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Carboxyl-terminal mutants of the large tumor antigen of simian virus 40: A role for the early protein late in the lytic cycle

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ABSTRACT **Simian virus 40 (SV40) mutants dl1066 and dl1140 contain deletions within the region encoding the carboxyl terminus of the large tumor (T) antigen. Although these mutations have little effect on the efficiency of viral DNA replication, they decrease the yield of infectious virus particles by 3–4 orders of magnitude [Pipas, J. (1985) *J. Virol.* 54, 569–575]. Here we show that the level of late RNA is lower by a factor of 5–15 in CV-1P monkey cells infected with these mutants compared to cells infected with wild-type SV40. Consistent with this decrease in RNA, synthesis of late viral structural proteins VP1 and VP3 decreases by a factor of 5–15. In contrast, the synthesis of SV40 agnoprotein decreases by a factor >100. Intercistronic complementation of these mutants with pm1493 and dl121, two SV40 mutants that are defective in agnoprotein but encode wild-type T antigen, results in an increased synthesis of agnoprotein in the infected cells. These results suggest that the carboxyl-terminal portion of T antigen participates in the posttranscriptional regulation of agnoprotein.**

Simian virus 40 (SV40) is a small DNA tumor virus that has served as a model system for studies of eukaryotic gene regulation (1). The virus produces a lytic infection of African green monkey kidney cells in which its genes are expressed temporally in two phases. Prior to the onset of DNA replication, an early set of genes encoding large (T) and small (t) tumor antigens is expressed. After the onset of DNA replication the major transcriptional program shifts to the expression of the late genes, which encode the agnoprotein and the viral structural proteins VP1, VP2, and VP3 (1–3). While the function of the 61 amino acid agnoprotein is not entirely clear, it appears to play a role in encapsidation, in assembly of the mature viral particles, or in release of virus particles from infected cells (4–6). In addition, the late agnoprotein may play a role in regulation of late gene expression (7–9).

The role of T antigen in activating the late phase of the lytic cycle relates in part to its autoregulation of early gene transcription and the initiation of rounds of DNA replication that increases the SV40 template number. Recent studies have suggested that in the absence of DNA replication, T antigen also plays a significant role, both direct and indirect, in activating late transcription (10–13).

Mutations in the carboxyl terminus of T antigen (14, 15) have an interesting phenotype in that, although a minimal decrease in the efficiency of viral DNA replication is observed, under appropriate conditions they decrease the yield of infectious virus particles by 3 orders of magnitude (14). This reduction of plaque formation occurs on BSC-40 cells at 32°C or on CV-1P cells at 32°C or 37°C. The present study was conducted to determine the effect of these T-antigen mutants on the levels of late viral RNA or capsid protein.

MATERIALS AND METHODS

Cells and Viruses. The cells used in these experiments were established CV-1P and BSC-40 African green monkey kidney cells. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Wild-type SV40 strain 776 was the parent of dl1066 and dl1140 mutants. Mutant pm1493, in which the ATG translational start codon at nucleotide 335 was changed to TTG, was obtained from T. Shenk (Princeton University). Mutant dl121 has a 52-base-pair (bp) deletion extending from nucleotide 360 to 413. Except for the immunofluorescence studies, semiconfluent monolayers of cells were infected with 25 plaque-forming units (pfu) per cell in medium containing 2% serum. After 2 hr of adsorption at 37°C, the cells were fed with the same medium and incubated at 37°C for 48 hr. For immunofluorescence analysis cells were infected at a multiplicity of 1.0 pfu/cell.

RNA Preparation and Analysis. Total cytoplasmic RNA was isolated from infected cells by a modification of the Nonidet P-40 procedure (16). For nuclease S1 analysis, 30 μg of total cytoplasmic RNA was coprecipitated with the specific DNA probe (80,000 cpm), heated at 85°C for 5 min, and incubated overnight at the desired temperature in 80 mM Pipes [1,4-piperazinebis(ethanesulfonic acid)], pH 6.4/400 mM NaCl/1 mM EDTA/80% (vol/vol) formamide. Hybrids were treated with 800 units of nuclease S1 (Bethesda Research Laboratories) for 45 min at 37°C according to conditions described previously (17). S1-resistant DNA fragments were analyzed by electrophoresis in 6% polyacrylamide/7 M urea denaturing gels and autoradiography. DNA probes were end-labeled with bacteriophage T4 polynucleotide kinase to 10^7 cpm/pmol (16). For primer-extension analysis, 15 μg of total cytoplasmic RNA was hybridized to end-labeled oligonucleotides under the conditions described above. Hybrids were treated with 20 units of reverse transcriptase in the presence of 1 mM deoxynucleoside triphosphates at 42°C (18). Primer-extension products were electrophoresed in 6% polyacrylamide/urea denaturing gels following denaturation in 80% formamide sample buffer. *Hin*fI-digest fragments of SV40 DNA were used as size markers.

Protein Analysis. Protein samples to be immunoblotted were extracted (12) from SV40-infected cells, denatured by heating at 100°C for 2 min in $NaDodSO_4$ sample buffer, and resolved by electrophoresis in 10% or 15% polyacrylamide gels containing $NaDodSO_4$ (19). Following electrophoretic transfer for 3 hr onto a nitrocellulose membrane in the

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; pfu, plaque-forming unit(s).

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presence of 0.1% $NaDodSO_4$, the membrane was incubated for 2 hr in 10 mM Tris hydrochloride, pH 7.5/150 mM NaCl (TN buffer) containing 2% (wt/vol) bovine serum albumin to block nonspecific binding sites, rinsed briefly with TN buffer, and then incubated for 4 hr with TN buffer containing specific antiserum. The membrane then was washed several times with TN buffer, treated with ^{125}I-labeled protein A (Amersham), and visualized by autoradiography (20).

Immunofluorescence. SV40-infected cells and mock-infected CV-1P cells grown on 100-mm dishes were rinsed, dried, and fixed with acetone. The cells were incubated with rabbit anti-SV40 serum, stained with fluorescein isothiocyanate, and observed by fluorescence microscopy (6, 9).

RESULTS

Analysis of the Effect of T-Antigen Deletion on Late Gene Transcription. In these experiments we employed two SV40 mutants, dl1066 and dl1140, that have been characterized and described in detail (14). Mutants dl1066 and dl1140 carry deletions of the carboxyl-terminal coding sequence of the large T antigen, at nucleotide positions 2809–2730 and 2792–2763, respectively. We employed the BSC-40 line as permissive cells and the CV-1P line as nonpermissive cells. All experiments were performed at 37°C, which elicits the differences in mutant growth on these two cell lines. CV-1P and BSC-40 cells were infected with either wild-type SV40 or mutants dl1066 and dl1140 separately at a multiplicity of infection of approximately 25 pfu/cell. After incubation for 48 hr at 37°C, cytoplasmic RNA was prepared by the Nonidet P-40 procedure (16). These RNAs were then analyzed by nuclease S1 protection and primer extension. In the nuclease S1 analysis, a 499-nucleotide *Bgl* I–*Hpa* I fragment (SV40 nucleotides 1–499), 5′-end-labeled at the *Hpa* I site (SV40 nucleotide 499), was used as a probe. In primer-extension analysis, two oligonucleotides extending from nucleotide 460 to 520 and from nucleotide 565 to 610 were used as primers. In both assays the DNA probe was present in excess. As seen in Fig. 1*A*, the two mutants produce 1/5th to 1/15th as much SV40-specific RNA in CV-1P cells when compared to the wild-type SV40 virus. The levels of SV40-specific RNA are reduced by a factor of 2–4 in BSC-40 cells at 37°C. These relative ratios were determined by densitometric analysis of the strongest bands corresponding to the start-site at nucleotide 325. Primer-extension analysis demonstrated that in spite of the significant quantitative differences in viral RNA produced by the mutants and wild-type SV40, the initiation sites of late RNAs were qualitatively similar (Fig. 1 *B* and *C*). Thus, we conclude that the major late RNA species are reduced in monkey kidney cells (especially in CV-1P cells) infected with the carboxyl-terminal T-antigen mutants dl1066 and dl1140 at 37°C. There appears to be little or no change in the 5′ end of these RNA species.

Analysis of Late Viral Proteins Synthesized in CV-1P and BSC-40 Cells After Infection with Mutant or Wild-Type Virus. We next examined the major late SV40 proteins, in particular VP1, for its accumulation in cells infected by mutant and wild-type SV40. CV-1P and BSC-40 cells were infected with either wild-type SV40, dl1066, or dl1140 (multiplicity of infection, 25). After 48 hr, total cellular proteins were harvested and analyzed by electrophoretic immunoblot with a polyclonal anti-SV40 serum (12, 20). Fig. 2 shows that the levels of the major capsid protein, VP1, correlate closely with the relative levels of late SV40 RNA observed in Fig. 1. The levels of VP3 appear to be similarly affected, although the precise ratios are difficult to determine because this protein is present at significantly lower levels than VP1. The viral capsid polypeptide VP2 comigrates with VP1 and cannot be assayed by this procedure.

Synthesis of the Agnoprotein in CV-1P and BSC-40 Cells After Infection with Mutant or Wild-Type SV40. Another late

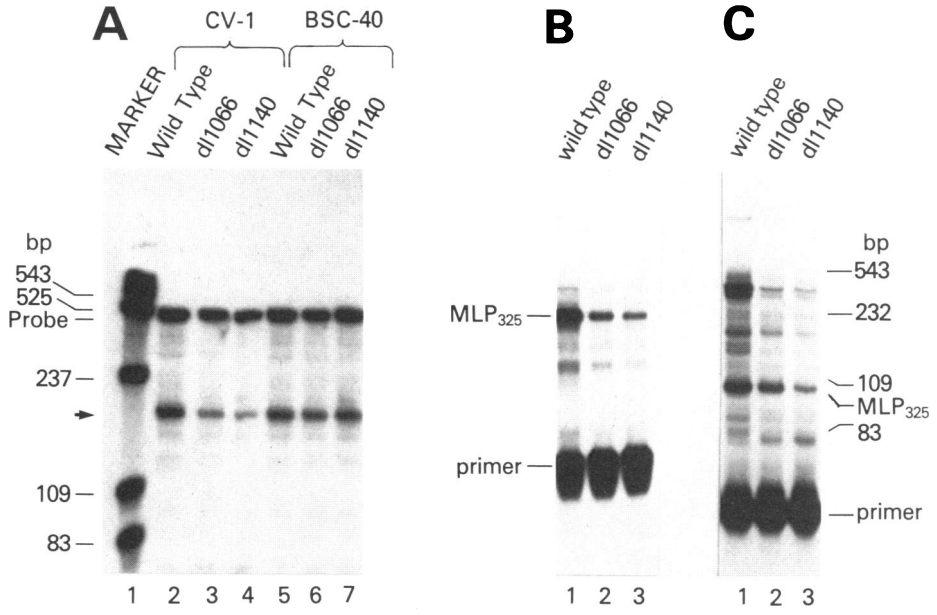

FIG. 1. Analysis of late mRNAs produced in cells infected with mutant or wild-type SV40. CV-1P and BSC-40 cells were infected with wild-type SV40 or T-antigen mutant dl1066 or dl1140 at a multiplicity of infection of 25 pfu per cell. Forty-eight hours later, total RNAs were extracted for nuclease S1 (*A*) or primer-extension (*B* and *C*) analysis. (*A*) For S1 analysis, a 499-bp *Bgl* I–*Hpa* I fragment (nucleotides 1–499) of the SV40 genome was end-labeled, hybridized to RNAs in solution, and treated with nuclease S1 for 45 min. Protected DNA fragments were resolved by electrophoresis in a 6% polyacrylamide/7 M urea gel. (*B* and *C*) For primer-extension analysis of late RNAs, oligonucleotides corresponding to SV40 nucleotides 460–520 (*B*) and 565–610 (*C*) were used as probes. After hybridization of DNA probes to RNA, the hybrids were treated with avian myeloblastosis virus reverse transcriptase for 1 hr and the products were resolved as in *A*. Lanes 2–4 in *A* and lanes 1–3 represent analysis of RNA from CV-1P cells infected with wild-type, dl1066, and dl1140 viruses, as indicated. Lanes 5–7 in *A* represent analysis of RNA from infected BSC-40 cells. End-labeled *Hin*fI fragments of SV40 DNA provided size markers. MLP_{325}, major late SV40 RNA initiation site (nucleotide 325).

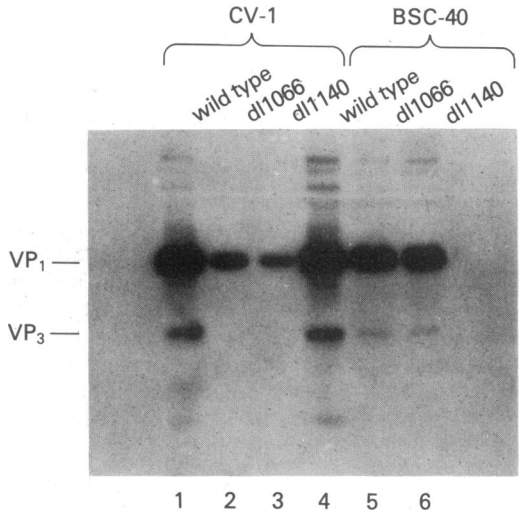

FIG. 2. Analysis of viral capsid proteins produced in mutant- and wild-type-infected CV-1P and BSC-40 cells. Approximately 10^6 cells were infected at a multiplicity of 25 pfu per cell. At 48 hr after infection, total proteins were extracted and samples containing the same amount of protein were resolved by $NaDodSO_4$/10% polyacrylamide gel electrophoresis. After transfer to nitrocellulose, the proteins were treated first with a rabbit polyclonal anti-SV40 serum and then with ^{125}I-labeled protein A and visualized by autoradiography. Lanes 1–3: proteins from CV-1P cells infected with wild type, dl1066, and dl1140, respectively. Lanes 4–6: proteins from BSC-40 cells infected with wild type, dl1066, and dl1140.

viral polypeptide, agnoprotein, is encoded by the leader of the major late 16S RNA. This protein is thought to play a role in maturation and/or encapsidation of SV40 particles (4, 5, 7, 8, 21, 22). To analyze late agnoprotein accumulation, cells were infected with mutant or wild-type SV40 virus as previously described. At 48 hr postinfection, protein extracts were prepared and examined for late agnoprotein by electrophoretic immunoblot using a polyclonal anti-agnoprotein serum (Fig. 3). In dl1066- or dl1140-infected BSC-40 cells, the level of agnoprotein was reduced by a factor of 10–15 relative to that in BSC-40 cells infected with wild-type virus (Fig. 3, lanes 4–6). The level of agnoprotein synthesis in mutant-infected CV-1P cells was less than 1/100th of that seen after wild-type SV40 infection (lanes 1–3). Thus, the synthesis of agnoprotein is more reduced relative to wild type after mutant virus infection of the nonpermissive CV-1P cell line. In addition, the decrease in agnoprotein during infections of CV-1P cells with carboxyl-terminal T-antigen mutants (factor of 100) is greater than the decrease of VP1 synthesis in these same cells (factor of 15; cf. Figs. 2 and 3).

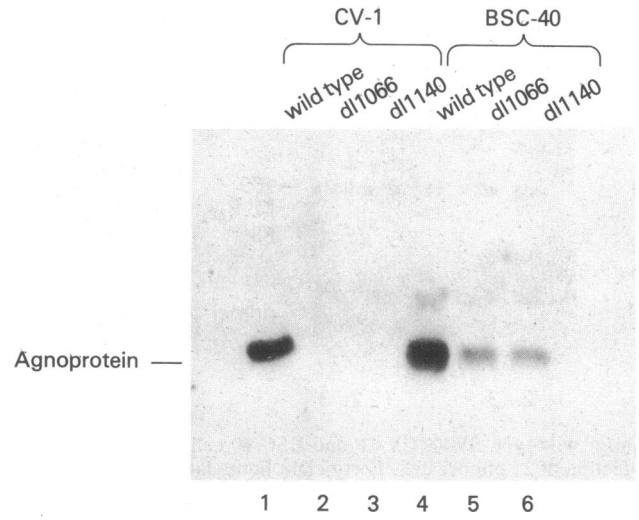

FIG. 3. Synthesis of agnoprotein. CV-1P or BSC-40 cells were infected with wild-type, dl1066, or dl1140 virus. Cells were harvested 48 hr after infection and proteins were analyzed by $NaDodSO_4$/15% polyacrylamide gel electrophoresis followed by electrophoretic immunoblot analysis using rabbit anti-agnoprotein antibody.

Localization of VP1 in CV-1P Cells Infected with Wild-Type or Carboxyl-Terminal Mutant Virus. It was recently reported that SV40 agnoprotein may affect the nuclear localization of structural protein VP1 (6, 21). It was of interest, therefore, to determine whether major capsid protein VP1 localizes to the nucleus. In this experiment, CV-1P cells were infected at a multiplicity of 1 pfu per cell and analyzed 48 hr later by immunofluorescence assays with a rabbit anti-SV40 antibody. In this analysis, we also examined mutant pm1493, in which the ATG translational start site at nucleotide 335 in wild-type SV40 has been changed to TTG. Mutant pm1493 does not produce agnoprotein in infected CV-1P cells (see Fig. 5), and most of the VP1 made by this mutant, unlike that of wild-type SV40, remains in the cytoplasm of infected cells (Fig. 4). Similarly, in dl1066- or dl1140-infected CV-1P cells,

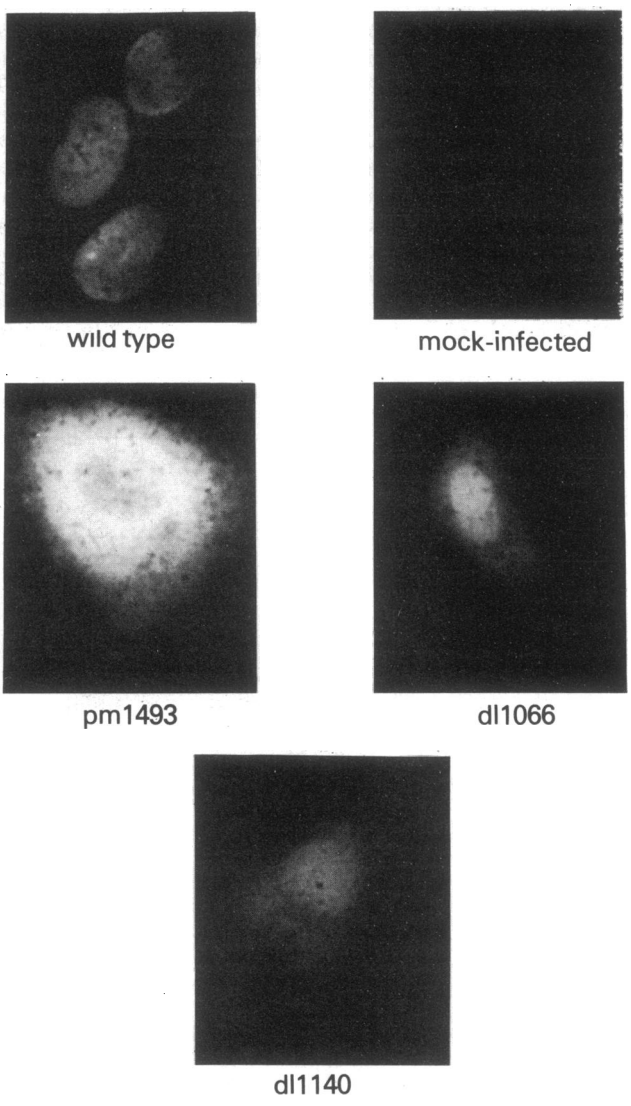

FIG. 4. Visualization of VP1 in infected CV-1P cells by indirect immunofluorescence. CV-1P cells were infected with either wild-type SV40, dl1066, dl1140, or pm1493 at a multiplicity of 1.0 pfu per cell. Infected cells were harvested at 48 hr and prepared for indirect immunofluorescence with rabbit anti-SV40 antibody as described (6, 9).

most of the newly synthesized VP1 capsid protein remains in the cytoplasm. Thus, in these mutants a block in transport of VP1 to the nucleus may contribute to the low yield of infectious virus particles in CV-1P cells. It is of interest that agnoprotein mutants, such as pm1493, grow on CV-1P cells but carboxyl-terminal T-antigen mutants do not.

Complementation of Carboxyl-Terminal T-Antigen Mutants with Agnoprotein Mutants pm1493 and dl121. To demonstrate that a functional coding sequence for SV40 agnoprotein was present in the carboxyl-terminal T-antigen mutants, we coinfected pairs of mutants containing nonoverlapping mutations and monitored late agnoprotein synthesis in infected cells. The T-antigen mutants, dl1140 and dl1066, were coinfected with two agnoprotein mutants, pm1493 and dl121, separately. Mutant dl121 has a 52-bp deletion between nucleotides 362 and 413 and is incapable of producing agnoprotein. At 48 hr postinfection, cells were harvested and proteins were analyzed for agnoprotein and viral capsid protein VP1 by immunoblot analysis. Consistent with the results presented above, in the single infections of CV-1P cells, none of the mutants produced agnoprotein (Fig. 5, lanes 1–4). In contrast, parallel analysis of coinfection of CV-1P cells with a carboxyl-terminal T-antigen mutant and agnoprotein mutant pm1493 showed a significant production of agnoprotein in the infected cells (lanes 8 and 9). Similar results were obtained when agnoprotein mutant dl121 was coinfected with either of the carboxyl-terminal T-antigen mutants (lanes 6 and 7). Furthermore, the production of agnoprotein facilitates cytoplasmic/nuclear transport of VP1 in coinfected cells (data not shown). Production of agnoprotein in the infected cells demonstrates that the two carboxyl-terminal mutants have the potential to express an agnoprotein in the presence of intact T antigen. These experiments suggest that the carboxyl-terminal region of T antigen may contribute to the translation or stability of agnoprotein.

DISCUSSION

Our studies indicate that a reduction in late viral RNA and a concomitant reduction in synthesis of the major capsid protein, VP1, may be partly responsible for the lower titers produced by infection of dl1066 and dl1140 in CV-1P cells at 37°C. While viral DNA synthesis is only marginally affected by these mutations, the synthesis of late RNA and capsid protein is 1/5th to 1/15th that of wild-type SV40. Nuclease S1 and primer-extension analyses demonstrate no major differences in the structure of late viral RNAs, although a number of minor species may not be appropriately evaluated by either technique. The reduction in late RNA synthesis of dl1066 and dl1140 mutants suggests that the carboxyl-terminal domain of T antigen, aside from its role in DNA replication, may play a role in the stabilization of late RNAs or in activation of the late transcription unit during the late phase of the lytic cycle. It is not clear whether the reduction in levels of the major late structural protein, VP1, can completely account for a reduction in viral titer of approximately 3 orders of magnitude (14). In accord with our observations, Tornow and Cole (23) have demonstrated that mutant dlA2459, which lacks 14 bp of the carboxyl-terminal coding sequence of T antigen (nucleotides 2785–2798), is unable to produce a viable virus in CV-1P cells. In this mutant, viral DNA replication, late RNA transcription, and processing of late RNAs appear normal.

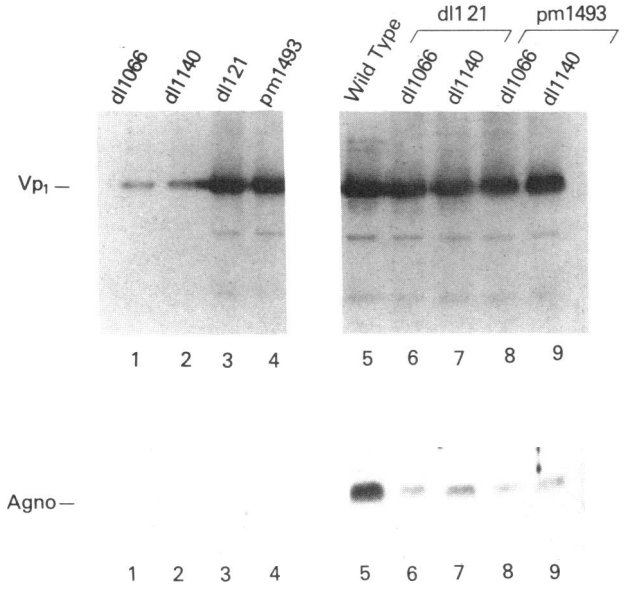

FIG. 5. Analysis of viral proteins in CV-1P cells after coinfections with carboxyl-terminal T-antigen and agnoprotein mutants. CV-1P cells were singly (lanes 1–4) or doubly (lanes 6–9) infected with mutant viruses, as indicated, at a multiplicity of 25 pfu per cell. Total proteins were isolated 48 hr postinfection and analyzed by an electrophoretic immunoblot procedure using rabbit polyclonal anti-SV40 serum or rabbit anti-agnoprotein antibody as described (12).

Our results also demonstrate that one of the viral proteins, agnoprotein, is not produced in CV-1P cells infected with dl1066 or dl1140. Agnoprotein apparently mediates the efficient localization of the major capsid protein, VP1, to the nucleus and further facilitates cell-to-cell spread of virus (6, 21). The data presented in Fig. 4 demonstrate inefficient localization of VP1 to the nuclei in CV-1P cells infected with either dl1066 or dl1140. Thus, the absence of agnoprotein (at least at the detectable level) in cells infected with dl1066 or dl1140 may delay nuclear localization of VP1 and assembly of viral particles. The mechanism by which agnoprotein induces cytoplasmic/nuclear transport of VP1 remains unclear, although there is ample evidence indicating an interaction of these two viral proteins (4, 22). It is also possible that agnoprotein mediates an interaction that prevents VP1 from leaking out of the nucleus once it is transported.

Our data further suggest that the carboxyl-terminal domain of T antigen may play a role in agnoprotein translation, induction of cellular mRNA whose product impacts on agnoprotein translation, or stabilization of agnoprotein. Interestingly, a role for T antigen in posttranscriptional control has been proposed in other systems (24, 25). Several laboratories have demonstrated that adenoviruses cannot grow productively in monkey cells (1). Coinfection of cells with adenovirus and SV40 can complement the defect for adenovirus production in CV-1 cells (25). In addition, there is strong genetic evidence suggesting that the carboxyl-terminal domain of T antigen participates in productive infection of CV-1 cells with adenovirus (23, 26). It is interesting that the block predominantly occurs at the level of translation of adenovirus late RNAs (24). These observations are reminiscent of our findings on the role of the carboxyl-terminal domain of SV40 T antigen on translation of the first open reading frame of SV40 16S RNA. At present, we do not understand the molecular basis for the role T antigen might play in translation of adenovirus and SV40 late RNAs.

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