Mutants Deleted in the Agnogene of Simian Virus 40 Define a New Complementation Group

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Analysis of the DNA sequence of the late leader region of simian virus 40 indicates that it might encode a 61-amino acid, highly basic protein, LP-1. Mutants deleted in this region are viable, but they produce infectious progeny more slowly than wild-type virus in established monkey cells. On the basis of the rates of appearance and the sizes of mixed plaques formed after cotransfections with pairs of mutants, we found that mutants defective in the synthesis of LP-1 complemented mutants in all known complementation groups of simian virus 40. Complementation was also observed in infections with virions and was bidirectional. Therefore, these mutants define a new complementation group, group G. In addition, a protein of the appropriate molecular weight for LP-1 (approximately 8×10^3) was synthesized by wild-type virus-infected cells but not by mock-infected or group G gene mutant-infected cells. This protein, whose identity has been established definitively by Jay et al. (*Nature (London)* 291:346-349, 1981), was synthesized at a high rate at late times after infection, was present predominantly in the cytoplasmic fraction of cells, possessed a fairly short half-life, and was absent from mature virions. Once formed, virions of group G gene mutants behaved biologically and physically like virions of wild-type virus. On the basis of these findings and other known properties of LP-1 and mutants defective in LP-1 synthesis, we hypothesize that LP-1 functions to facilitate virion assembly, possibly by serving as a nonreusable scaffolding protein.

The late leader region of the simian virus 40 (SV40) genome encodes several interesting functions. These include (i) the promoters for early-strand RNA synthesis (2, 7a, 8, 11; L. A. Trimble and J. E. Mertz, unpublished data) and late-strand RNA synthesis (7a; J. E. Mertz, L. A. Trimble, T. J. Miller, and G. Z. Hertz, manuscript in preparation), (ii) the 5' ends of the late-strand mRNAs (39), and (iii) the signals involved in splicing the 5'-terminal leader regions onto the "bodies" (i.e., the main protein-encoding regions) of the late mRNAs (39). In addition, Dhar et al. (7) noted that the late leader region also contains an "agnogene" that might encode a 61-amino acid, highly basic protein (see Fig. 1).

The first reports of mutants defective in the late leader region of the SV40 genome were by Mertz and Berg (25), Carbon et al. (4), and Shenk et al. (31). More recently, numerous additional mutants with deletions spanning various segments of this region have been isolated in several laboratories (7a; 11, 12, 16, 34; Trimble and Mertz, unpublished data). Surprisingly, all

of these mutants are viable unless they lack sequences essential for viral RNA synthesis or viral DNA replication. These viable mutants are all somewhat defective in the rate of production of infectious virions in established lines of African green monkey kidney (AGMK) cells, as indicated by both (i) the delayed appearance and smaller sizes of the plaques which they produce (12, 25, 34) (see Table 1) and (ii) lower rates of production of PFU during single cycles of growth (1, 31). The severity of the defectiveness varies considerably from mutant to mutant (25) and does not correlate in a clearly discernible manner with either the size or position of the deletion (1). In addition, since the kinetics and rates of synthesis of viral DNA in single cycles of growth are similar to those observed in wild-type virus-infected cells (1), the defect(s) in these mutants occurs in the late part of the lytic cycle.

There have been several reports concerning the effects of a variety of mutations in the late leader region on the structures of the viral mRNAs synthesized in infected monkey cells (10, 10a, 12, 28, 40). The main conclusion of these studies has been that both the 5' ends and the patterns of splicing of the late mRNAs are

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altered in complex ways. Unfortunately, no clearly discernible pattern has yet emerged from the mass of data. However, a working model consistent with these results has been proposed recently (10a, 23).

The data presented here are concerned with the existence and possible function(s) of the 61-amino acid protein encoded by the late leader region of SV40. Using a modification of a previously developed complementation test (24; J. E. Mertz, Ph.D. thesis, Stanford University, Stanford, Calif., 1975), we found that mutants deleted in this region define a new complementation group, group G. The polypeptide that may be responsible for the observed complementation behavior of these mutants was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of appropriately radiolabeled proteins obtained from monkey cells infected with wild-type virus and leader region mutants of SV40. Based upon the known properties of the 61-amino acid protein and mutants defective in its synthesis, we propose that this protein may function to facilitate virion assembly, possibly by serving as a nonreusable scaffolding protein.

(Preliminary accounts of the genetic and protein aspects of this work were presented at the Tumor Virus Meetings on SV40, Polyoma, and Adenoviruses held at Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., during the summers of 1978 and 1980. Subsequently, we learned that Jay et al. [16], Jackson and Chalkley [15], P. Southern and P. Berg [personal communication], and R. Margolskee and D. Nathans [personal communication] have also independently identified this virus-encoded protein in SV40-infected monkey cells.)

MATERIALS AND METHODS

Cell lines. CV-1P, MA-134, and CV-1 are established lines of AGMK cells. These cell lines were grown as described previously (24) in medium supplemented with 5% fetal bovine serum. Primary AGMK cells were purchased from Flow Laboratories and were grown in medium containing 10% fetal bovine serum.

Viruses and viral DNAs. Wild-type strain 776 was obtained from S. Weissman. WT800 is a plaque-purified derivative of SV40 strain Rh-911 (24).

The late leader region deletion mutants *dl*-802, *dl*-805, *dl*G806, *dl*-809, and *dl*G810 are naturally arising mutants of WT800. The selection, propagation, growth characteristics, and sequences of these mutants have been described previously (1, 25). Altered restriction mutant *ar*1077, which has a single base change at nucleotide residue 348, was generously provided by D. Nathans. The isolation and growth characteristics of this mutant have been described previously (32). SV-L, which was obtained from H. Ozer, is a temperature-sensitive, host range, large-plaque variant of SV40 (27, 35).

The temperature-sensitive mutants *ts*A58 (38) and

*ts*B201, *ts*C219, and *ts*D202 (5) were provided by P. Tegtmeier and R. G. Martin, respectively. Mutant *dl*E1226 (6) was obtained from C. Cole.

Virus stocks of each of these mutants were prepared and titrated as described previously (24). Unless indicated otherwise, viral DNA was isolated directly from infected cells by the procedure of Hirt (14). The supercoiled DNA remaining in the supernatant was purified by two cycles of equilibrium centrifugation in CsCl-ethidium bromide gradients (29). After removal of ethidium bromide by repeated extraction with isopropanol, the viral DNA was precipitated with NaCl and ethanol and suspended in 10 mM Tris (pH 7.6)–1 mM EDTA–10 mM NaCl.

Enzymes. Restriction enzymes were obtained from commercial sources and were used as suggested by the manufacturers.

Complementation tests with viral DNAs. Solutions of Tris-buffered saline (TBS) (17) (0.2 ml/60-mm dish) containing varying dilutions of DNA of the mutant being tested, 4 ng of DNA from a known complementation group (= helper), and 100 μ g of DEAE-dextran were used to transfect freshly confluent monolayers of CV-1P cells as previously described (24). After transfection, the cell monolayers were overlaid with agar medium (24) containing 4% fetal bovine serum and incubated at 40.5°C, and 3 days later the cells were fed with an additional overlay of agar medium supplemented with 1% fetal bovine serum. At 6 days after infection, agar medium containing 0.01% neutral red was added, and the dishes were transferred to 39.5°C for subsequent incubation. Each day thereafter, the plaques visible to the unaided eye were counted, and their diameters were measured. Two mutants were presumed to complement when the plaques observed in the mixed infection appeared sooner and were larger than the plaques observed when cells were infected with either mutant by itself.

Complementation tests with virions. When a conditionally lethal mutant could serve as the lawn of potentially complementing helper virus, tests were performed essentially as described previously (24; Mertz, Ph.D. thesis). Briefly, freshly confluent monolayers of CV-1P cells (1×10^6 to 2×10^6 cells per 60-mm dish) were coinfecting with 0.1-ml portions of serial dilutions of the mutant being tested and 0.1-ml portions of the mutant of known complementation properties (2×10^5 to 1×10^6 PFU/ml). After incubation for 2 h at 37°C with occasional rocking of the dishes, the cells were overlaid with agar medium and treated subsequently as described above.

When both mutants were viable although poorly growing (e.g., *dl*G806 and *dl*G810), complementation tests with virions were performed essentially as described by Brockman and Nathans (3). Briefly, CV-1P cells were infected at a multiplicity of infection of approximately 8 PFU of each mutant per cell and incubated at 37°C in medium containing 2% fetal bovine serum. The next day, the infected cells were removed from the dish with trypsin, serially diluted in liquid medium containing 10% fetal bovine serum, and plated onto new dishes. After incubation at 37°C for 4 h to enable the cells to attach to the dish, uninfected CV-1P cells (5×10^5 cells per 60-mm dish) were added to create a monolayer of cells. Incubation was continued in liquid medium at 37°C for 1 additional day. The monolayers of cells were then washed once with TBS,

overlaid with agar medium, and treated subsequently as described above.

Agarose gel electrophoresis. DNA samples were electrophoresed at room temperature in horizontal gels (thickness, 1 to 2 mm) of 1.0 to 1.5% agarose in 4× TA buffer (0.16 M Tris-acetate, pH 8.3, 0.08 M sodium acetate, 8 mM EDTA) at 1.5 to 2 V/cm until the bromophenol blue dye had migrated 3 to 6 cm. The gels were stained by incubation for 20 to 40 min in 4× TA buffer containing 0.5 µg of ethidium bromide per ml, destained by incubation for 15 to 30 min in water, and photographed by using Tri-X or Polaroid type 57 film, an orange filter, and a short-wavelength UV light box.

Isolation and purification of [³H]lysine-labeled SV40 virions. MA-134 cells were infected with 20 PFU of WT800 virus per cell and incubated at 37°C in medium supplemented with 2% fetal bovine serum. After 34 h the medium in each 100-mm dish was replaced with 5 ml of medium containing one-fifth the normal concentration of lysine, 2% dialyzed fetal bovine serum, and 0.1 mCi of L-[4,5-³H]lysine (40 Ci/mmol) per ml; 12 h later, 5 ml of complete medium containing 2% fetal bovine serum was added to the medium already present in each dish. Incubation was continued at 37°C. The cells were harvested 118 h after infection by scraping them off the dishes into TBS with a rubber policeman. The cells were disrupted by three cycles of freezing and thawing, extraction with chloroform, and sonication. After centrifugation at 5,000 rpm for 10 min at 4°C, the resulting supernatant was collected and made 0.75% Nonidet P-40. SV40 virions were purified from this extract by sedimentation (SW41 rotor, 35,000 rpm, 4 h, 15°C) through 1.5 ml of 15% (wt/vol) sucrose into 3 ml of CsCl (density, 1.33 g/cm³) in TBS

lacking calcium and magnesium (26), followed by centrifugation (SW50.1 rotor, 30,000 rpm, 40 h, 4°C) to equilibrium in a density gradient of CsCl (average density, 1.33 g/cm³). After 100 µg of bovine serum albumin was added, the purified virions were dialyzed against 10 mM NaPO₄ (pH 7.2)–0.1 M NaCl–0.1 mM EDTA and stored at 4°C.

Determination of ratios of virion particles to PFU. SV40 virions were purified as described above from mutant-infected and wild-type virus-infected cells. After CsCl equilibrium density gradient centrifugation, each band of purified virions was collected separately. A portion of each purified virion preparation was diluted 100-fold into TBS containing 2% fetal bovine serum and was stored frozen until it was titrated to determine the number of PFU per milliliter as described previously (10). The number of virion particles per milliliter was determined by spectroscopy, assuming 1 U of absorbance at 260 nm of SV40 virions was equivalent to 6.5 × 10¹² physical particles per ml (41).

RESULTS

Biological and physical properties of late leader region mutants. Some of the biological and physical properties of late leader region mutants *dl*-801 through *dl*-810 have been described previously (1, 10, 10a, 25; Mertz, Ph.D. thesis). In this paper we describe experiments performed with these mutants that were directed specifically toward trying to define the function(s) of the agnogene.

Figure 1 shows the precise map location of the 61-amino acid protein that could be encoded by

FIG. 1. Nucleotide sequence of the late leader region of the SV40 genome. Nucleotide residues are numbered according to the system of Tooze (39), starting from the center of the palindrome in the origin of viral DNA replication. The arrows indicate the locations of the 5' ends of prominent species of late mRNAs, 80 to 90% of which map to residue 325 (9). The brackets indicate the locations of donor and acceptor splice sites used in the production of the 16S RNAs and the three spliced classes of 19S RNAs (9). met and term indicate the locations of triplet codons that may function as initiators and in-phase terminators of translation. LP-1 and VP-2 indicate the AUG codons used in the synthesis of the 61-amino acid product of the agnogene and VP-2, respectively.

TABLE 1. Summary of physical, biological, and genetic properties of late leader region mutants

Mutant ^a	Size of deletion (nucleotides)	Nucleotide residues deleted ^b	Mean plaque diam (mm) 11 days after infection of:		Induction of synthesis of LP-1 ^c
			CV-1P cells	Primary AGMK cells	
Wild-type strain 776			2.5	ND ^d	+ ^e
WT800	8 (21) ^f	179–186 (251'–271') ^g	2.4	1.8	+ ^h
<i>dl</i> -802	80	344–423 ⁱ	0.9	1.8	ND
<i>dl</i> -805	187	331–517 ⁱ	1.0	2.1	ND
<i>dl</i> G806	138	222–359 ⁱ	0.1	0.8	ND
<i>dl</i> -809	170	299–468 ⁱ	1.2	1.6	– ^h
<i>dl</i> G810	175	297–471 ⁱ	0.4	1.1	– ^h
<i>ar</i> 1077		348 (G→A) ^j	ND	ND	+ ^h

^a Strain 776 and WT800 are naturally occurring wild-type strains of SV40. G indicates that the mutant belongs to complementation group G; a dash indicates that the complementation group has not been determined yet.

^b The numbering system used here is the system shown in Fig. 1 for wild-type strain 776. Therefore, WT800 is indicated as having a substitution even though it differs only in the precise region of the genome that is repeated in tandem. Where there is ambiguity in the precise endpoints of a deletion due to a repeated sequence at its ends, the largest possible residue numbers are given.

^c Determined as described in the legend to Fig. 4. +, Presence; –, absence.

^d ND, Not determined.

^e Data from references 15 and 16.

^f The number in parentheses indicates the size of an insertion.

^g Trimble and Mertz, unpublished data. The residue numbers in parentheses indicate the second copy of the sequence that is present in place of the deleted nucleotide residues.

^h Data from this paper.

ⁱ These mutants were derived from WT800; consequently, although not indicated, they all have the substitution relative to wild-type strain 776 of residues 251' to 271' in place of residues 179 to 186, as well as the deletions shown (1).

^j Mutant *ar*1077 contains a single base pair change of G·C to A·T at nucleotide residue 348 (32).

the late leader region of the SV40 genome. Based upon the nucleotide sequences of their genomes (1) (Table 1), mutants *dl*-801 through *dl*-810 should all be defective in the synthesis of this protein; whereas *dl*-802 contains a frame-shift mutation that should result in premature termination of translation, the other mutants all lack the translation initiation codon that begins at nucleotide residue 335. Therefore, some of these mutants were selected for further study.

The proposed product of the agnogene is a highly basic protein containing seven lysine residues and eight arginine residues (Fig. 1). Consequently, this histone-like protein might be expected to function in association with SV40 minichromosomes or as a component of virions.

SV40 virions containing mutant proteins frequently exhibit altered physical or biological properties (39). For example, incubation of mutant SV-L virions at 50°C for 2 h in TBS containing serum reduces their infectivity 10⁴-fold (36). However, when we treated virions of WT800 and mutants *dl*-801 through *dl*-810 in an analogous manner, they were inactivated less than fivefold (data not shown). Similarly, mutants in the minor capsid proteins VP-2 and VP-3 produce virions with very low specific infectivities because they are defective in adsorption and

uncoating, respectively (39). On the other hand, the ratios of particles to PFU for *dl*-806 and *dl*-810 virions were within threefold of the ratio observed with WT800 virions (data not shown). Therefore, we conclude that the virions produced by these late leader region mutants are like the virions of wild-type virus with respect to physical integrity and adsorption, penetration, and uncoating.

Some mutants of SV40 exhibit phenotypes that vary with the host cell type. For example, mutant SV-L forms plaques in primary AGMK cells 500-fold more efficiently than in established AGMK cells (37). Therefore, we also compared the abilities of wild-type virus and mutants *dl*-801 through *dl*-810 to form plaques on freshly confluent monolayers of CV-1P and primary AGMK cells that were infected in parallel with appropriately diluted virus stocks and incubated at 37°C in agar medium. As expected, the leader region mutants produced plaques in CV-1P cells that were smaller than the plaques made by wild-type virus (Table 1), but at efficiencies that were equal to those observed in primary AGMK cells (data not shown). Surprising, therefore, was the finding that all mutants except *dl*-806 and *dl*-810, the two poorest growing mutants, formed wild-type-sized plaques in primary AGMK cells (Ta-

TABLE 2. Rates of appearance of plaques in mixed infections^a

Infecting DNAs		% of maximum no. of plaques on the following days after infection:				
Mutant	Helper	7	8	9	10	11
<i>dl</i> -810	None	3	10	29	69	100
<i>dl</i> -810	<i>tsA</i> 58	67	96	100	100	
<i>dl</i> -810	<i>tsB</i> 201	54	92	100	100	
<i>dl</i> -810	<i>tsC</i> 219	65	99	100	100	
<i>dl</i> -810	<i>tsD</i> 202	60	98	100		
<i>dl</i> -810	<i>dIE</i> 1226	69	100	100		
<i>tsB</i> 201	<i>tsA</i> 58	60	96	99	100	
<i>tsB</i> 201	<i>tsD</i> 202	86	100	100		
<i>tsB</i> 201	<i>dIE</i> 1226	93	100	100		

^a CV-1P cells in 60-mm dishes were cotransfected with 0.04 to 0.2 ng of mutant DNA and 4 ng of helper DNA in 0.2 ml of TBS containing 500 μ g of DEAE-dextran per ml and treated subsequently as described in the text. The values presented are the averages from duplicate sets of infected cell monolayers, each of which contained 30 to 90 plaques by 11 days after infection.

ble 1). This result indicates that, although virions of these mutants are indistinguishable from those of wild type with respect to adsorption, penetration, and uncoating, these mutants are nevertheless defective in at least one function that is not important for efficient growth in primary AGMK cells. The finding that mutants *dl*-806 and *dl*-810 form, even in primary AGMK cells, somewhat smaller plaques than wild type does probably indicate that these two mutants possess one or more additional defects that are not compensated for in either primary AGMK or CV-1P cells.

Mutants *dl*-806 and *dl*-810 define a new complementation group of SV40, group G. If the agnogene encodes a protein that is synthesized in SV40-infected cells, these late leader region mutants may be defective in a function that can

be supplied in *trans* by a coinfecting helper virus. To test this hypothesis, complementation tests were performed by using a modification of a mixed-plaque assay that has been described previously (24; Mertz, Ph.D. thesis). Monolayers of CV-1P cells were cotransfected with serial dilutions of DNA of a late leader region mutant and 4 ng of DNA of a mutant from each of the known complementation groups (= helper). The cells were incubated at 40.5°C, a temperature that is nonpermissive for the growth of the temperature-sensitive helpers. In addition, although mutants *dl*-806, *dl*-810, and *dIE*1226 are viable, on average their plaques appear later and grow much more slowly than the plaques of wild type (Table 1) (6). Consequently, most of the plaques that are visible within 8 days after infection are likely to be mixed plaques resulting from two complementing mutants.

Tables 2 and 3 show the type of data obtained from one experiment of this kind. When cells were transfected with mutant *dl*-810 DNA by itself, only 10% of the plaques were visible with an unaided eye within 8 days. On the other hand, more than 90% of the plaques were visible by this time when DNA from complementation groups A, B, C, D, or E was included. Complementation with mutants defective in small t antigen was not tested because these group F mutants make plaques that are only slightly smaller than those of wild type. Nevertheless, we assume that late leader region mutants can also be complemented by group F mutants since the latter are defective in a function expressed early rather than late in the lytic cycle (39).

The total numbers of plaques that eventually appeared with and without helper DNA were similar (data not shown). This result was expected since 4 ng of helper DNA per 60-mm dish of cells is enough to infect all of the approximately 2% of the cells that are susceptible to transfection by the DEAE-dextran technique which we

TABLE 3. Rates of growth of plaques resulting from mixed infections^a

Infecting DNAs		Mean sizes (mm) of visible plaques on the following days after infection:			
Mutant	Helper	8	9	10	11
<i>dl</i> -810	None	0.3 (0.1–0.7) ^b	0.3 (0.1–0.7)	0.5 (0.1–1.8)	0.6 (0.1–1.5)
<i>dl</i> -810	<i>tsA</i> 58	1.0 (0.2–2.0)	1.6 (0.4–2.7)	1.8 (1.2–2.8)	
<i>dl</i> -810	<i>tsB</i> 201	0.8 (0.3–1.6)	1.4 (0.4–2.8)	1.9 (1.1–3.0)	
<i>dl</i> -810	<i>tsC</i> 219	0.6 (0.2–1.2)	1.3 (0.5–2.2)	1.5 (1.0–2.2)	
<i>dl</i> -810	<i>tsD</i> 202	1.0 (0.2–1.5)	1.6 (0.5–3.2)		
<i>dl</i> -810	<i>dIE</i> 1226	0.8 (0.2–1.7)	1.3 (0.6–2.1)		
<i>tsB</i> 201	<i>tsA</i> 58	1.1 (0.3–2.2)	2.0 (1.3–3.0)	2.4 (1.8–3.5)	
<i>tsB</i> 201	<i>tsD</i> 202	1.2 (0.3–2.0)	1.4 (0.8–2.3)		
<i>tsB</i> 201	<i>dIE</i> 1226	1.9 (0.8–3.0)			

^a The values presented were obtained from the same set of infections described in Table 2, footnote a; each number is the average of the diameters of 6 to 12 plaques selected at random for measurement.

^b The numbers in parentheses are ranges.

used (24). Consequently, few, if any, of the plaques observed in the cotransfections should have arisen from cells receiving only *dl*-810. This expectation was confirmed by the finding that, except for the data at 8 days, the ranges of plaque sizes observed in the cotransfections were always outside the mean plaque sizes observed in the absence of helper (Table 3). Even the one apparent exception actually followed this rule since, given that only 10% of the *dl*-810 plaques were visible at 8 days (Table 2), the real, as opposed to observed, mean plaque size in the absence of helper was less than 0.1 mm. Therefore, as measured both by the rates of appearance and by the sizes of the plaques, mutant *dl*-810 complemented mutants in all of the other known complementation groups of SV40.

Several types of experiments were performed to confirm that these results were interpreted correctly and that the apparent complementation did not have trivial, uninteresting explanations. First, complementation tests were performed in parallel with *tsB201*. The resulting data (Tables 2 and 3) were very similar to the data obtained with *dl*-810, although the means and ranges of plaque sizes may have been slightly larger with *tsB201*. Therefore, we conclude that *dl*-810 complements the mutants in other complementation groups almost as well as or equally as well as *tsB201*, which is known to complement other mutants excellently as determined by a variety of complementation assays, including the classical ones (39).

Second, data similar to the data obtained with *dl*-810 were also obtained in complementation tests performed in an analogous fashion with mutant *dl*-806 (data not shown). The one apparent difference observed in these two sets of experiments was that whereas the helpers increased the rates of appearance and sizes of *dl*-810 plaques by 3 to 4 days (Tables 2 and 3), they increased those of *dl*-806 by only 2 to 3 days. This latter finding indicates that the helpers compensated for only some of the defects of *dl*-806.

Third, complementation tests between *dl*-806 and *dl*-810 were also performed. However, because these mutants are viable, the monolayer of cells would have been destroyed by transfection with 4 ng of DNA of either one of them per 60-mm dish. Consequently, the tests were performed with virions by coinfecting cells at a multiplicity of infection of approximately 8 PFU of each mutant per cell and then diluting and replating the infected cells with fresh monolayers of uninfected cells. As a control, mixed infections were also done with stocks of *dl*-810 and *tsA58* virus. Coinfection with *dl*-810 and *tsA58* virus indicated that mixed plaques appeared and grew at rates similar to the rates

observed in the DNA transfections performed with the same mutants (data not shown). Nevertheless, the plaques from the coinfection with *dl*-806 and *dl*-810 were similar to those observed when cells were infected with either mutant by itself. Therefore, we conclude that (i) mutants *dl*-806 and *dl*-810 are defective in the same *trans*-complementable function, and (ii) as opposed to what is observed with group D mutants (39), these late leader region mutants complement as efficiently when they are packaged into virions as when they are in the form of purified DNA, thus confirming the finding that they are not defective in adsorption, penetration, or uncoating.

Theoretically, the plaques observed in the experiments described above could have been produced by (i) wild-type recombinants that arose in the mixedly infected cells or (ii) unidirectional complementation in which the late leader region mutants, being viable, supplied the helpers with all of the viral products needed for efficient multiplication, but the helpers were unable to improve the slow rate of growth of the viable leader mutants. If either of these possibilities had actually occurred, we would have expected the virus that grew out of the cotransfections to contain either (i) at least some wild-type SV40 genome or (ii) only a small percentage of leader mutant genomes.

To rule out these possibilities, plaques observed in experiments similar to those summarized in Tables 2 and 3 were picked and used to produce small stocks of viral DNA. A portion of each of these DNA stocks was incubated with *Hpa*II only or *Hpa*II plus *Hae*II and then electrophoresed in agarose gels. Since our late leader region mutants are resistant to cleavage by *Hpa*II (25), whereas *dIE1226* is resistant to cleavage by *Hae*II (6), these reactions enabled us to distinguish among leader mutant, *dIE1226*, and wild-type genomes.

The data obtained from one experiment of this type are shown in Fig. 2. Since these DNA stocks were grown from the small amounts of virus present in single plaques, any wild-type recombinants produced in the initial infections should have had sufficient opportunity to outreplicate the mutants and become the predominant species. Nevertheless, no wild-type DNA was detected in the DNAs grown from plaques originating from the cotransfections. Instead, these DNA stocks consisted of approximately equimolar amounts of the two mutants. Similar results were also obtained with plaques produced from cotransfections with *dl*-810 and *dIE1226* (data not shown). Therefore, we conclude that the plaques observed in these complementation tests were indeed mixed plaques.

To eliminate definitively the possibility that

FIG. 2. Analysis of the viral genomes present in DNA stocks grown from mixed plaques. Plaques obtained in experiments similar to those described in Tables 2 and 3 were picked with a Pasteur pipette and placed in TBS containing 2% fetal bovine serum. The virus was released from the infected cells present in each plaque by three cycles of freezing and thawing and was used to infect confluent monolayers of CV-1 cells at a multiplicity of infection of 10^{-2} to 10^{-3} PFU/cell. The cells were then incubated at 37°C in medium supplemented with 2% fetal bovine serum. When more than 50% of the cells exhibited cytopathic effect, viral DNA was harvested by the procedure of Hirt (14) and purified as described in the text. All of the DNA samples were incubated with restriction endonuclease *HpaII*. One-half of each sample was also incubated with restriction endonuclease *HaeII* (lanes 2, 4, 6, 8, and 10). The patterns of digestion were determined by electrophoresis in a 1.0% agarose gel. I, II, and L indicate the positions of supercoiled, nicked circular, and unit length linear SV40 DNAs, respectively; 0.91 and 0.09 mark the positions of the DNA fragments that resulted from digestion of wild-type DNA with both *HpaII* and *HaeII*. Lanes 1 and 2 and lanes 3 and 4, DNA samples from plaques picked from cells infected as controls with *dlG806* and WT800, respectively; lanes 5 and 6 and lanes 7 to 10, DNA samples from plaques obtained from mixed infections with *dlE1226* plus *tsA30* and *dlE1226* plus *dlG806*, respectively.

complementation was unidirectional, we also looked for changes either intracellularly or in virions in the relative molar ratios of coinfecting viruses during single cycles of growth. Figure 3 shows that in cells mixedly infected with *dl-806* and wild type (i) the percentage of the intracellular viral DNA that was mutant remained constant during the course of the infection and (ii) the *dl-806* genomes were packaged into virions as efficiently as the wild-type genomes were. The first of these conclusions was confirmed by a Southern blot analysis (33) of the relative molar ratios of the two genomes at earlier times after infection; we found (data not shown) that even at 21 h the percentage of the intracellular viral DNA that was *dl-806* was approximately

70%. Results similar to these were also obtained in experiments involving cells coinfecting with *dl-810* and wild-type virus (data not shown). Therefore, we conclude that these late leader region mutants (i) do not possess a *cis*-acting defect in either viral DNA replication or virion assembly and (ii) truly benefit from the presence of a complementing helper.

In summary, the findings described above clearly indicate that mutants *dl-806* and *dl-810* define a new complementation group of SV40. We propose that this new complementation group be named group G.

Synthesis of the agnogene protein in monkey cells infected with wild type and late leader region

FIG. 3. Analysis of the viral genomes present intracellularly (cell) and in virions (viral) at different times after coinfection with *dlG806* and WT800. CV-1P cells were mixedly infected with approximately 10 PFU each of *dlG806* and WT800 per cell and incubated at 37°C. At different times thereafter, batches of infected cells were harvested both by the method of Hirt (14) and by the virion isolation procedure described in the text. Intracellular viral DNA was purified from the Hirt lysates by extraction with phenol and chloroform, treatment with pancreatic RNase, and precipitation with ethanol. Viral DNA was obtained from purified virions by incubation at 65°C for 0.5 h in 50 mM Tris (pH 7.6)–50 mM NaCl–10 mM EDTA, followed by treatment for 2 h at 37°C with 100 µg of proteinase K per ml, extraction with phenol-CHCl₃-isoamyl alcohol (25:25:1), and precipitation with ethanol. The resulting viral DNA samples were incubated with restriction endonuclease *HpaII* and electrophoresed in a 1.3% agarose gel. To check that the digestions had gone to completion, pBR322 DNA was included in each reaction. The gel was photographed by using Tri-X film, and the relative amount of DNA in each band was quantified by densitometry. The fraction of the viral DNA that was *dlG806* was determined as follows: (I + II)/(I + II + L).

mutants of SV40. To look directly for synthesis of a 61-amino acid leader-encoded protein, which we propose to name LP-1 (LP standing for leader-encoded protein), monkey cells were infected with *dl*-809, *dl*G810, *ar*1077, or wild type, radiolabeled 40 to 48 h later with [3 H]leucine, [3 H]lysine, or [35 S]methionine for 0.5 h, and then fractionated into cytoplasmic and nuclear components. The resulting extracts were electrophoresed in gradient polyacrylamide-sodium dodecyl sulfate gels and examined by fluorography.

The data from one such experiment (Fig. 4) indicate that (i) a protein of the appropriate molecular weight for LP-1 (approximately 8×10^3) was synthesized at a high rate by WT800-infected cells but not by mock-infected or *dl*-810-infected cells and (ii) whereas a 0.5 h of pulse-labeling was sufficiently long for most of the newly synthesized VP-1, VP-3, and cellular histone proteins to have been transported to the nucleus, most of the radiolabeled 8-kilodalton virus-induced protein was found in the cytoplasmic fraction. In addition, the fact that the *dl*-810-infected cells synthesized VP-1 and VP-3 at rates comparable to the rates observed in WT800-infected cells indicates that the failure of these cells to have made the 8-kilodalton protein could be attributed specifically to the mutation in the late leader region.

Other experiments (data not shown) indicated that (i) whereas mutant *ar*1077, which theoretically contains a missense mutation in LP-1, induced the synthesis of the 8-kilodalton protein, mutant *dl*-809 did not and (ii) the 8-kilodalton protein was radiolabeled by [3 H]lysine and by [3 H]leucine, but not by [35 S]methionine. This latter finding is consistent with the amino acid composition of the protein as predicted from the DNA sequence and indicates that the only methionine of the protein, which is located at the amino terminus, is removed intracellularly.

Finally, preliminary data (S. A. Sedman and J. E. Mertz, unpublished data) indicated that an 8-kilodalton protein was also synthesized in large amounts when wild-type SV40-specific RNA selected by hybridization to SV40 DNA was included in a micrococcal nuclease-treated rabbit reticulocyte lysate *in vitro* translation system. Together with the data presented above, this finding leads us to conclude that the 8-kilodalton protein is probably LP-1, the product of the agnogene.

Analysis of virion proteins. To determine whether the 8-kilodalton virus-induced protein was incorporated into virions, SV40 virions were purified from [3 H]lysine-labeled, WT800-infected monkey cells and electrophoresed in a sodium dodecyl sulfate-polyacrylamide tube gel. The presence and relative amounts of the vari-

FIG. 4. Electrophoretic analysis of the [3 H]leucine-labeled proteins found in the nuclei and cytoplasm of *dl*G810- and WT800-infected monkey cells. CV-1 cells in 100-mm dishes were infected with approximately 20 PFU of *dl*G810 or WT800 per cell and incubated at 37°C in medium containing 4% fetal bovine serum. After 48 h, the cells were washed once with TBS and once with medium lacking leucine and then were incubated at 37°C for 30 min in 1 ml of leucine-free medium containing 4% dialyzed fetal bovine serum and 300 μ Ci of L-[4,5- 3 H]leucine (57 Ci/mmol) per ml. The cells were then washed twice with TBS at 0°C and scraped off the dish in 1 ml of TBS containing 0.5% Nonidet P-40 and 300 μ g of phenylmethylsulfonyl fluoride per ml. After incubation at 0°C for 5 min, the nuclei were pelleted by centrifugation at $2,000 \times g$ for 10 min, washed twice with TBS, suspended in 2 ml of sample loading buffer (21), and boiled for 5 min with frequent blending with a Vortex mixer. The supernatant from the first centrifugation (cytoplasm) was diluted directly into 2 $\times$ sample loading buffer; 100,000 cpm of each sample was electrophoresed for 3 h at 125 V in a 15 to 20% gradient acrylamide-sodium dodecyl sulfate gel prepared by the method of Laemmli (21). The gel was stained with Coomassie brilliant blue to visualize the molecular weight markers (chymotrypsinogen A, 25,700; lysozyme, 14,300; cytochrome c, 12,300; insulin, ~3,000) and then processed for fluorography (22). Lanes 3 and 6 contained nuclear and cytoplasmic extracts, respectively, of cells processed in parallel as controls but without virus present in the infection. (The slightly faster mobility of the VP-1 synthesized in the *dl*G810-infected cells than the VP-1 synthesized in the WT800-infected cells was probably due to a second-site mutation mapping in the VP-1 gene that arose during serial passage of the mutant in monkey cells [Barkan and Mertz, manuscript in preparation].)

ous radiolabeled proteins were determined by scintillation spectroscopy after slicing the gel into 125 sections, each 2 mm thick. This experiment (data not shown) indicated that, whereas 1,570 cpm was present in the fraction containing the largest amount of cellular histone protein H4, fewer than 30 cpm was present in any of the fractions expected to contain LP-1. Since 7 of the 61 amino acid residues in LP-1 are lysine (Fig. 1), whereas H4 is also composed of approximately 11% lysine residues and is present at a level of 20 to 25 copies per virion (39), we calculated that on average, the SV40 virions contained fewer than two copies of LP-1. The conclusion that LP-1 is probably absent from mature virions agrees both with the failure of LP-1 to accumulate in large amounts in the nucleus (Fig. 4) and with the finding that once formed, virions of the late leader region mutants behave biologically and physically like virions of wild-type SV40.

DISCUSSION

The main findings of this work are that mutants deleted in the major late leader region both define a new complementation group and fail to induce the synthesis of an 8-kilodalton protein that is made in wild-type SV40-infected cells. After we completed these studies, Jay et al. (16) demonstrated directly by amino-terminal sequence analysis that this protein is indeed the 61-amino acid protein LP-1 whose sequence is shown in Fig. 1. Other workers (15; Margolskee and Nathans, personal communication; Southern and Berg, personal communication) have also independently found an 8-kilodalton protein in SV40-infected cells and have identified it by a variety of techniques, including mutational analysis and determination of which amino acids can and cannot be used to radiolabel it. Therefore, we conclude that our late leader region mutants fail to make the 8-kilodalton virus-encoded protein LP-1 because they lack the nucleotide residues required for its synthesis.

What is the reason for the complementation properties of mutants *dl*-806 and *dl*-810? The conclusion stated above provides a likely explanation. However, these mutants are also defective in "attenuation" of late-strand RNA synthesis (13, 25a), the efficiencies of splicing and precise locations of the 5' ends of the late-strand mRNAs (10, 10a, 12, 28, 40; A. Barkan and J. E. Mertz, manuscript in preparation), and the precise rates of synthesis in virus-infected cells of the virion proteins VP-1, VP-2, and VP-3 (Barkan and Mertz, manuscript in preparation) and, possibly, other leader-encoded proteins (see below). Consequently, experiments with mutants containing single nucleotide pair changes rather

than deletions will need to be done to answer this question definitively.

Properties and function(s) of LP-1. The data presented here and elsewhere (13, 15, 16) indicate that LP-1 (i) is a 61-amino acid, highly basic, acid-soluble, phosphorylated protein whose amino terminus is an unblocked valine residue; (ii) is synthesized from 16S mRNA at a high rate at late times in the lytic cycle, but has a half-life of only 2 to 3 h; (iii) binds to single-stranded and double-stranded DNA in 0.1 M NaCl; (iv) is helpful, but not essential, for a late step in the viral lytic cycle; (v) can function *in trans* to aid the growth of mutants defective in its synthesis; (vi) is also encoded, although with a somewhat different sequence, by BK virus, a related human papovavirus; (vii) may exist in association with SV40 minichromosomes or intermediates in virion assembly, but is not present in mature virions; (viii) is found predominantly in the cytoplasmic fraction of cell extracts, although possibly because of leakage from the nucleus during the fractionation procedure; and (ix) may enable VP-1 to perform one of its functions more readily (Margolskee and Nathans, personal communication; Barkan and Mertz, manuscript in preparation).

One hypothesis consistent with all of these findings and the known properties of mutants defective in the synthesis of LP-1 is that a function of LP-1 may be to facilitate virion assembly. Possibly, this could be accomplished by LP-1 serving as a nonreusable scaffolding protein which displaces H1 from the minichromosomes and then promotes the condensation of the virion proteins onto the viral DNA. If LP-1 molecules were degraded during or as a consequence of this reaction, this hypothesis would explain both their short half-life and their predominantly cytoplasmic location, i.e., the cytoplasmic molecules are the ones that have not yet participated in the reaction. Consistent with this proposed role for LP-1 is the fact that the cellular histone protein H1, although present intracellularly on SV40 minichromosomes, is probably missing from virions (39).

If our hypothesis is true, it would provide a good rationalization as to why SV40 encodes LP-1 on the same mRNAs used in the synthesis of VP-1; by making polygenic mRNAs, SV40 may have developed a simple mechanism for guaranteeing production of these two proteins at the constant relative rates at which it utilizes them. In addition, other plausible functions of LP-1 that should be considered include roles in (i) control of initiation of late-strand RNA synthesis, (ii) attenuation of late-strand RNA synthesis (13), and (iii) regulation of translation start codon selection on these polygenic viral mRNAs.

Are other proteins also encoded by the late leader region of SV40? The findings discussed above demonstrate definitively that the AUG codon at nucleotide residues 335 to 337 can be utilized *in vivo* for initiation of translation. Since 30 to 50% of the 19S viral mRNAs present in wild-type SV40-infected monkey cells lack nucleotide residues 374 to 557 (9), it is likely that nucleotide residues 335 to 337 can also function for the synthesis of a 30-amino acid polypeptide.

Eight additional AUG codons are present between nucleotide residues 39 and 256 of the late leader region (Fig. 1). Preliminary findings (Barkan and Mertz, unpublished data) have indicated that some of these codons may be utilized in the synthesis of small (i.e., 11- to 20-amino acid) polypeptides. Experiments are in progress to test this hypothesis. If correct, it would provide an explanation for the finding that the 5' ends of the late SV40 mRNAs are situated throughout this region. In addition, it would indicate that attenuation of late-strand SV40 RNA synthesis (13, 25a) may function as a mechanism for enabling these possibly functional late-strand leader region polypeptides to be synthesized early in the lytic cycle or at higher rates than the capsid proteins are.

Implications for the mechanism of translation start codon selection in eucaryotic mRNAs. Together with what is presently known about the primary structures of the SV40 late mRNAs (9), the conclusion that LP-1 is synthesized *in vivo* indicates that VP-1, VP-2, and VP-3 are made starting from the second, third, or even fourth of several functional AUG codons present in these polygenic mRNAs. We have recently found that VP-2, VP-3, and, possibly, VP-1 can be synthesized *in vivo* from the same spliced species of 19S mRNAs (Barkan and Mertz, manuscript in preparation). These findings are clearly inconsistent with the original "scanning model" for translation start codon selection in eucaryotic mRNAs of Kozak (18, 19). Recently, Kozak has modified the model to allow ribosomes to initiate at a downstream site provided that all of the upstream AUGs are flanked by unfavorable sequences such that some 40S ribosomes can get through (20). Whether synthesis of the proteins encoded by the polygenic SV40 mRNAs actually occurs by this or some other mechanism, such as direct internal translational initiation or sequential synthesis of two or more proteins by the same ribosome, remains to be determined.

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