last 8 h of the infection. PKR was immunoprecipitated from the extracts prepared from 5×10^6 cells by using a polyclonal antiserum raised against human PKR. PKR from CV-1p cells (A) was immunoprecipitated by using a polyclonal antibody raised against baculovirus-expressed human PKR which also recognizes monkey and mouse PKRs (4). PKR from 293 cells (B) was immunoprecipitated by using a polyclonal antiserum raised against bacterially expressed human PKR, a kind gift of M. B. Mathews, Cold Spring Harbor Laboratory. The immunoprecipitated proteins were then analyzed on SDS-PAGE gels. The immunoprecipitates shown in panel A were run on SDS–12.5% PAGE gels, whereas those shown in panel B were run on SDS–20% PAGE gels. However, it is to be noted that in both instances, the immunoprecipitated protein migrated as expected at about 68 kDa. Arrowheads indicate the position of PKR. The positions of the protein molecular weight markers are indicated on the left (numbers are in thousands).

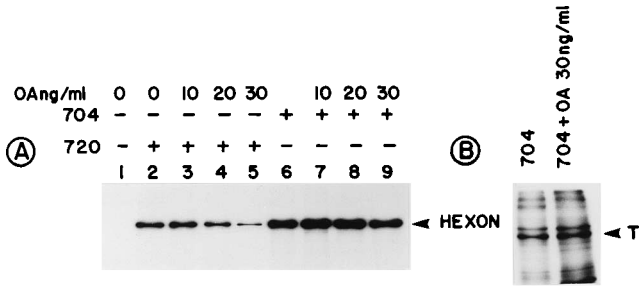

FIG. 5. OA reverses the complementation phenotype of the SV40 large-T antigen in COS cells. (A) Complementation of the VA-negative mutant phenotype by the large-T antigen in COS cells. COS cells were infected with *dl*704 or *sub*720 and treated with OA (0 to 30 ng/ml) as described in Materials and Methods. Infection was allowed to proceed for 20 h. The cells were labeled with [^{35}S]methionine during the last hour of infection, harvested, and lysed. The hexon was immunoprecipitated (lanes 1 to 9) as described in Materials and Methods. +, virus present; −, virus absent. (B) Quantitation of the large-T antigen in COS cells treated with OA. Large-T antigen in the left and right lanes was immunoprecipitated from the same extracts used for lanes 6 and 9, respectively, of the gel shown in panel A. T, large-T antigen.

immunoprecipitated by using a polyclonal antiserum raised against human PKR. The phosphorylated proteins were then analyzed by SDS-PAGE. Figure 4 shows these results. Note that the immunoprecipitates in the two panels of Fig. 4 were analyzed in two different gels under different electrophoretic conditions (see Fig. 4 legend). In agreement with previous results, 293 cells infected with *sub*720 showed a large increase in the phosphorylation of PKR (Fig. 4B). Consistent with this, CV-1p cells infected with *sub*720 also showed a large increase in the levels of phosphorylated PKR (Fig. 4A). Thus, this manifestation of the VA-negative phenotype in monkey cells is consistent with that seen to occur in human cells (37, 54) (Fig. 4B). Contrary to expectations, a large increase in the levels of phosphorylated PKR was also observed with monkey cells coinfected with *sub*720 and WT SV40 (Fig. 4A). The levels of phosphorylated PKR in these cells were comparable to those in cells infected with *sub*720, indicating that the large-T antigen did not block the activation of PKR. These results were also substantiated in in vitro experiments. A partially purified PKR preparation was preincubated with purified large-T antigen in vitro and then incubated with double-stranded RNA. Control experiments without large-T antigen and with preincubation with VAI RNA were also carried out in parallel. Large-T antigen did not block the activation of PKR in vitro, whereas VAI RNA blocked the activation of PKR effectively (data not shown). We conclude that SV40 large-T antigen does not rescue the translational defect of *sub*720 by blocking the activation of PKR. The mechanism by which SV40 large-T antigen rescues the translational defect appears to be quite distinct from the one employed by VAI RNA in that it acts downstream of the PKR activation step.

An important question is whether, in monkey cells coinfected with SV40 and *sub*720, eIF-2 is phosphorylated to the same extent as it is in cells infected with *sub*720 alone. In human cells infected with VA-negative Ad mutants, eIF-2 is phosphorylated to levels much higher than those in cells infected with WT Ad as a consequence of PKR activation (46, 50). This in turn halts protein synthesis. To date, our repeated attempts to determine the phosphorylation status of eIF-2 in monkey cells infected with *sub*720 or *sub*720 and WT SV40 have been unsuccessful (see Discussion). One mechanism by which SV40 large-T antigen may rescue the translational defect despite a large increase in the phosphorylation of PKR may be by dephosphorylation of eIF-2. Because the levels of activation of PKR of the *sub*720 infection were unaltered during an SV40 WT coinfection, it was possible that the SV40 large-T antigen was acting through a phosphatase function that was directed downstream of the p68 kinase step in the protein synthesis initiation pathway, presumably by dephosphorylation of eIF-2. It has been shown that protein phosphatase 2A (PP2A), a cellular phosphatase, has the capacity to dephosphorylate the α subunit of eIF-2 at Ser-51 in in vitro experiments (40).

Phosphorylation of Ser-51 of eIF-2α results in the cessation of protein synthesis. We used OA, an inhibitor of PP2A (11), to determine whether PP2A may be involved in the large-T-mediated rescue of the translational defect. Results of experiments in which we treated CV-1p cells coinfected with SV40 and *sub*720 were difficult to interpret because we found that OA, at low concentrations, significantly inhibited replication of SV40, consistent with published reports (33) (data not shown). As a result, large-T antigen accumulated to much reduced levels which were not adequate for complementation (data not shown). Therefore, we studied the effect of OA using COS cells, a CV-1 cell line which constitutively expresses the WT large-T antigen (19). We previously showed that COS cells infected with *sub*720 do not show the translational defect phenotype (51). COS cells were infected with *sub*720 or WT Ad and then treated with increasing concentrations of OA. Cells were radiolabeled with [^{35}S]methionine for 1 h at 19 h postinfection and then lysed, and hexon was immunoprecipitated as described above. Figure 5A shows that at a concentration of 30 ng/ml, there was significantly reduced synthesis of hexon in COS cells infected with *sub*720 in comparison with synthesis in untreated cells (compare lane 2 with lane 5), indicating that the function provided by the large-T antigen is inhibited by OA.

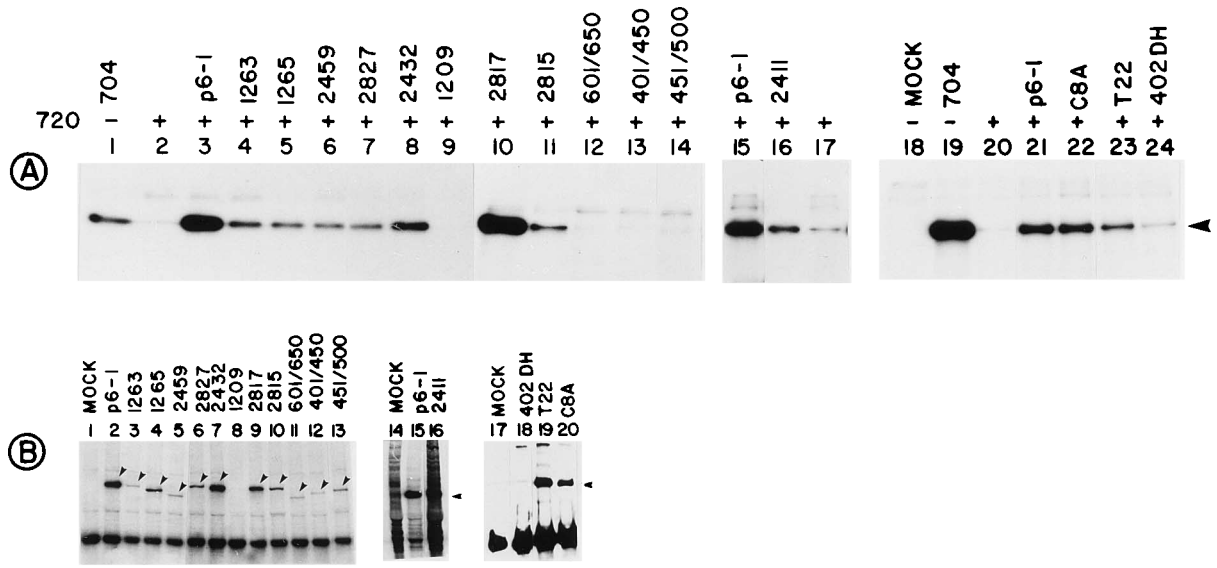

FIG. 6. Localization of the domain of large-T antigen which is important for the complementation function. (A) Ability of the various SV40 mutants to complement the VA RNA-negative phenotype of Ad. CV-1p cells were transfected with 10 μg of the indicated mutants with mutations in the large-T gene. Sixty hours after transfection, the cells were infected with *sub*720 (+) or left without *sub*720 infection (−) as indicated. Infection was allowed to proceed for 20 h. During the last hour of infection, the cells were labeled with [^{35}S]methionine, harvested, and lysed and the hexon polypeptide (arrowhead) was immunoprecipitated from an equal number of cells. Lane 1 shows the amount of hexon synthesized in a WT Ad infection, and lane 2 shows the amount synthesized in a *sub*720 infection. See Fig. 7 for the positions of the mutations in the large-T gene. (B) The steady-state levels of the large-T protein were determined by using the same lysates from which the hexon was quantitated in the experiment shown in panel A. A Western blot analysis was performed as described in Materials and Methods. The position of the large-T protein is indicated by small arrowheads. Each mutant was assayed at least three times. One representative experiment is shown.

Immunoprecipitations of large-T antigen performed in parallel with the same extracts did not show any decrease in large-T levels (Fig. 5B). Thus, the reduced protein synthesis in *sub*720-infected cells in the presence of OA is not the result of reduced synthesis of large-T antigen. OA did not affect WT Ad protein synthesis because the amounts of hexon protein synthesized in the presence of 30 ng of OA per ml were comparable to those synthesized without OA (Fig. 5A, compare lane 9 with lane 6). This concentration of OA was sufficient to inhibit the large-T-mediated complementation effect (Fig. 5A, compare lane 5 with lane 2). These results also indicate that the reduced protein synthesis in *sub*720-infected cells in the presence of OA is not the result of inefficient entry of the mutant virus into the cell. It is also interesting that levels of hexon synthesized by *dl*704 are slightly higher than those synthesized by *sub*720. Perhaps SV40 large-T antigen does not substitute completely for the VAI function. In summary, these results suggest that, perhaps, a phosphatase may be involved in large-T-mediated complementation of VAI RNA function.

Identification of a domain in large-T antigen that is responsible for the complementation function. The large-T antigen is a 90-kDa protein which is essential for the successful completion of the virus life cycle. At least 15 functions and activities have been assigned to or associated with the large-T antigen, and these activities are distributed at different locations in the molecule. Some of these functions are targeted toward regulating the life cycle of the virus, while others regulate the cellular events that transpire in the host cell after infection (reviewed in reference 16). To determine the domain in the large-T antigen that is responsible for the complementation function, we analyzed a number of previously characterized large-T mutants in the transient transfection assay described above. The large-T gene in all these mutants was expressed from its own promoter. CV-1p cells were transfected with WT or mutant large-T plasmids and after 60 h were infected with the VA-negative mutant, *sub*720. Twenty hours after infection, synthesis of hexon polypeptide was quantitated by immunoprecipitation of radiolabeled proteins. Because the stabilities of the mutant large-T proteins vary considerably, the steady-state levels of large-T protein synthesized from the transfected gene in the same extract were quantitated by Western blot, as described in Materials and Methods. Quantitation of steady-state levels of the large-T protein in the cell was considered more relevant for our assay than was the determination of transfection efficiency because the capacities of the large-T mutants to complement the translational defect of *sub*720 correlate with the steady-state levels of large-T antigen. A total of 26 mutants with mutations in the large-T gene were assayed for their capacity to complement the VA RNA-negative Ad phenotype, and only those mutants that produced detectable amounts of stable large-T protein were used to evaluate complementation ability. Complementation assays were carried out at least three times with independently grown plasmid preparations, and similar results were obtained. The results of one such experiment are shown in Fig. 6. The large-T mutants used in this study and their capacities to complement the translational defect are schematically shown in Fig. 7.

It has been reported that the SV40 large-T antigen provides a function which allows efficient late protein synthesis in Ad-infected monkey cells. This phenomenon, referred to as the Ad helper function of SV40, is known to reside in the extreme C-terminal region of the large-T antigen (14, 35, 38). The helper function has proven to be very complicated and is thought to act at multiple levels, including splicing, nucleocytoplasmic transport of viral messages, and transcription rate and transcriptional termination (references 27 and 31 and references cited in those papers). It seemed possible that the translation complementation phenotype of the large-T antigen for Ad mutants in monkey cells was yet another facet of this complex function. To resolve this, three large-T mutants,

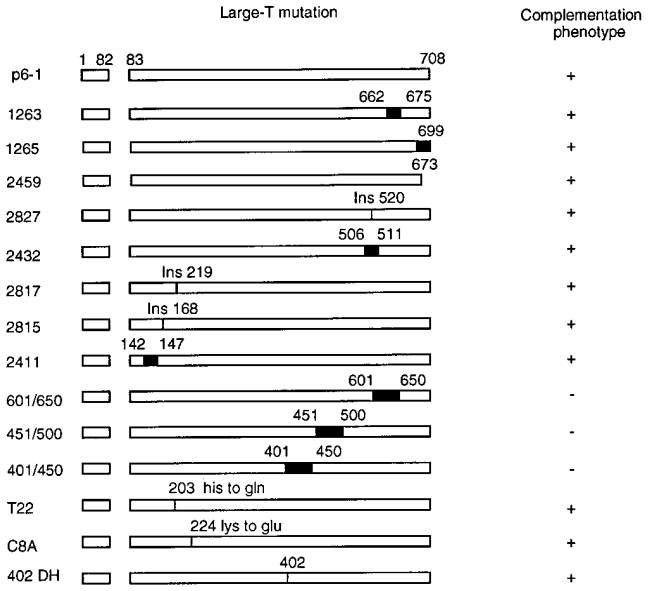

FIG. 7. Diagrammatic representation of the various large-T mutant genes employed in determining the complementation domain and their capacity to rescue the translational defect specific for the VA-negative Ad mutant. The WT large-T protein has 708 amino acids. Black boxes denote deletions between the indicated amino acid positions in the various mutants. Insertion mutations (Ins) are shown by vertical lines. *dl*1265 lacks the last 9 amino acids of the large-T protein but has 4 new amino acids not normally present in the protein (57). Not shown is mutant 1209, which lacks the entire large-T coding region (14). Because the stabilities of the mutant large-T proteins vary considerably, the steady-state level of large-T protein synthesized from the transfected gene was quantitated by Western blotting. A mutant which showed detectable levels of large-T antigen in the Western analysis was scored as either positive or negative depending on the detection of the hexon protein (see Fig. 6).

*dl*1263, *dl*1265, and *dl*2459, which are defective for the Ad helper function (21), were assayed in the transient assays described above. Of these three mutants, *dl*2459 was severely defective for the helper function, whereas *dl*1263 and *dl*1265 retained 33 and 8% of WT helper activity, respectively (14, 21). As shown in Fig. 6A, all three mutants complemented the VA RNA-related translational defect significantly and the levels of complementation among the three mutants were comparable. The complementation capacities of *dl*1263 and *dl*1265 were also assayed by coinfecting mutant viruses which harbored these mutant genes with *sub*720 and analyzing viral polypeptides as previously described (51). These results, shown in Fig. 8, confirmed the data obtained in the transient assays. The decreased protein synthesis in CV-1p cells infected with VA-negative Ad (Fig. 8A, compare the 722 and 720 lanes with the 704 lane) was restored to normal levels by coinfection with WT SV40 (Fig. 8B, WT lanes). Both *dl*1263 and *dl*1265 efficiently corrected the VA-negative defect (Fig. 8B, compare 1265 and 1263 lanes with the WT lanes). Complementation assays with *dl*2459 could not be carried out at the virus level, as stocks of sufficiently high titer could not be obtained for this mutant. In addition, we could not see any complementation when large-T plasmid was replaced with an expression plasmid which expressed a mutant form of the 72-kDa DBP (Ad2hr404) (data not shown). Mutant Ad2hr404 has been shown to overcome the block to growth of Ad in monkey cells (32). Together, these results show that the SV40 large-T protein is capable of complementing the VAI function independently of the Ad helper function.

Large-T antigen is known to encode an ATPase function which resides in the C-terminal half of the protein between amino acids 415 and 630 (12). Mutants *dl*2827 and *dl*2432 lack this function (12). In our transient assays, both mutants synthesized significant quantities of large-T antigen (Fig. 6B, lanes 6 and 7) and complemented the VA RNA function significantly (Fig. 6A, lanes 7 and 8), indicating that the large-T-mediated rescue of the translational defect is independent of the ATPase function. SV40 large-T antigen has been shown to bind to tumor suppressor gene product p53, and the region that is essential for this binding has been mapped to the domain between amino acids 272 and 517 (reviewed in reference 16). Mutant 402DH is defective for large-T antigen's stable association with p53 (50a). In transient assays, this mutant

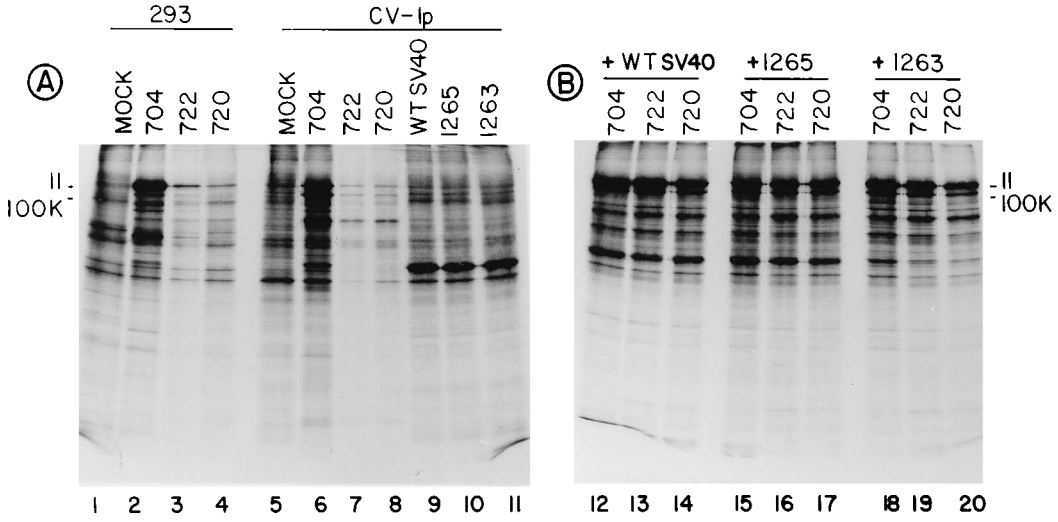

FIG. 8. The helper-defective SV40 mutants complement the Ad VA-negative defect as efficiently as WT SV40. (A) Translation defect of the VA RNA-negative mutants in 293 and CV-1p cells. 293 and CV-1p cells were infected with either WT Ad (*dl*704) or the VA-negative mutants (*sub*722 and *sub*720) at 10 PFU per cell. Viral polypeptides synthesized 20 h postinfection were analyzed on denaturing gels. (B) Complementation of the VA-negative phenotype of Ad by a coinfection with WT SV40, *dl*1263, or *dl*1265. CV-1p cells were coinfected with the indicated Ad and SV40 mutants at 10 PFU per cell each for 20 h, and the viral polypeptides were analyzed as described in Materials and Methods. II and 100K represent hexon and the 100-kDa protein, respectively.

synthesized barely detectable amounts of large-T antigen (Fig. 6B, lane 18); however, hexon protein was detected in the complementation assays (Fig. 6A, lane 24). These results suggest that the translational complementation function is independent of large-T antigen's association with p53. Several mutants with deletion, substitution, and insertion mutations in the N-terminal half of the second exon region were also found to complement the VAI RNA function in our transient assays. These include two insertion mutants, 2815 and 2817 (Fig. 6A, lanes 11 and 10, respectively), which contain in-frame insertions after amino acids 168 and 219, respectively, and a deletion mutant (*dl*2411) in which amino acids 142 to 147 are deleted (Fig. 6A, lane 16). Two substitution mutants, T22 and C8A, in which histidine at position 203 and lysine at position 224 are substituted by glutamine and glutamic acid, respectively, also complemented the VAI RNA function (Fig. 6A, lanes 23 and 22, respectively). All these mutants synthesized significant amounts of hexon (Fig. 6A).

Of all the mutants tested, mutants *dl*601/650, *dl*401/450, and *dl*451/500, which contain deletions of amino acids between positions 601 and 650, 401 and 450, and 451 and 500, respectively, were found to be defective for the complementation function (Fig. 6A, lanes 12, 13, and 14, respectively). These mutants did synthesize detectable amounts of large-T antigen, as evidenced by Western blot analysis (Fig. 6B, lanes 11, 12, and 13). Failure of these mutants to complement the translational defect of *sub*720 is not the result of insufficient quantities of large-T antigen because mutants *dl*1263 and *dl*2459 also accumulated comparable amounts of large-T antigen under identical conditions (Fig. 6B, compare lanes 3 and 5 with lanes 11, 12, and 13); nevertheless, mutants *dl*1263 and *dl*2459 complemented the VAI RNA function to a significant level (Fig. 6A, compare lanes 4 and 6 with lanes 12, 13, and 14). Similarly, mutant 402DH synthesized barely detectable amounts of large-T antigen (Fig. 6B, lane 18) yet complemented the translational defect of *sub*720 to a detectable level (Fig. 6A, lane 24). Thus, we conclude that a domain between amino acids 401 and 650 is important for the translational complementation function.

DISCUSSION

The cellular defense mechanisms that block a successful virus infection usually center around the posttranscriptional steps of the control of gene expression and are mediated in several instances by PKR. Viruses have developed molecular mechanisms which make it possible for them to circumvent the host-cell-mediated block to their successful replication (reviewed in references 28, 36, and 44). The VA RNAs of Ad have evolved as the viral mechanism that circumvents the host-cell-mediated block during Ad infection (reviewed in references 37, 47, and 54). Our previously published results (51) and the present studies show that mutants of Ad which lack VA RNAs manifest the translational-defect phenotype in monkey cells, a phenomenon identical to that observed for human cells. In human cells, PKR that is activated as a consequence of infection by a VAI-negative virus (*sub*720 in this case) will phosphorylate eIF-2, which then will bind to and trap the guanine nucleotide exchange factor GEF that is present in limiting amounts. As a consequence, initiation of protein synthesis is blocked (46, 50). In CV-1p cells infected with a VA-negative mutant, both early and late Ad polypeptides are synthesized at much reduced levels, indicating that the phenotype that results from the deletion of the VAI gene from Ad is identical in both human and monkey cells. This translational defect is reversed when the cells are coinfected with WT SV40 and the phenotypic rescue occurs at the level of translation (51). In this study, we have attempted to address two important questions related to this translational complementation phenomenon, namely, the mechanism by which SV40 complements the Ad VA RNA function and the identification of the SV40 gene product responsible for this complementation.

In this investigation, we successfully employed a combination of transient transfection and a VA-negative virus infection assay to study the complementation. Using these assays, we have shown that the SV40 large-T gene product is responsible for this complementation and that this complementation occurs at the level of the protein. No detectable complementation was observed when plasmid pR137, in which the AUG of the large-T open reading frame was mutated into a chain termination codon, was used in the complementation assay. Unlike Ad, SV40 does not encode an RNA polymerase III gene product. However, SV40 has been reported to synthesize a low-molecular-weight RNA, designated SAS-RNA, which is believed to be a cleavage product of late transcripts (2). Because a plasmid which synthesizes only large-T antigen can complement the VAI RNA function, and also because cells transfected with pR137 contain amounts of large-T (and small-t) mRNA comparable to those in cells transfected with the WT plasmid, we can rule out the possibility that the SAS-RNA or the mRNAs that encode the two early gene products are responsible for this complementation. Cloned cDNA that encodes WT large-T antigen can complement the VA-negative defect as well as the large-T antigen synthesized in SV40 infection can, indicating that this transient assay can be used to study the domain in the large-T protein responsible for complementation (see below). In our studies, a plasmid that expresses only small-t antigen did not complement, suggesting that this function does not overlap with those described for small-t antigen.

As predicted, in CV-1p cells infected with the VA-negative mutant *sub*720, PKR is phosphorylated to high levels, a result that is consistent with its phenotype in human cells. The most surprising observation, however, is that in CV-1p cells coinfected with *sub*720 and WT SV40, PKR is activated to the same levels as in cells infected with *sub*720 alone. This indicates that the translational rescue does not involve the prevention of activation of PKR but functions at a step downstream of it. This raises the important question of whether eIF-2 is phosphorylated to the same levels in cells infected with *sub*720 and in cells coinfected with *sub*720 and WT SV40. Our numerous attempts to determine the levels of phosphorylated eIF-2 in monkey cells infected with *sub*720 have not been successful. We used an isoelectric focusing immunoassay (48) and two-dimensional PAGE followed by immunoblotting (24) to evaluate the phosphorylation status of eIF-2α in mock- and *sub*720-infected CV-1p cells. Although we could detect eIF-2α in these assays, we could not separate the phosphorylated and unphosphorylated forms of the protein. Our attempts to monitor the levels of eIF-2α phosphorylation by direct immunoprecipitation from in vivo ^{32}P-labeled CV-1p cell lysates using a polyclonal antibody specific for human eIF-2α have also been unsuccessful.

Because the kinase remains activated in an SV40-*sub*720 coinfection, as it does in a *sub*720 infection, it has the capacity to phosphorylate eIF-2. However, because protein synthesis proceeds as normal in these cells, it would seem that the eIF-2 would occur in its natural unphosphorylated state. One possible mechanism by which large-T antigen could neutralize the effects of PKR would be by dephosphorylation of eIF-2. Protein synthesis would then continue at normal levels, despite the activation of PKR. For example, by activating a phosphatase,

large-T antigen could directly or indirectly dephosphorylate the eIF-2 as it is phosphorylated by PKR. Although several functions have been attributed to the large-T protein, a phosphatase function is not included in its repertoire. As shown in Fig. 6 and 7, and as will be discussed below, the translation complementation function localizes to a large portion of the C-terminal half of the large-T protein molecule (amino acids 401 to 650). It is possible that the large-T protein itself possesses a phosphatase function that we have localized to this region, or that the large-T antigen is acting via an intracellular phosphatase by increasing either its activity or expression. Our studies with the protein phosphatase inhibitor OA indicate that treatment of COS cells 1 h after *sub*720 infection, providing sufficient time for infection to take place, causes inhibition of the large-T-mediated translational complementation phenotype. OA can inhibit both PP2A and PP1 in vitro, depending on the concentration (11). Because it is extremely difficult to quantitate the intracellular concentrations of OA in our experimental system, we cannot at this point define the source of the phosphatase activity in our experiments based on OA inhibition. Also, the fact that OA treatment does not hamper protein synthesis of WT Ad indicates that normal cellular translation is not affected by OA treatment, while the SV40 large-T-mediated translational control is affected. While these results hint at the possibility that a phosphatase may be involved, our data at present are not strong enough to argue that this may be the sole basis for the translational complementation by the large-T antigen.

Another mechanism that could explain large-T-mediated translational complementation is based on the phosphorylation of GEF. This is a large, 5-subunit complex present at very low stoichiometric concentrations in the cell. It functions in the exchange of GDP for the GTP moiety on the α subunit of eIF-2 at the end of every round of protein synthesis initiation (reviewed in reference 42). Phosphorylation of the largest subunit of GEF results in an increase in the exchange activity of the GEF complex (22). It is thus possible that, during SV40 infection, phosphorylation of the GEF subunit might cause the few free GEF complexes to function at increased efficiency, thereby compensating for the bulk of GEF that is in the form of inactive complexes with the phosphorylated eIF-2 in the cell. Large-T antigen has also been shown to have an RNA helicase function in vitro (45). During the initiation of translation, eIF-4A, eIF-4B, and eIF-4F are thought to function as a complex in the unwinding of the secondary structure of the mRNA before it complexes with the ribosome (26). Currently, it is not possible to determine the role of RNA helicase activity in the translation complementation function because no information is available for the RNA helicase domain.

Our mutational analysis of the SV40 large-T gene indicates that the C-terminal half of the large-T protein is important for the manifestation of the translation complementation phenotype. Because the translational complementation function of large-T antigen maps to a reasonably large region of the protein, it is likely that this function may depend on the protein conformation in this region or on multiple activities of the large-T antigen. However, it does not overlap with the Ad helper function because the C-terminal deletion mutants that lack the Ad helper function complement for the VAI RNA function in transient transfection assays. The 82 N-terminal amino acids of the protein do not seem to be important, as the complementation occurs in the absence of small-t antigen. Also, a large part of the N-terminal region of large-T antigen may not be involved in the complementation function because a number of mutants with deletion, substitution, and insertion mutations in this region are able to rescue the *sub*720-specific translational defect. Mutants with mutations encompassing amino acids 401 to 650 were defective in the complementation assay, indicating that the complementation function maps to this region. Although the amount of hexon that we see in the complementation assays is related to the amount of large-T protein present in the cell during the assay, lack of complementation for mutants 601/650, 451/500, and 401/450 cannot be explained by the lack of sufficient quantities of T antigen in the cell. Mutants *dl*1263 and *dl*2459 also synthesize large-T antigen in quantities comparable to those of *dl*601/650, *dl*401/450, and *dl*451/500 (Fig. 6B), yet they are able to rescue the translational defect (Fig. 6A). Thus, we believe that these three deletion mutants are truly defective for the complementation function. Several important functions of the protein reside in this region, including ATPase activity, ATP binding, p53 binding, and DNA helicase activity (16). Mutants *dl*2827 and *dl*2432 are defective in ATPase activity yet can complement the *sub*720 phenotype, indicating that ATPase activity is not involved in the complementation function. The observation that large amounts of phosphorylated PKR can be detected in cells coinfected with *sub*720 and SV40, despite the ATPase activity of large-T antigen, is consistent with this finding. The activation of PKR involves ATP-dependent autophosphorylation. If the ATPase activity of large-T antigen had any role in the complementation function, it is possible that this would have impacted upon the autophosphorylation of PKR.

The region of large-T antigen that is essential for complementation also binds to p53. However, we believe that the translation complementation function is not related to large-T antigen's association with p53. Of the three mutants which fail to complement, mutants 401/450 and 601/650 do not bind p53 whereas mutant 451/500 does (30). Mutant 402DH, which carries a point mutation at amino acid 402, also does not bind p53 (50a) yet suppresses the *sub*720 phenotype, commensurate with its poor stability. Another activity associated with this region is DNA helicase activity. It is unlikely that the DNA helicase function would be important in translational regulation. The role of the RNA helicase function of the large-T antigen in complementation cannot be addressed genetically because this function has not yet been mapped to a domain on the protein. With the helper-defective deletion mutants, we have also shown that the translational regulation property of the large-T antigen is not a part of the Ad helper function of SV40, suggesting that the translational complementation function is a novel function which is not associated with previously described functions of large-T antigen.

Cellular defense mediated by interferon-induced PKR is a problem that viruses must overcome in order to establish successful infection. Different viruses use different strategies to overcome the PKR effect (28, 36, 47). Several strategies have been described, and many more probably exist. Viruses use both proteins and RNAs to antagonize the effects of PKR. As discussed above, VAI RNA directly binds to and blocks the activation of PKR. The Epstein-Barr virus-encoded EBERs (13) and the human immunodeficiency virus TAR RNA (20) can stimulate translation in vitro in reticulocyte lysates, presumably by inhibiting the activation of PKR. EBERs can complement the VAI RNA function in the Ad chromosome, indicating that they may function like VAI RNA (5, 7), although rigorous proof that they are functionally equivalent to VA RNAs is lacking (52). Human immunodeficiency virus type 1 tat protein reduces the amounts of PKR by an unknown mechanism (41), whereas poliovirus degrades PKR (8). Influenza virus can inhibit PKR activation indirectly via a host protein (34). Vaccinia virus-encoded E3L (1, 10) and reovirus sigma 3 (25) gene products have the capacity to bind to double-

stranded RNA and thus are able to inhibit the activation of PKR. In all these cases, the end result is reduction of active PKR in the cell. Our results show that the SV40 large-T gene product counteracts the inhibitory effects of PKR downstream of the PKR activation step, possibly by activation or induction of phosphatases that may dephosphorylate eIF-2. The other possibility, that the large-T protein may mimic the α subunit of eIF-2 and thus inhibit phosphorylation of eIF-2, cannot be ruled out at present. Indeed, such a mechanism has been proposed for the vaccinia virus-encoded K3L gene product in the neutralization of the PKR effect (15). Further work will be necessary to define the molecular mechanism by which large-T antigen may antagonize the deleterious effects of the activation of PKR.

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