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Missense Mutations in the VP1 Gene of Simian Virus 40 That Compensate for Defects Caused by Deletions in the Viral Agnogene

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Simian virus 40 mutants lacking sequences in the late leader region are viable but produce smaller plaques than does wild-type virus. Within three passages at low multiplicities of infection, virus stocks of several such mutants accumulated variants that synthesized an altered form of the major virion protein, VP1, having a slightly faster mobility in sodium dodecyl sulfate-polyacrylamide gels than did the wild-type protein. Because these variants overgrew the original virus stocks, we consider them to be second-site revertants. By construction and characterization of a series of recombinants, the second-site mutations were shown to map to at least two different regions of the VP1 gene. Nucleotide sequence analysis indicated that single-amino-acid changes were responsible for the rapid mobility of VP1. When combined in *cis* with either a wild-type or mutant leader region, these VP1 mutations sped up by 10 to 20 h the time course of accumulation of infectious progeny but not of viral DNA or VP1. LP1, the protein encoded by the agnogene, was shown previously to be necessary for the efficient transport of the virion proteins to the nucleus or for their efficient assembly with viral minichromosomes. The VP1 missense mutations reported here compensate for the lack of LP1 by facilitating this process. On the basis of these findings and findings reported previously by us and others, we hypothesize that LP1 facilitates the formation of infectious particles by inhibiting the polymerization of VP1 molecules until the time they interact with viral minichromosomes; the VP1 mutations reported here compensate for the loss of LP1 by lessening the potential of VP1 for self-polymerization.

The late leader region of simian virus 40 (SV40) encodes the 5′ termini of the late-gene RNAs, sites used in RNA splicing, regulatory signals affecting synthesis, processing, and translation of the late mRNAs (for a review, see reference 32), and LP1 (also called agnoprotein), a 61-amino-acid basic protein (12, 13, 16, 21) whose function and mode of action have yet to be determined definitely. Mutants with deletions in this region are viable. However, they produce smaller plaques than does wild-type virus (5, 9, 19, 26, 28) and have lower rates of synthesis during single cycles of growth of infectious progeny (2, 26) but not lower rates of synthesis of viral DNA, the late RNAs, or the virion proteins (2, 4, 24; A. Barkan, Ph.D. thesis, University of Wisconsin, Madison, 1983; J. E. Mertz, X.-M. Yu, C. J. Hussussian, and G. Gelembiuk, unpublished data).

Because the late leader region encodes several overlapping functions, it has been difficult to assign particular functions to specific sequences within it. One approach that can be helpful in defining interactions between virally encoded macromolecules involves the isolation and characterization of mutants and their second-site revertants (i.e., pseudorevertants). We describe here the isolation and characterization of pseudorevertants of leader region deletion mutants. These pseudorevertants contain second-site mutations in the VP1 gene, the gene encoding the major structural protein of SV40 virions. Margolskee and Nathans (16) have isolated a complementary class of pseudorevertants in which second-site mutations affecting LP1 compensate for a primary lesion altering VP1. Taken together, these data indicate that VP1 and LP1 probably interact during the viral lytic cycle. On the basis of the genotypes and phenotypes of LP1$^-$ mutants and their pseudorevertants, we hypothesize that LP1 interacts with VP1 molecules to inhibit their self-polymerization until the time they interact with viral minichromosomes to form virions.

A preliminary account of this work was submitted by A. Barkan in partial fulfillment of the requirements for the Ph.D. degree from the University of Wisconsin, Madison, 1983.)

MATERIALS AND METHODS

Cells and viruses. The origin, growth characteristics, and biological properties of mutants *dl*-802, *dl*-805, *dl*-806, *dl*-809, and *dl*-810 have been reported previously (2, 19, 21; Fig. 1). MA-134 and CV-1P cells are established lines of African green monkey kidney (AGMK) cells. Primary AGMK cells were obtained from Flow Laboratories, Inc. Virus stocks were prepared by infecting MA-134 cells with approximately 0.01 PFU per cell and allowing the infection to proceed until more than 80% of the cells exhibited cytopathic effect (12 to 15 days); viral DNA stocks were prepared concurrently from these infected cells by the procedure of Hirt (11). Virus was plaque purified, and the titers of virus stocks were determined in CV-1P cells as described previously (20).

Construction of recombinants. Each parental DNA was cleaved with restriction enzymes at two sites (see Fig. 3). The resulting restriction fragments were fractionated in agarose gels and recovered by electroelution. Recombinants were made by ligation at a DNA concentration of 5 μg of equimolar amounts of the indicated fragments from each parent per ml. Ligations of the individual DNA fragments by themselves were performed as controls. Afterward, the DNAs were transfected into CV-1P cells by a previously described modification (10) of the DEAE-dextran procedure of McCutchan and Pagano (18). In each instance, the specific infectivity of the individual DNA fragments was less than 1% of that of the mixed fragments. Three plaques that arose

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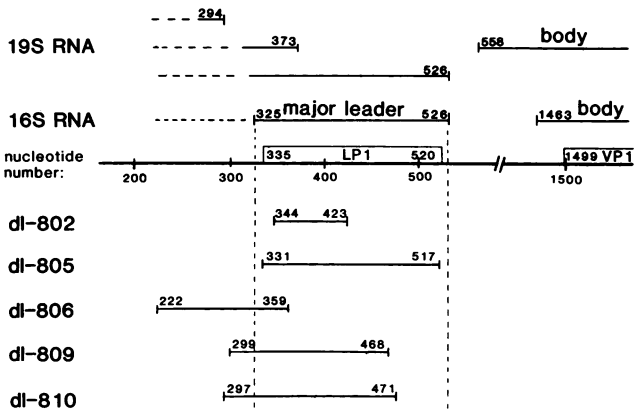

FIG. 1. Locations of deletions in leader region mutants. The nucleotides deleted in each mutant are indicated by bar lines (2). The sequences encoding LP1, the product of the agnogene, are shown. The alternative leader segments of the late mRNAs are also indicated by bar lines, with the broken lines representing their 5'-terminal heterogeneity (7). The nucleotide numbering system is from Tooze (32).

from transfection with each recombinant ligation mixture were picked and used to prepare virus stocks.

Analysis of VP1 phenotype. Cells were harvested 40 to 45 h after infection of CV-1P cells with virus at approximately 20 PFU per cell. One hour immediately before being harvested, the cell proteins were radiolabeled with [^{35}S]methionine by incubation of the cells in RPMI 1640 medium lacking methionine and supplemented with [^{35}S]methionine (50 μCi/ml; 1,300 Ci/mmol; Amersham Corp.). Nuclear lysates were prepared by incubation of the cells for 5 min at 0°C in Tris-buffered saline containing 0.5% Nonidet P-40 and 1% aprotinin. Afterward, the nuclei were collected by centrifugation at 2,000 × *g* for 5 min and lysed by the addition of sodium dodecyl sulfate (SDS) sample buffer (15). Alternatively, whole-cell lysates were prepared by suspension of the cells in hypotonic buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 1.5 mM $MgCl_2$, 1% aprotinin) and lysis by the addition of one-fifth volume of lysis buffer (50 mM sodium phosphate [pH 7.2], 0.75 M NaCl, 5% Triton X-100, 2.5% sodium deoxycholate, 0.5% SDS) (33).

VP1 was immunoprecipitated from the lysates by incubation for 1 to 2 h at 0°C with an excess of antiserum specific for VP1 (horse anti-SV40 serum; Flow Laboratories). Inactivated *Staphylococcus aureus*, prepared as described by Kessler (14), was then added, and the mixtures were incubated for an additional 30 min at 0°C. After three rinses with RIPA buffer (8), the bacteria were suspended in SDS sample buffer (15), incubated at 100°C for 5 min, and pelleted by centrifugation. The proteins in the supernatants were separated by electrophoresis at approximately 3 V/cm in 12% polyacrylamide (0.15% bisacrylamide) gels containing SDS (15). Afterward, the gels were prepared for fluorography with En^{3}Hance (New England Nuclear Corp.), dried, and exposed to X-ray film (Eastman Kodak Co.).

Virus stocks and recombinants were routinely assayed for their VP1 phenotype by fluorographic analysis of immunoprecipitated, electrophoresed, radiolabeled VP1 obtained from virus-infected or viral DNA-transfected whole-cell lysates (Fig. 2B). However, detection of unlabeled VP1 by simply staining the gels with Coomassie brilliant blue (see Fig. 5) or analysis of the virion proteins obtained from nuclear lysates (Fig. 2A) yielded similar results.

RESULTS

Variants possessing second-site mutations affecting VP1 accumulate in leader region deletion mutant virus stocks. The late leader region deletion mutants *dl*-802, *dl*-805, *dl*-806, *dl*-809, and *dl*-810 are naturally arising, viable mutants which make smaller plaques than does wild-type virus (19). The precise endpoints of their deletions are indicated in Fig. 1. Each mutant is defective in the synthesis of LP1 because it lacks much of the sequence that encodes this protein.

Within three serial passages after plaque purification at low multiplicities of infection (approximately 0.01 PFU per cell), virus stocks of these mutants were frequently overgrown with variants that synthesized altered forms of the major virion protein, VP1. An example of this phenomenon is illustrated in Fig. 2A. In this particular experiment, cells infected with mutants *dl*-805 and *dl*-809 contained an abundant protein with a mobility slightly more rapid than that of wild-type VP1. The virus stocks of *dl*-805 and *dl*-809 used for these infections were from the third serial passage of plaque-purified virus. Virus stocks from earlier passages did not exhibit this phenotype (Fig. 2B). The variants responsible for this phenotype were plaque purified and named *dl*-805-R1 and *dl*-809-R1, respectively. Virus stocks from subsequent serial passages of mutants *dl*-802 and *dl*-810 also exhibited this phenotype (Barkan, Ph.D. thesis, and Fig. 2A, which shows that the *dl*-810-P3 virus stock induced the synthesis of a mixture of wild-type and R1 VP1). Of the five leader region

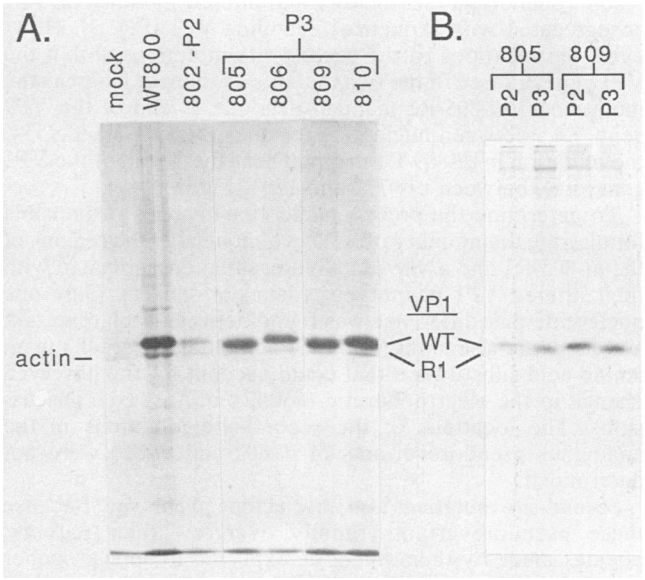

FIG. 2. Gel-fractionated proteins from wild-type (WT)- and mutant-infected cells. (A) Fluorogram of radiolabeled nuclear proteins. CV-1P cells were infected with 10 PFU of virus per cell obtained from the second (P2) or third (P3) serial passage after plaque purification. Forty-four hours later the infected cells were incubated for 1 h with [^{35}S]methionine. Nuclear protein was then extracted and fractionated by electrophoresis in a 12% polyacrylamide gel containing SDS. The gel was prepared for fluorography, dried, and exposed to X-ray film. (B) Fluorogram of radiolabeled protein from whole-cell lysates immunoprecipitated with VP1 antiserum. CV-1P cells were transfected as described previously (10) with mutant viral DNA obtained at the passages indicated, incubated at 37°C for 40 h, and radiolabeled with [^{35}S]methionine for 2 h. Immediately thereafter, whole-cell lysates were prepared. VP1 cross-reactive proteins were immunoprecipitated from the lysates and analyzed by polyacrylamide gel electrophoresis followed by fluorography.

deletion mutants that have been serially passaged at least three times in our laboratory, only mutant *dl*-806 has never been overgrown by this type of variant. Nevertheless, we have never observed such variants in virus stocks of WT800, the parental strain of these mutants.

The novel, abundant protein found in cells infected with these variants was shown to be closely related to wild-type VP1 both by tryptic peptide analysis (Barkan, Ph.D. thesis) and by its ability to be immunoprecipitated by antisera specific for VP1 (Fig. 2B). Therefore, under the conditions used for growing these mutant viruses, there was a strong selective advantage for virus that had acquired second mutations affecting VP1. This implies that these new mutations compensate at least partially for defects caused by the original mutations in the leader region.

Second-site mutations are single-base-pair changes that map to at least two locations within the VP1 gene. We first attempted to map the mutations responsible for altering the electrophoretic mobility of VP1 by the S1 nuclease mapping technique of Shenk et al. (27). However, S1 nuclease failed to cleave efficiently heteroduplexed DNAs formed between mutant and parental genomes (data not shown). This indicated that the second-site mutations were probably single-base-pair changes rather than larger alterations.

To localize the mutations, we therefore constructed the series of recombinants between the wild-type and the double-mutant genomes indicated in Fig. 3. As expected, in every instance the small-plaque phenotype cosegregated with the deletion in the leader region, whereas the sharp-plaque phenotype (see below) and altered mobility of VP1 cosegregated with sequences encoding VP1 (Fig. 3). However, the locations of the second-site mutations within the VP1 gene differed in the two mutants examined: whereas the mutation in *dl*-805-R1 mapped near the 3′ end of the VP1 gene, i.e., between nucleotide residues (nts) 1993 and 2534, the mutation in *dl*-809-R1 mapped near the 5′ end of the VP1 gene, i.e., between nts 771 and 1783 (Fig. 3).

To determine the precise nucleotide changes responsible for altering the mobility of VP1, we sequenced the regions of the *dl*-805-R1 and *dl*-809-R1 genomes that cosegregated with their altered VP1 phenotypes (data not shown). Only one nucleotide-pair difference was found between each revertant and its parental mutant (Fig. 3). Each mutation results in an amino acid substitution that could account for the observed change in the electrophoretic mobility of VP1 (see Discussion). The locations of the second-site mutations in the analogous pseudorevertants of *dl*-802 and *dl*-810 were not determined.

Second-site mutations also alter plaque phenotype. Because these pseudorevertants rapidly overgrew their parents, plaques made by them might be expected to appear sooner and grow more rapidly than those made by the parental mutants. In fact, plaques made by the pseudorevertant *dl*-805-R1 did just that, although they were still somewhat smaller than those made by WT800 (Fig. 4 and Table 1). However, plaques produced by the pseudorevertant *dl*-809-R1, although visible slightly earlier (Fig. 4A), did not grow more rapidly than those of its parent *dl*-809 (Fig. 4B and Table 1). Nevertheless, plaques produced by mutants that contained these VP1 mutations always had a sharp appearance (Fig. 4 and Table 1). Therefore, these pseudorevertants exhibit altered plaque phenotypes. Since these altered plaque phenotypes cosegregated with the altered mobility of VP1 (Fig. 3), the single-base-pair changes within the VP1 gene identified here must be responsible for the altered plaque phenotypes, as well as the altered VP1 phenotypes.

Activity of the VP1 mutation 805-R1 in other genetic backgrounds. To determine whether the VP1 mutation present in *dl*-805-R1 (i.e., 805-R1) compensates specifically for defects of late leader region mutants or confers a selective advantage in other genetic backgrounds as well, recombinants were constructed in which the smaller *Eco*RI-*Bam*HI restriction fragment from *dl*-805-R1 (encoding the VP1 mutation) was placed into WT800 and mutant *dl*-806 genomes. The data (Fig. 4 and Table 1) show that the second-site mutation 805-R1 increased significantly the size of the plaques made by the otherwise wild-type virus WT(805-R1) (recombinant a.2 in Fig. 3). Therefore, the effect of the VP1 mutation 805-R1 on plaque size is not restricted to leader region mutants. However, the VP1 mutation 805-R1 failed to increase significantly the size of the plaques produced by mutant *dl*-806 [mutant *dl*-806(805-R1) in Table 1]. Therefore, VP1 mutation 805-R1 is unable to compensate significantly for the severe defects caused by the deletion in *dl*-806. This finding may account for our failure to date to obtain pseudorevertants of *dl*-806 with second-site mutations altering VP1.

VP1 mutation 805-R1 confers a selective advantage under some growth conditions. The fact that the double mutant *dl*-805-R1 rapidly overgrew its parent, *dl*-805, indicates that the former has a selective advantage for growth over the latter. To demonstrate that the VP1 mutation can also confer, under some conditions, a selective growth advantage to virus with a wild-type leader region, we infected MA-134 cells at a low multiplicity of infection (approximately 0.01 PFU per cell) with a 9:1 mixture of WT800 and WT(805-R1) virus. Afterward, the cells were incubated at 37°C until approximately 80% exhibited cytopathic effect. A virus stock (P1) was prepared from these cells and used at a low multiplicity of infection to prepare a second virus stock, P2. The VP1 phenotype of each virus stock was then determined (Fig. 5). As expected, the initial virus stock (P0) consisted predominantly of virus with a wild-type VP1 phenotype. However, the relative amount of the rapidly migrating form of VP1 encoded by WT(805-R1) increased significantly with each serial passage, with wild-type VP1 no longer detectable after the second passage. This rapid overgrowth of wild-type virus by the recombinant containing the VP1 mutation shows clearly that the VP1 mutation 805-R1 does indeed confer a strong selective advantage under our cell culture conditions even to otherwise wild-type virus.

VP1 mutation 805-R1 facilitates transport of the virion proteins or assembly of virions. The selective advantage conferred by the VP1 mutation 805-R1 could, theoretically, result from (i) a faster initiation of the virus infection, (ii) more rapid assembly of infectious progeny, (iii) an increased yield of infectious progeny, or (iv) quicker initiation of subsequent cycles of infection (e.g., via faster release of the progeny from infected cells). To examine these possibilities, we first determined the effect of the VP1 mutation of 805-R1 on the rate of accumulation of infectious progeny during a single cycle of growth. The presence of this mutation in the $LP1^-$ mutant *dl*-805 resulted in the time course of accumulation of infectious progeny being shifted approximately 20 h earlier without alteration of the final yield (Fig. 6). This VP1 mutation placed in a wild-type background also resulted in a similar, although not quite so dramatic, shift in the time course of appearance of PFU (Fig. 6). Therefore, the presence of this VP1 mutation in either the $LP1^-$ mutant *dl*-805 or the wild-type speeds up production of infectious progeny by 10 to 20 h.

Several plausible mechanisms could account for the

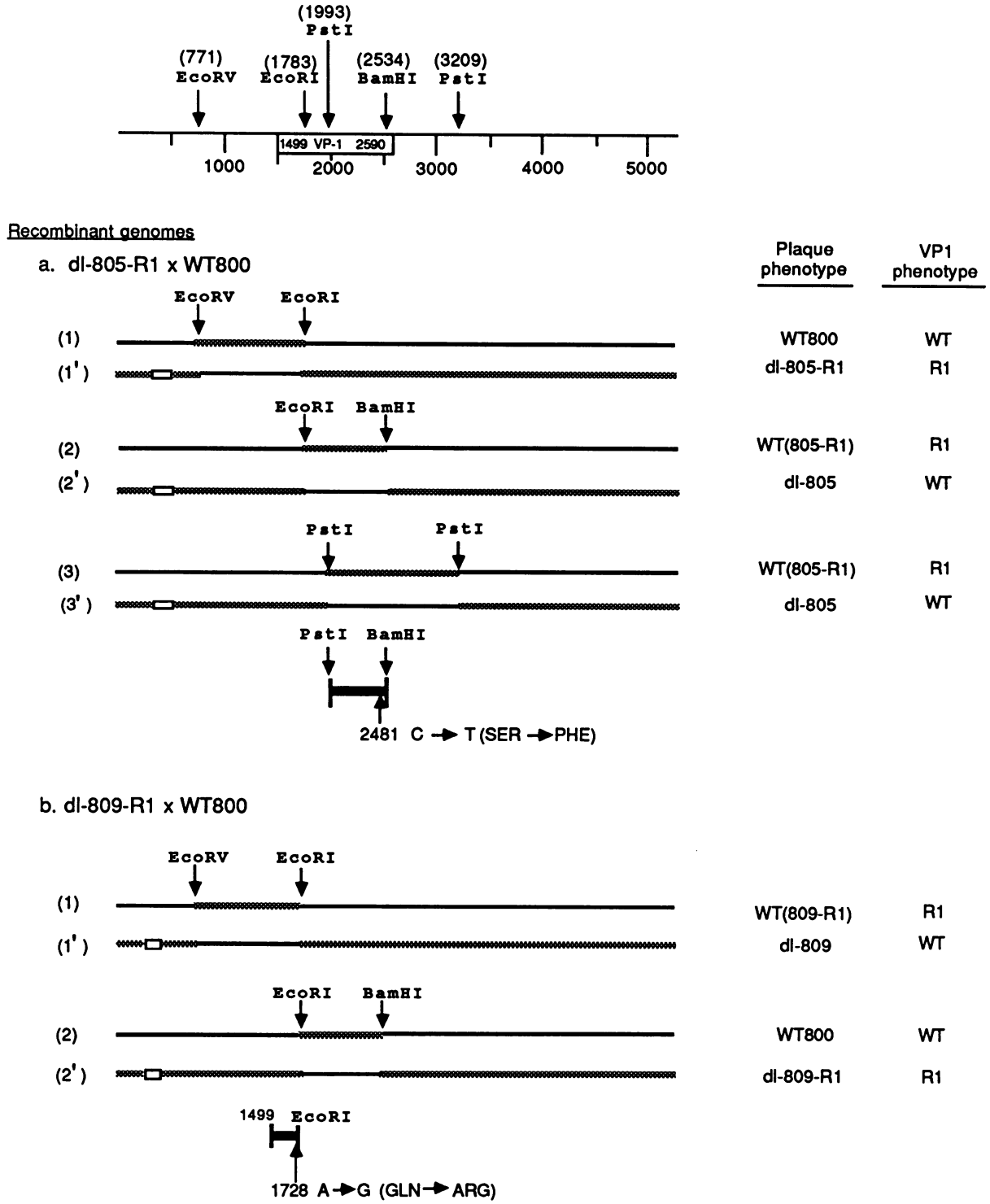

FIG. 3. Localization and identification of second-site mutations in the pseudorevertants *dl*-805-R1 and *dl*-809-R1 responsible for the altered plaque and VP1 phenotypes. The circular SV40 genome is depicted as a linear molecule with its ends chosen arbitrarily to be at the origin of viral DNA replication. WT800, *dl*-805-R1, and *dl*-809-R1 DNAs were cleaved at the following sites: *Eco*RV and *Eco*RI (1), *Eco*RI and *Bam*HI (2), and *Pst*I (3). Recombinants were made by ligation of the wild-type (WT) restriction fragments to the complementary mutant fragments. Sequences derived from WT800 are designated by solid lines; sequences derived from *dl*-805-R1 (a) and *dl*-809-R1 (b) are designated by hatched lines. The rectangles indicate the locations of the previously known deletions in *dl*-805-R1 and *dl*-809-R1. The results obtained from assays of the plaque and VP1 phenotypes for each pair of reciprocal recombinants are indicated on the right: the plaque phenotypes were determined by comparison of the sizes and sharpness of the plaques made by the recombinants on monolayers of CV-1P cells with those made by known mutants (Fig. 4); the VP1 phenotypes were determined as described in Materials and Methods. Indicated by a thick line below each set of recombinants is the region deduced from these data to encode the novel mutation responsible for the altered plaque and VP1 phenotypes. For each mutant, the nucleotide sequence of this entire region was determined by the method of Maxam and Gilbert (17); the only difference from the parent noted in each instance is indicated. In addition, both the parental mutant and the pseudorevertant genomes contained the following differences from the published sequence of SV40 (32). (i) *dl*-805 and *dl*-805-R1 have an A in place of C at nt 2384 and a G in place of T at nt 2239. Neither substitution affects the coding of an amino acid. (ii) *dl*-809 and *dl*-809-R1 have a C in place of A at nt 1756; this results in the substitution of aspartate for glutamate.

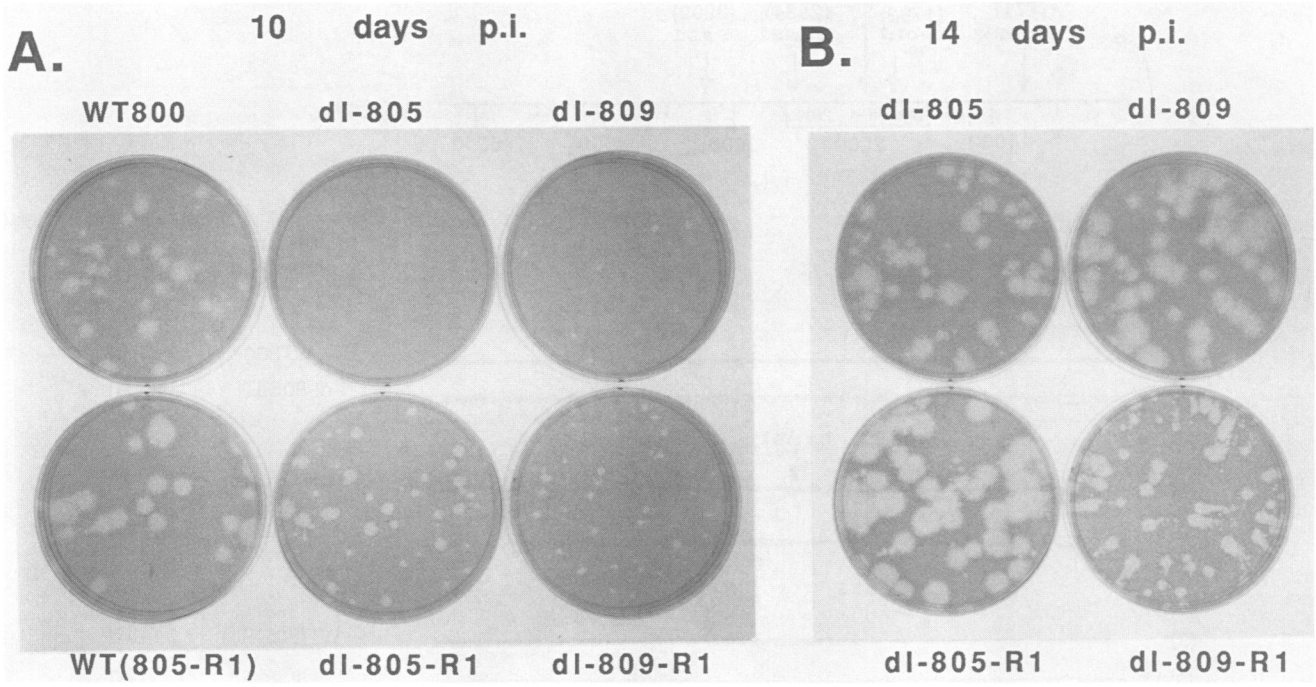

FIG. 4. Morphology of plaques formed by WT800 and mutants *dl*-805, *dl*-809, WT(805-R1), *dl*-805-R1, and *dl*-809-R1. Monolayers of CV-1P cells were infected with each of the mutants, overlaid with agar medium, and incubated at 37°C as described previously (20). Neutral red was added at 8 days after infection, and the dishes were photographed 2 (A) and 6 (B) days later (10 and 14 days postinfection [p.i.], respectively). Each of the dishes exhibited 40 to 50 plaques by 12 days after infection, except for the one labeled WT(805-R1), which exhibited 21 plaques.

finding discussed above. For example, the protein encoded by the mutant VP1 gene might act at one or more early steps in the lytic cycle by facilitating virus adsorption, penetration, or uncoating. If it did, all subsequent steps in the lytic cycle would be shifted to earlier times. Alternatively, the mutant protein might accumulate faster in the cell or be utilized more efficiently in the production of virions. The first of these possibilities was eliminated by determination of the amounts of viral DNA present at various times after infection with the VP1 mutant WT(805-R1) versus its parent, WT800 (Fig. 7A). Excision and counting by scintillation spectroscopy of the bands obtained from Southern blot analyses of the viral DNA samples from two independent experiments indicated unequivocally that the mutant- and wild-type-infected cells accumulated viral DNA at identical rates. Protein samples harvested in parallel with the viral DNA samples were analyzed by probing immunoblots with a polyclonal antiserum specific for VP1 (Fig. 7B). The data indicated that the cells infected with the VP1 mutant WT(805-R1) accumulated VP1 at rates similar to or, possibly, slightly slower than those observed in the cells infected with WT800 virus. Therefore, the VP1 mutation 805-R1 must act to speed up a step in the lytic cycle that occurs after the synthesis of VP1, i.e., the transport of VP1 molecules to the nucleus or their association with viral minichromosomes to form infectious particles.

TABLE 1. Mean diameters of plaques at 11 days after infection with mutants, pseudorevertants, and recombinants

Virus	Mean diameter (mm) of plaques on cells[a]:	
	CV-1P	Primary AGMK
WT800	2.9	1.8
dl-805	0.4	2.1
dl-806	0.2	0.8
dl-809	0.8	1.6
dl-805-R1	2.4[b]	
dl-809-R1	0.8[b]	
WT(805-R1)[c]	5.0[b]	
dl-806(805-R1)[c]	0.2[b]	
dl-805-R1(WT)[c]	0.4	

[a] Monolayers of CV-1P and primary AGMK cells were infected with 5 to 20 PFU per 60-mm dish and incubated at 37°C as described previously (20) in parallel with the monolayers shown in Fig. 4. Mean plaque diameters are average sizes of 10 to 20 plaques selected at random for measurement as described previously (19).

[b] The plaques produced by these mutants had edges that were more sharply defined than those typically produced by wild-type SV40 (Fig. 4).

[c] Recombinants containing the VP1 genotype of the virus indicated in parentheses in the background of the virus listed outside the parentheses. These recombinants were constructed by recombination in vitro of the smaller and larger of the two *Eco*RI-*Bam*HI restriction fragments derived, respectively, from the two parental genomes. The structures of WT(805-R1) and *dl*-805-R1(WT) are presented in Fig. 3 as recombinants a.2 and a.2′, respectively. The smaller *Eco*RI-*Bam*HI restriction fragment of *dl*-805-R1 encodes the VP1 mutation (Fig. 3).

DISCUSSION

We showed that single-base-pair changes within the VP1 gene can compensate in part for defects caused by deletions in the late leader region and can also accelerate the rate of growth of wild-type virus (Fig. 6). The two second-site mutations mapped are located at opposite ends of the VP1 gene and result in different amino acid substitutions (Fig. 3). A serine codon is replaced by a phenylalanine codon in the 805-R1 mutation, whereas a glutamine codon is replaced by one for arginine in the 809-R1 mutation. Substitutions of

nonpolar or basic amino acids (such as phenylalanine or arginine) for polar ones (such as serine or glutamine) increase protein mobility in SDS-polyacrylamide gels because hydrophobic and basic amino acids bind more SDS than do polar ones (6). We also obtained pseudorevertants of two other LP1$^-$ mutants in which the electrophoretic mobility of VP1 was similarly affected. Therefore, it is likely that these mutants have also acquired second-site mutations mapping within sequences encoding VP1.

We believe that the mutations in the VP1 gene are responsible for the growth advantages of these second-site revertants because (i) stocks of four different LP1$^-$ mutants were each independently overgrown by double mutants with alterations affecting VP1, (ii) the ability of both *dl*-805-R1 and WT(805-R1) to produce plaques larger and sharper than those of their parents cosegregated with the VP1 mutation (Fig. 3 and 4 and Table 1), and (iii) the VP1 mutant WT(805-R1) rapidly overgrew its parent, WT800, during serial passage of a mixed virus stock at low multiplicities of infection (Fig. 5).

Naturally occurring large-plaque variants of SV40 have been noted previously (for example, see reference 29). Our finding that the large-plaque mutant WT(805-R1) rapidly overgrew WT800 raises the interesting question of why variants with VP1 genes similar to those described here do not arise frequently in wild-type virus stocks. Part of the answer may be that the relative advantage of these mutations in a wild-type background is not so great as it is in an LP1$^-$ background (Fig. 6). Second, whereas these VP1 mutations

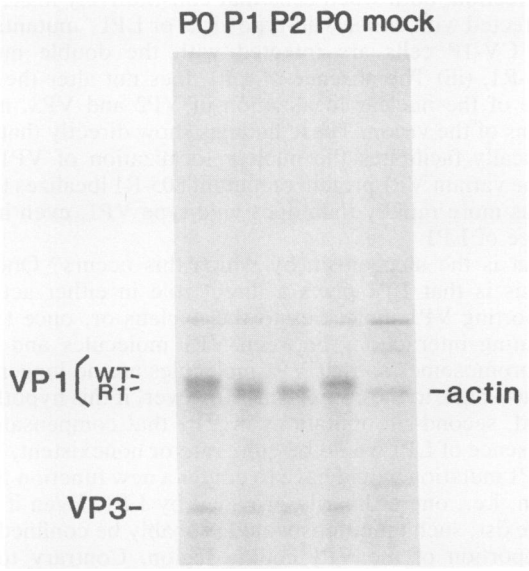

FIG. 5. Electropherogram of nuclear proteins from cells infected with serially passaged mixtures of wild-type (WT) and WT(805-R1) virus. An initial mixed virus stock (P0) was made by the addition of one part WT(805-R1) virus to nine parts WT800 virus. MA-134 cells were infected with this mixed virus stock at a low multiplicity of infection (approximately 0.01 PFU per cell) and incubated at 37°C until approximately 80% of the cells exhibited cytopathic effect. A virus stock (P1) was prepared from these cells and used at a low multiplicity of infection to prepare virus stock P2. CV-1P cells were then infected in parallel with each of these virus stocks at multiplicities of infection of approximately 10 PFU per cell. Mock-infected cells received no virus. Nuclear protein was prepared at 40 h after infection and was fractionated by electrophoresis in a 12% polyacrylamide gel containing SDS (15). Shown is a photograph of the gel stained with Coomassie brilliant blue.

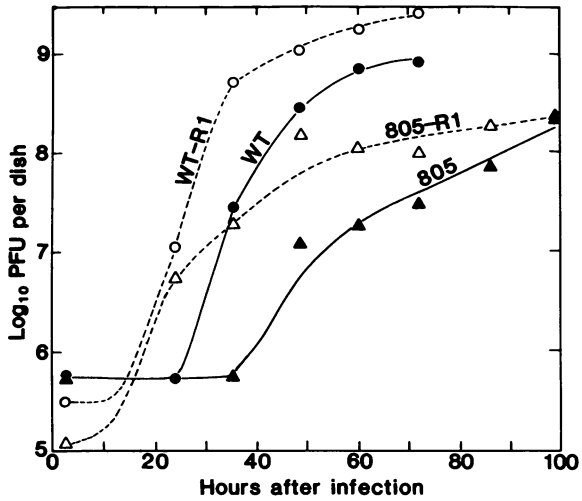

FIG. 6. Time courses of accumulation of PFU of WT800 (●), WT(805-R1) (○), *dl*-805 (▲), and *dl*-805-R1 (△) virus during single cycles of growth. Monolayers of CV-1P cells (2×10^6 per 60-mm dish) were infected in parallel at multiplicities of infection of approximately 30 PFU per cell with the indicated virus. After removal of unadsorbed virus, the cells were incubated at 37°C for the times shown. The total number of PFU in the cells plus culture medium contained in each dish was then determined as described previously (2, 20).

enable LP1$^-$ mutants to accumulate progeny with kinetics closer to those observed with the wild type (Fig. 6), by themselves they may under some conditions cause viral DNA to be assembled too rapidly into virions to the detriment of progeny yield. Third, the selective advantage conferred by these mutations may exist during growth in some cell lines (e.g., CV-1P) but not in others (e.g., primary AGMK [Table 1] and BSC-1 [24] cells). Lastly, these amino acid changes in VP1 may result in the production of virions that are more susceptible to inactivation than are those of the wild type (see below). For example, whereas wild-type virions are still infectious after incubation at 50°C for 2 h, those of the large-plaque variant SV-L no longer are (30).

Although the pseudorevertant *dl*-809-R1 accumulated rapidly during serial passage of mutant *dl*-809 in MA-134 cells, it did not make plaques on CV-1P cells that were significantly larger than those made by its parent (Table 1). Possible reasons for this finding include the following: (i) small differences in growth rates are amplified during the numerous cycles of infection that occur during serial passages at low multiplicities, (ii) plaque size is not determined solely by the rate of production of infectious progeny, and (iii) the cell lines and culture conditions used for the preparation of virus stocks were different from those used for plaque assays. Possibly, the double mutant has a greater selective advantage for growth in MA-134 cells than it does in CV-1P cells. Consistent with this hypothesis is the fact that the leader region deletion mutants studied here exhibited markedly different plaque phenotypes on monolayers of primary AGMK cells than on monolayers of CV-1P cells (Table 1).

Margolskee and Nathans (16) have independently isolated spontaneous revertants of a VP1 mutant with second-site suppressing mutations mapping within sequences encoding LP1. It is notable that the mutation in the VP1 gene reported by them (i) maps only 8 base pairs away from the mutation in our VP1 mutant 809-R1 and (ii) also causes a sharp-plaque

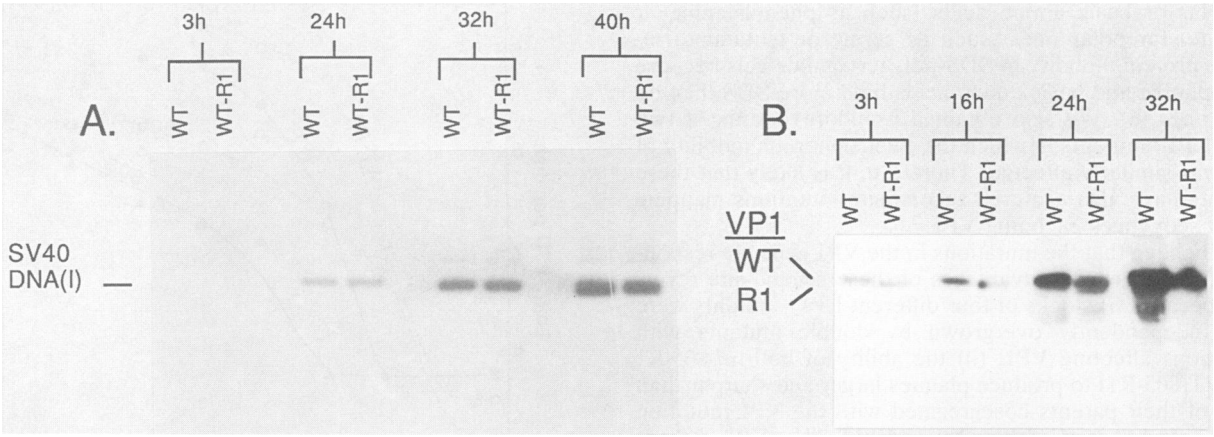

FIG. 7. Time courses of accumulation of viral DNA (A) and VP1 (B) in cells infected with WT800 (WT) and mutant WT(805-R1) (WT-R1). (A) Autoradiogram of a Southern blot. CV-1P cells were infected as described in the legend to Fig. 6. At each of the times indicated, viral DNA was prepared by the procedure of Hirt (11) and purified by extraction with phenol-chloroform-isoamyl alcohol (25:25:1), precipitation with ethanol, and incubation with RNase A. Afterward, 1% of each sample was electrophoresed in a 0.8% agarose gel, transferred to nitrocellulose paper (34), and detected by hybridization with wild-type SV40 DNA sequences cloned in pBR322 that had been radiolabeled with ^{32}P by nick translation. (B) Autoradiogram of an immunoblot. CV-1P cells were infected as described in the legend to Fig. 6. At each of the times indicated, whole-cell lysates were prepared as described in Materials and Methods. The proteins in each lysate were separated by electrophoresis in a 12% polyacrylamide gel containing SDS and were analyzed by immunoblotting essentially as described by Baichwal and Sugden (1) by using a polyclonal antiserum specific to VP1 (a gift from S. A. Sedman) followed by ^{125}I-protein A (Amersham).

phenotype of the type observed in our study (Fig. 4 and Table 1). Therefore, our finding that the growth of LP1$^-$ mutants is enhanced by second-site mutations in the VP1 gene is probably closely related to the phenomenon they observed. Taken together, these data suggest that VP1 and LP1 probably interact. However, how can a VP1 mutation that accelerates the production of both *dl*-805 (LP1$^-$) and WT800 (LP1$^+$) progeny (Fig. 6) be explained by specific, direct interactions between VP1 and LP1?

Ng et al. (22) have reported steady-state and pulse-chase labeling experiments indicating that the kinetics of virion assembly is much slower in cells infected with the LP1$^-$ mutant *dl*-805 than in ones infected with wild-type virus. We (2; Barkan, Ph.D. thesis; J. E. Mertz, X.-M. Yu, C. J. Hussussian, and G. Gelembiuk, unpublished data), Carswell and Alwine (4), and Resnick and Shenk (24) have performed numerous experiments which indicate that the kinetics of early-gene expression, viral DNA replication, and late viral mRNA and virion protein synthesis is similar in LP1$^-$- and WT-infected CV-1P cells. Data presented here show that the presence of the VP1 mutation 805-R1 did not alter the kinetics of viral DNA (Fig. 7A) or VP1 (Fig. 7B) accumulation even though it increased quite significantly the rate of appearance of infectious progeny during single cycles of growth (Fig. 6). Taken together with results reported previously (2, 9, 12, 13, 16, 19, 21–23, 26), these findings suggest that LP1 facilitates a step in the lytic cycle after the synthesis of the virion proteins, i.e., the transport of VP1 molecules to the nucleus or their association with viral minichromosomes to form infectious particles.

After completion of the work reported here, we learned of relevant studies performed recently by Carswell et al. (4, 5) and Resnick and Shenk (24). Their novel findings include the following. (i) As assayed by immunofluorescence staining, VP1 localizes gradually between 40 and 60 h postinfection, first to the perinuclear region and, subsequently, to the nuclear region of wild-type-infected CV-1P cells; this nuclear localization occurs more slowly and to a lesser extent in CV-1P cells infected with LP1$^-$ mutants. (ii) Perinuclear and nuclear localization of VP1 is already apparent by 30 h postinfection, both when cells that constitutively make LP1 are infected with either wild-type virus or LP1$^-$ mutants and when CV-1P cells are infected with the double mutant *dl*-805-R1. (iii) The absence of LP1 does not alter the time course of the nuclear localization of VP2 and VP3, minor proteins of the virion. These findings show directly that LP1 specifically facilitates the nuclear localization of VP1 and that the variant VP1 protein of mutant 805-R1 localizes to the nucleus more rapidly than does wild-type VP1, even in the absence of LP1.

What is the mechanism by which this occurs? One hypothesis is that LP1 plays a direct role in either actively transporting VP1 molecules to the nucleus or, once there, facilitating interactions between VP1 molecules and viral minichromosomes so that VP1 molecules are no longer free to diffuse back to the cytoplasm. However, if this hypothesis is valid, second-site mutations in VP1 that compensate for the absence of LP1 would be quite rare or nonexistent, since the VP1 mutation would have to confer a new function to the protein, i.e., one ordinarily provided by LP1. Even if they could exist, such mutations would probably be confined to a small portion of the VP1 coding region. Contrary to the prediction of this model, mutations arose at a high frequency and map to at least two regions of the VP1 gene (Fig. 3). Therefore, these compensatory VP1 mutations probably reduce or eliminate a normal interaction rather than create a novel one. In addition, Wychowski et al. (35) have recently reported that the first 11 amino-terminal amino acids of SV40 VP1 are sufficient to target a fused polypeptide to the nucleus, even in the absence of LP1, VP2, and VP3. Nevertheless, the two mutations mapped here lie outside of this nuclear migration sequence (Fig. 3). Taken together, these findings suggest that models in which LP1 plays a direct role in transport (4, 24) or virion assembly (13, 16, 21–23) are unlikely to be valid.

As an alternative model, we hypothesize that LP1 facili-

tates the formation of infectious particles by inhibiting, via direct interaction with VP1, the polymerization of VP1 molecules until the time they interact in the nucleus with viral minichromosomes to form virions. In addition to the findings presented above, other facts consistent with this hypothesis include the following. (i) VP1 molecules of the related papovavirus, polyomavirus, self-assemble into capsidlike structures and related polymorphic aggregates (25). (ii) The synthesis of LP1 and VP1 occurs concurrently from a functionally bigenic mRNA; this enables the virus to regulate coordinately the production of these two proteins (3). (iii) Polyomavirus, which lacks a gene analogous to the SV40 LP1 gene, is much less efficient than SV40 in producing infectious progeny, producing instead large quantities of empty capsids and pseudovirions (for a review, see reference 31). Our model requires only that the variant VP1 proteins reported here be defective in polymerization in the absence of viral minichromosomes. This hypothesis explains the observations that (i) cells infected with the VP1 mutant WT (805-R1) accumulated infectious progeny more rapidly than did ones infected with WT800 (Fig. 6), (ii) the variant VP1 protein of the double mutant *dl*-805-R1 localizes to the nucleus in the absence of LP1 more rapidly than wild-type VP1 does in its presence (4), and (iii) mutants with these properties arise with high frequency. Quite likely, numerous amino acids situated throughout the VP1 protein can be altered to generate the phenotype seen with the VP1 mutant 805-R1. Although these mutants overgrew wild-type virus under the laboratory conditions used in this study (Fig. 5), their virions are probably more susceptible than those of the wild type to inactivation under some conditions. Both this prediction and the one that cells infected with $LP1^-$ mutants should produce abnormally large quantities of empty capsids are readily testable. In addition, it should be possible to test directly the validity of our hypothesis by the examination in vitro of the ability of VP1 to self-aggregate in the presence and absence of LP1.

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