JOURNAL OF VIROLOGY, May 1974, p. 1101-1109
Vol. 13, No. 5
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Complementation Analysis of Simian Virus 40 Mutants

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Received for publication 28 January 1974

Seventy six new temperature-sensitive mutants of simian virus 40 have been isolated. A simple modification of the standard plaquing technique permits complementation analyses to be performed readily. By using this technique the new mutants have been divided into four complementation groups.

A number of temperature-sensitive mutants of simian virus 40 (SV40) have been isolated and partially characterized (2, 3, 6, 7, 10–12). Several years ago we started an extensive search for such mutants with the object of saturating the genetic map. We now report the first of a series of studies on 76 mutants.

To facilitate the genetic analysis of such an extensive series of mutants, it was first necessary to develop a relatively rapid technique for complementation analysis. This paper describes how a simple modification of the standard plaquing technique can be used for complementation analysis, and it applies this modification to the new SV40 mutants.

MATERIALS AND METHODS

Virus and cells. The virus stock and CV-1 cells used have been described (4).

Media and plaque assay. Cells were grown to confluence at 37 C on monolayers in nutrient mixture F-12 (Schwarz/Mann, Orangeburg, N.Y.) buffered at pH 7.5 with 50 mM tricine (Calbiochem) and 0.5 g of $NaHCO_3$ per liter, and enriched with 10% fetal calf serum. For plaque assay at 40 C, this medium was removed 2 to 4 days prior to adsorption of the virus, replaced with modified Eagle media enriched with 5% fetal calf serum, 3E5 (6), and incubated at 40 C. No adaptation—incubation of the monolayer with 3E5 prior to virus adsorption—of the cells was required for plaque assay at 34 C. After adsorption at the appropriate temperature for 2 to 4 h the virus suspension was removed, and 1.2% melted Noble agar in 3E5 medium was added and allowed to gel. The addition of fresh medium in agar was not required at 34 C but was required at 3 and 7 days after infection at 40 C. Neutral red (0.04%) in 1.2% Noble agar was added on day 13 after infection, and plaques were counted on day 14.

Mutagenesis. Hydroxylamine mutagenesis was carried out as previously described (6). Portions of the virus-hydroxylamine suspension were removed at various times, treated with acetone in 3E5 media, and titered at 34 C. A reduction in the titer of 100-fold was generally achieved in 18 to 24 h. These stocks were stored at −20 C and used for mutant isolation. Ethyl methane sulfonate (EMS) mutagenesis was carried out as follows. To a fresh solution of 0.2 M EMS in 0.2 M Tris, final pH ~ 7, an equal volume of virus suspension was added and incubation carried out at 37 C. At various times portions were removed, diluted 1 to 10 into 3E5 media, and titered. A reduction in the titer of 10-fold was achieved in 2.5 h. Only one mutant, *201*, was isolated from an EMS-treated stock of virus.

Plaques were picked with sterile Pasteur pipettes, and the agar plug was suspended in 2 ml of phosphate buffered saline (6, 11).

Screening for mutants. Tissue culture plates (Linbro Chemical Co. New Haven, Conn.) containing 24 wells of 16-mm diameter were used. Monolayers of CV-1 cells were grown to confluence and adapted where necessary as described above. Approximately 0.2 ml of the buffered suspension of the agar plugs from discrete plaques was added to each well after the medium had been removed. After adsorption the virus suspension was removed and 1 ml of 3E5 medium in 1.2% agar was added. Identical plates were prepared and incubated at 40 and 34 C. The plates incubated at 40 C were fed with 0.5 ml of 3E5 medium in agar at 3 and 7 days. All plates received 0.2 ml of 0.04% neutral red in agar at 13 days. Wells in which the cells were extensively lysed at 34 C but contained few or no plaques at 40 C were recorded and the corresponding original stocks were then titered accurately at 34 and 40 C. A single plaque from the plates titered at low temperature was then picked and used to prepare a master stock. In this way each mutant master stock was plaque purified twice. Each master stock was again titered at 40 and 34 C, and isolates with greater than 100× difference in titer at the two temperatures were given stock numbers.

Standard complementation. Cells were coinfected for 3 days at 40 C, then lysed by freezing and thawing, and the number of virions was assayed at 34 C (2, 12).

Agar-slant complementation. The 24-well Linbro plates were also used for complementation analysis. After the cells had reached confluence and had been adapted for 2 to 4 days in 3E5 medium at 40 C, the medium was removed and 0.8 ml of 3E5 agar medium was added. The plates were set at a 45° angle and the agar was allowed to gel (see Fig. 1). Next, a few drops of the appropriate stock(s) were added to each well

and, after 2 to 3 h at 40 C, 0.3 ml of additional agar in 3E5 was added. The cells were fed at 3 and 7 days with 0.5 ml of medium and were stained at 13 days.

RESULTS

Isolation of mutants. Plaque-purified low-multiplicity-passaged virus was incubated with hydroxylamine until the virus titers at 34 C were reduced 100-fold (see Materials and Methods). Approximately 10,000 single plaques were picked and tested for their temperature sensitivity. The 76 temperature-sensitive mutants isolated in this manner were further plaque purified and then titered at 34 and 40 C.

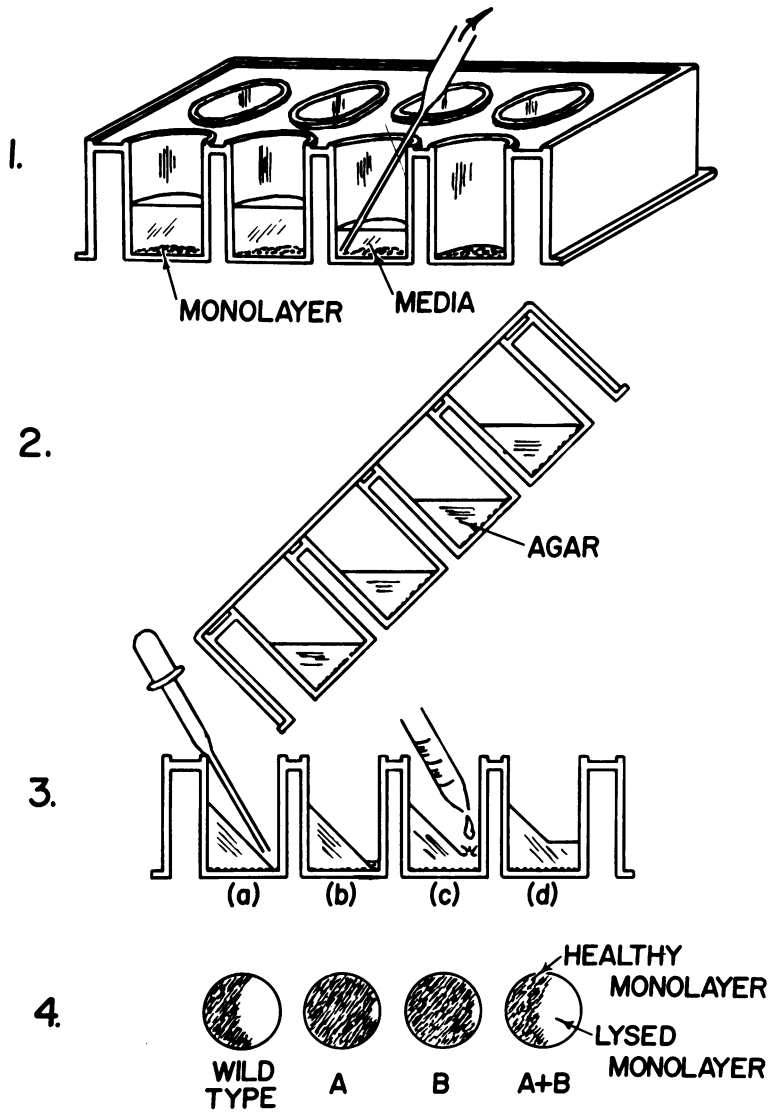

FIG. 1. *Confluent monolayers are prepared and the media are removed (step 1). Next, media with agar are added and allowed to gel while the plates are set at a 45° angle (step 2). A few drops of each of the two viruses to be tested are added (step 3a) and allowed to adsorb (step 3b). Additional agar is added (step 3c), allowed to gel (step 3d), and the plates are then incubated for 13 days at 40 C and stained with neutral red. Typical results for wild type, a mutant of the A and B complementation groups, and the combination are diagrammatically illustrated. There is usually a small area (less than 10% of the monolayer) which is lysed in the control wells containing a single temperature-sensitive mutant either because the cells in that area were destroyed by exposure to air while the agar was setting and/or because the viruses which have been adsorbed at an effective MOI of several hundred have resulted in cytopathic effects upon these cells. Leaky mutants will also lyse more than 10% of the monolayer and are hence unsuitable for this type of complementation analysis.*

The results are given in Table 1. A number of these mutants yield small or minute plaques even at the permissive temperature.

Complementation analysis by the agar-slant technique. The conventional complementation assay involves infection of confluent monolayers at an MOI ≈ 2 with the mutants individually or in combination followed by incubation at the nonpermissive temperature. After 42 (2) or 72 (12) h the monolayers are frozen and thawed, and the resulting lysates are titered at the permissive temperature. On the basis of this test three classes of SV40 mutants have been found (2, 7, 10, 12). Two of the classes, *A* and *B*, complement each other, but neither complement a mutant, *ts*101*, previously described (7). As a result, *101* had been considered to represent a third, noncomplementing class of temperature-sensitive mutants. Similar tests have been performed with some of the mutants listed in Table 1 (see below), but a complete analysis by this method involving a 76 × 76 matrix would be a Herculean task.

A simple technique for complementation analysis applicable to any animal virus was therefore developed (see Materials and Methods). Tissue culture plates having many wells per plate are inoculated with sensitive cells. When the cells have reached confluence, an agar slant covering all but a very small area

TABLE 1. *Temperature-sensitive mutants of SV40*

Strain	PFU			Strain	PFU		
	40 C	33 C	33/40 C		40 C	33 C	33/40 C
Wild type	9×10^7	9×10^7	1	*238*	10^3	1.6×10^7	10^4
101[a]	3×10^2	3.5×10^6	1.1×10^4	*239*	7	3.2×10^7	4×10^5
A30[a]	2×10^3	4×10^6	2×10^3	*240*	100	3×10^7	3×10^5
201	<10	10^7	>10^6	*241*	<10	3×10^7	>3×10^6
202	<10	7.4×10^6	>7×10^5	*242*	<10	1.6×10^7	>1.6×10^6
203	3×10^4	1.2×10^7	4×10^2	*243*	1.2×10^4	8×10^6	600
204	<10	1.2×10^7	>10^6	*244*	100	8×10^6	8×10^4
205	<10	1.6×10^6	>1.6×10^5	*245*	<10	7×10^6	>7×10^5
206	<10	1.6×10^6	>1.6×10^5	*246*	550	7×10^6	1.3×10^4
207	<10	3.5×10^6	>3.5×10^5	*247*	<10	5×10^6	>5×10^5
208	<10	8×10^6	>8×10^5	*248*	2	3×10^7	1.5×10^7
209	<10	1.8×10^6	>1.8×10^5	*249*	<10	1.4×10^6	>1.4×10^5
210	37	10^6	3×10^4	*250*	6	1.6×10^7	2.5×10^5
211	<10	3.2×10^7	>3.2×10^6	*251*	<100	1.3×10^7	>1.3×10^5
212	240	2.2×10^7	8.8×10^4	*252*	1.7×10^4	1.3×10^7	800
213	240	3.2×10^7	10^5	*253*	500	1.6×10^7	3×10^4
214	22	3.2×10^7	10^6	*254*	<10	2.4×10^6	>2.4×10^5
215	<100	3.2×10^7	>3×10^5	*255*	<10	1.3×10^7	>10^6
216	<10	3.2×10^7	>3×10^6	*256*	<100	1.6×10^7	>1.6×10^5
217	<10	3.2×10^7	>3×10^6	*257*	<10	6×10^6	>6×10^5
218	<10	3.2×10^7	>3×10^6	*258*	<100	1.6×10^7	>1.6×10^5
219	100	3×10^7	3×10^5	*259*	10^3	1.6×10^7	1.6×10^4
220	100	3×10^7	3×10^5	*260*	10^2	1.6×10^7	1.6×10^5
221	5	6×10^7	10^7	*261*	10^3	1.6×10^7	1.6×10^4
222	<10^3	1.6×10^7	>1.6×10^4	*262*	<10^2	1.6×10^7	>1.6×10^5
223	<10	3×10^7	>3×10^6	*263*	5×10^3	1.6×10^7	3×10^3
224	10^3	1.3×10^7	1.3×10^4	*264*	<10	1.6×10^7	>1.6×10^6
225	1.2×10^3	6×10^6	5×10^3	*265*	2	1.3×10^7	6×10^6
226	<100	5×10^6	>5×10^4	*266*	1.2×10^4	8×10^6	600
227	<100	5×10^6	>5×10^4	*267*	<10	9×10^6	>9×10^5
228	<10	10^7	>10^6	*268*	<10	8×10^6	>8×10^5
229	<10	3×10^6	>3×10^5	*269*	2×10^4	2×10^7	10^3
230	<10	4×10^6	>4×10^5	*270*	<10	4×10^6	>4×10^5
231	<10	8×10^6	>8×10^5	*271*	40	3×10^7	8×10^5
232	5	10^7	2×10^6	*272*	8×10^2	6×10^6	8×10^3
233	11	1.1×10^7	10^6	*273*	<100	2×10^7	>2×10^5
234	6×10^3	6×10^6	10^3	*274*	<10	2×10^7	>2×10^6
235	10^3	1.6×10^7	1.6×10^4	*275*	100	3×10^7	3×10^5
236	10^3	1.6×10^7	1.6×10^4	*276*	20	2×10^7	10^6
237	<10	3×10^6	>3×10^5				

[a] Strain *101* was previously isolated in this laboratory (6, 7); *A30* was a gift of Peter Tegtmeyer.

of the well bottom is allowed to form. The agar serves as a barrier to prevent adsorption of the virus in all but the uncovered area. After adsorption, additional agar is added and the plates are then incubated at the nonpermissive temperature and stained in the usual manner. Wild-type virions or two mutants which can complement at 40 C can grow, starting in the area where adsorption had not been prevented by the agar slant, and the infection can then spread under the agar by the release of virions to neighboring cells. A uniform healthy monolayer is found for any temperature-sensitive mutant or for any combination of noncomplementing mutants. Wild-type or two complementing SV40 mutants destroy approximately 50 to 60% of the monolayers after 13 days incubation at 40 C (see Fig. 1). The validity of this test was confirmed by the use of some temperature-sensitive mutants kindly provided by P. Tegtmeyer, e.g., *tsA* mutants, *A7* and *A30*, do not complement each other, although each complements *B8*. Applying this technique to the mutants described in Table 1, the matrix shown in Table 2 was obtained.

These results show that the mutants fall into four complementation groups. The mutants of one group (*207*, *209*, *239*, *241*, *255*, and *276*) are unable to replicate viral DNA after infection at the nonpermissive temperature (unpublished results). These mutants do not complement *A7* or *A30* but do complement *B8*. Thus, they belong to the *A* complementation group of Tegtmeyer (10). A second group of mutants (*202*, *222*, *238*, *263*, *270*, and *275*) also unable to replicate viral DNA at the nonpermissive temperature (unpublished results) will henceforth be referred to as group *D*. It will be shown below that the mutant, *101*, previously described as noncomplementing is a member of this group.

The pattern of complementation amongst late mutants, those capable of viral DNA replication at the nonpermissive temperature (unpublished results), appears to be somewhat more complex than previously described. Three groups of mutants can be distinguished: (i) *219*, *240*, *244*, *259*, *260*, *261;* (ii) *201*, *204*, *205*, *212*, *213*, *215*, *218*, *220*, *221*, *227*, *228*, *231*, *232*, *233*, *237*, *242*, *246*, *253*, *262*, *264*, *265*, *267*, *269*, *271*, *273;* and (iii) *206*, *208*, *210*, *211*, *214*, *216*, *217*, *223*, *226*, *229*, *230*, *245*, *247*, *248*, *249*, *250*, *251*, *254*, *256*, *257*, *258*, *268*, *272*, *274*. However, the complementation by these three groups is anomalous, the first and second groups complement each other and the early mutants normally, but fail to complement the third group. Nonetheless, the third group, although incapable of complementing other late mutants, complements normally with early mutants. In a strictly formal sense these results define two complementation groups, *B* and *C*. Mutant *219*, *240*, etc. belong to group *C*, mutants *201*, *204*, etc. belong to group *B* (since *B8* complements *219*), and mutants *206*, *208*, etc. belong to both complementation groups *B* and *C*. Possible explanations for the existence of the *BC* group of mutants are given in the Discussion.

Complementation analysis in liquid media. We previously reported that mutant *101* did not complement other mutants by the standard complementation test. However, this mutant does complement mutants of the *A*, *B*, and *C* groups by the new test.

To resolve this discrepancy between the two complementation techniques more detailed analyses were undertaken with representative temperature-sensitive mutants. Because repeated single-plaque isolates of *101* have yielded virus stocks which have been too leaky for reliable analyses, we have, in general, employed *202* as representative of the *D* complementation group. Tables 3 and 4 again demonstrate that, when complementation is assayed at 72 h by the standard technique, even when the group *A* or *B* partner is supplied at multiplicities of 50 PFU/cell, *D202* fails to complement. However, when the freezing and thawing is performed after 13 days of co-infection at the nonpermissive temperature (Table 5) *D202* complements and with the same pattern seen by the agar-slant technique. Similar results have been obtained after 6 days at the nonpermissive temperature. The results in Table 5 are not the result of multiple cycles of replication since the cells were infected at an MOI of approximately 5 PFU/cell so that essentially all cells were initially infected. Rather, the results seem to indicate that group *D* mutants exhibit delayed complementation. The reason for this delayed complementation is not known.

The data in Tables 4 and 5 further corroborate the existence of two late complementation groups, since *C219* complements *A*, *B*, and *D* mutants in the normal assay of both 3 and 13 days of co-infection.

DISCUSSION

Seventy-six temperature-sensitive SV40 mutants have been isolated. Since approximately 10,000 plaques were assayed the probability (P) that any strain contains two temperature-sensitive mutations is somewhat less than 1 in 100 ($P = 0.0076$). It is therefore not unlikely that at least one of the strains described has two temperature-sensitive mutations ($P = 0.44$),

TABLE 2. *Complementation analysis of SV40 mutants*

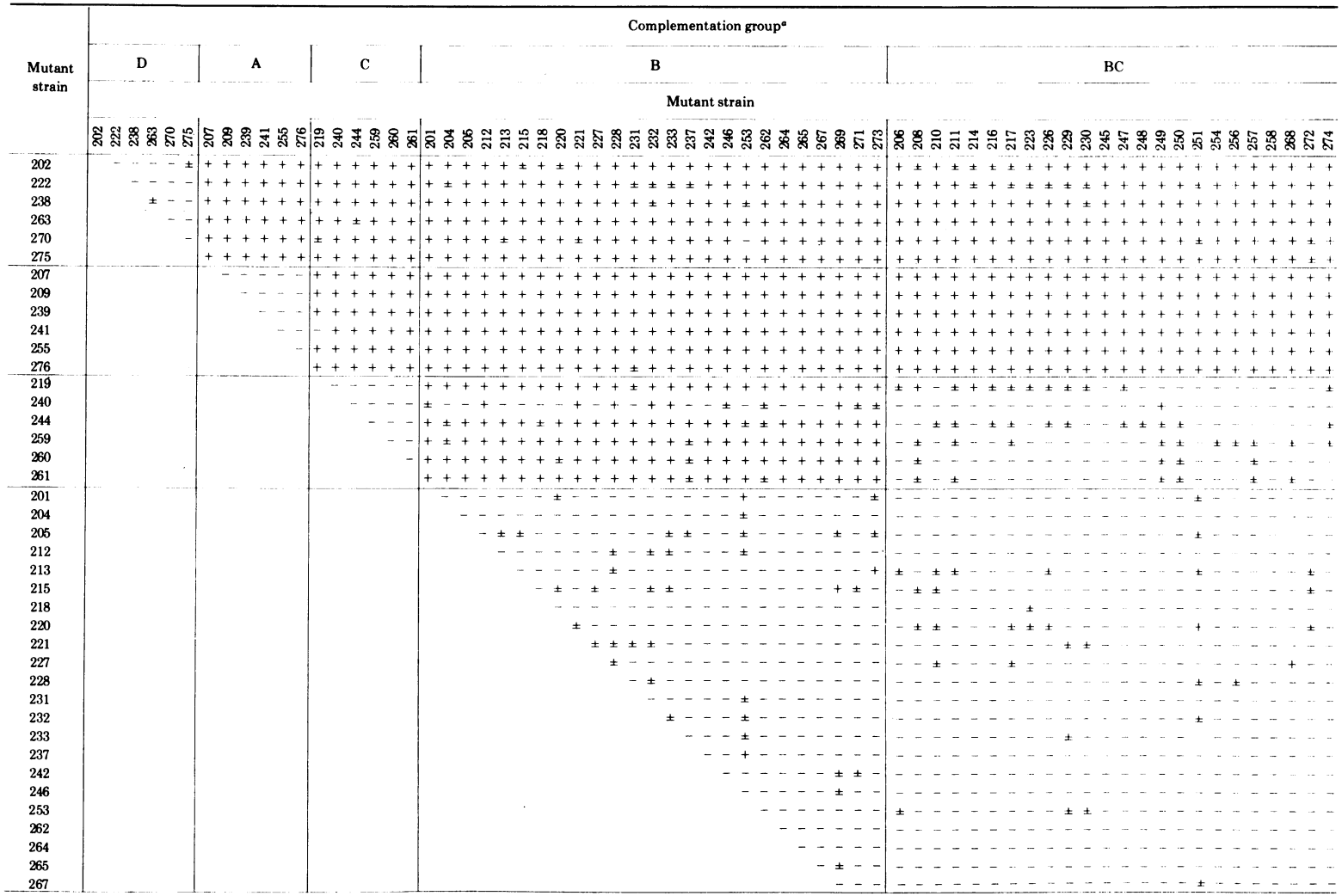

TABLE 2.—*Continued*

Mutant strain	Complementation group[a]																																										
	D						A						C						B																								
	Mutant strain																																										
	202	222	238	263	270	275	207	209	239	241	255	276	219	240	244	259	260	261	201	204	205	212	213	215	218	220	221	227	228	231	232	233	237	242	246	253	262	264	265	267	269	271	273
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268																																											
272																																											
274																																											
101	–						+	+											+	+	+																						
A7	+																																										
A30	+				+			–			–		+	+	+				+	+	+																						
B8	+				+			+			+		+	±	+				–	–	–																						

Mutant strain	BC																							
	206	208	210	211	214	216	217	223	226	229	230	245	247	248	249	250	251	254	256	257	258	268	272	274
269	–	±	–	–	–	–	±	–	–	–	–	±	–	–	±	–	+	±	–	–	+	±	–	–
271	–	–	–	–	±	–	±	–	–	–	–	±	–	–	–	–	–	–	–	–	–	–	–	–
273	–	±	–	–	±	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	±	–	–	±
206		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
208			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
210				–	–	–	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–	–	–
211					–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
214						–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
216							–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
217								–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
223									–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
226										–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
229											–	–	–	–	–	–	–	–	–	–	–	–	–	–
230												–	–	–	–	–	–	–	–	–	–	–	–	–
245													–	–	–	–	–	–	–	–	–	–	–	–
247														–	–	–	–	–	–	–	–	–	–	–
248															–	±	–	–	–	–	–	–	–	–
249																–	–	–	–	–	–	–	–	–
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257																					–	–	–	–
258																						–	–	–
268																							–	–
272																								–
274																								
101	+	+	+																					
A7	+																							
A30			+								+	+												
B8			–								±	±												

[a] Results of the agar slant complementation experiments are represented by: +, complete lysis of at least 30% of the monolayer when neither of the strains alone lysed more than 5 to 10% of the monolayer; –, lysis of less than 10% of the monolayer; and ±, any other result. Many, but not all combinations have been carried out in duplicate, and in such cases a positive (or negative) result in duplicate with an ambiguous result has been recorded as ±.

though it is highly unlikely that four or more are double mutants ($P = 0.0028$). The frequency of temperature-sensitive mutants in the unmutagenized stock was less than 1 in approximately 2,000.

Because of the laborious nature of the technique, prior complementation analyses were carried out on only a limited number of SV40 mutants (2, 7, 12), and only two complementation groups, *A* and *B*, were recognized.

A simple modification of the standard plaque assay technique permits rapid complementation analysis of any two viral mutants. The agar-slant assay corroborated the classification of representative mutants isolated by Tegtmeyer and his collaborators (10–12) into two groups. Of the 76 new mutants, 67 were sufficiently "non leaky" to permit complementation analysis. Of these, six appeared to be in complementation group *A*.

The agar-slant technique revealed the existence of a complementation group, *D*, composed of six new mutants similar to *101* (7). Although it was previously reported that *101* does not complement, the data in Tables 4 and 5 demonstrate that the prior failure to observe complementation is not a function of the mutation itself, but rather of the length of time required to achieve complementation. That is, group *D* mutants of which *101* and *202* are examples exhibit delayed complementation at the nonpermissive temperature.

The complementation analyses also established the existence of two "late" complementation groups, *B* (25 strains) and *C* (6 strains), as well as an anomalous class of mutants which behave as if they belong to both the *B* and *C* complementation groups. One possible explanation for the *BC* group is that all 24 of these strains are double mutants. However, the statistical probability of this is minuscule. Another explanation would assume that all of these strains contain a mutation in only one of the two late cistrons but that a secondary effect of these particular mutations was to reduce the pool of the other gene product. This might occur if, for

TABLE 3. *Virus yields after co-infection for 72 h at 40 C*

Second mutant	MOI	First mutant	MOI	PFU at 34 C	Complementation index[a]
		D101	1	1.8×10^5	
			50	2×10^6	
		D202	1	<10	
			50	1.4×10^4	
		B201	1	5×10^3	
			50	5×10^4	
		B204	1	1.2×10^3	
			50	6×10^3	
		A207	1	80	
			50	6×10^4	
		A209	1	<10	
			50	6×10^2	
D101	1	*D202*	1	1.9×10^5	1
			50	2×10^5	1
D101	1	*B201*	1	1.6×10^5	0.9
			50	4×10^4	0.2
D101	1	*B204*	1	1.6×10^5	0.9
			50	6×10^4	0.3
D101	1	*A207*	1	1.6×10^5	0.9
			50	1.6×10^5	0.8
D101	1	*A209*	1	1.6×10^5	0.9
			50	7×10^4	0.3
D202	1	*B201*	1	1.2×10^3	0.2
			50	2×10^4	0.4
D202	1	*B204*	1	1.6×10^3	1.4
			50	4×10^3	0.7
D202	1	*A207*	1	1.8×10^3	
			50	1.2×10^5	2
D202	1	*A209*	1	4×10^2	
			50	1.2×10^3	2

[a] Complementation index is defined as the number of PFU obtained after mixed infection divided by the sum of the PFU found in the singly infected controls.

example, the *B* and *C* cistrons were normally translated into a single polypeptide chain which was subsequently cleaved, and if the temperature-sensitive mutation affected the cleavage. A somewhat analogous reduction in the pool of an adjacent cistron occurs in polar mutations of bacteria (5). However, a direct analogy to bacterial aystems is not applicable since temperature-sensitive mutants harbor missense mutations and the latter are not polar in bacterial systems (5).

Another explanation for the *BC* group assumes that the *B* and *C* cistrons correspond to the major capsid proteins of SV40. Some members of the *BC* group would contain a mutation in the *B* cistron and others would contain one in the *C* cistron, their common characteristic being that the virions produced by these mutants would be highly sensitive to disruption at elevated temperature upon co-infection with other late mutants. Such strains might complement early mutants which provide the normal products of both late cistrons. Such strains, might, nonetheless, fail to complement late mutants of the heterologous cistron because the presence of even small amounts of the abnormal heterologous protein in some capsomeres would make the complemented virions hypersensitive to thermal disruption. Virions of the *BC* group strains are not themselves invariably hypersensitive to thermal inactivation (unpublished results).

Of the 67 mutants analyzed by complementation tests, 12 (18%) are in "early" functions. Current estimates by physical mapping of early and late mRNA suggest that the "early" region corresponds to 30 to 40% of the genome (1, 4, 9). Thus, early mutants appear to be under represented by about a factor of 2 (18% versus 30 to 40%) in our mutant collection. This result does not seem surprising in the least. Temperature-sensitive mutants are presumed to result from subtle changes in the ability of a protein to form the appropriate tertiary conformation as a function of temperature. Structural components,

TABLE 4. *Virus yields after co-infection for 72 h at 40 C*

Mutant	Mutant[a]			
	A209	*B205*	*C219*	*D202*
A209	5×10^2	9×10^{6b} (450)	4×10^6 (100)	4×10^4 (6.1)
B205		2×10^4	2.4×10^6 (40)	1.4×10^5 (5.4)
C219			4×10^4	6×10^4 (1.3)
D202				6×10^3

[a] All infections were carried out at an MOI of 5 PFU/cell of each virus type.

[b] Numbers not in parentheses are PFU determined at 34 C; numbers in parentheses are complementation indices.

TABLE 5. *Virus yield after co-infection for 13 days at 40 C*

Mutant	Mutant[a]								
	A207	*A209*	*A239*	*B201*	*B204*	*C219*	*D101*[b]	*D202*	*D238*[b]
A207	1.3×10^4	3.2×10^{3c} (0.3)[c]	5×10^4 (2)	1.4×10^6 (88)	1.6×10^6 (123)	1.3×10^6 (27)	1.2×10^7 (24)	1.4×10^6 (66)	10^7 (20)
A209		<10	2×10^4 (2)	4×10^5 (133)	4×10^5 (>10^4)	4×10^5 (11)	2×10^6 (4)	6×10^5 (75)	1.2×10^6 (2.4)
A239			10^4	8.6×10^5 (66)	1.5×10^6 (150)	10^6 (22)	1.3×10^6 (2.6)	2×10^6 (111)	8×10^6 (16)
B201				2.8×10^3	10^4 (3)	10^6 (26)	6×10^6 (12)	2×10^6 (185)	4×10^6 (8)
B204					<10	6×10^5 (17)	3×10^6 (6)	3×10^6 (380)	2×10^6 (4)
C219						3.5×10^4	10^6 (2)	10^6 (24)	1.4×10^6 (3)
D101							5×10^5	5×10^5 (1)	10^6 (1)
D202								8×10^3	4.7×10^5 (1)
D238									4.7×10^5

[a] All infections were carried out at an MOI of 5 PF /cell of each virus type.

[b] The relatively low complementation indices obta d with *D101* and *D238* reflect the relative leakiness o these mutants.

[c] Numbers not in parentheses are PFU determine t 34 C; numbers in parentheses are complementatio indices.

such as capsomeres, which are composed of relatively large numbers of identical subunits would be particularly sensitive to even the smallest conformational changes.

Although the total number of separate cistrons within SV40 has not been determined by this study, the fact that each complementation group now contains at least six representative mutants makes it unlikely that further mutant collection of the type described will reveal any additional cistrons. On a statistical basis it is highly probable that SV40 requires only four cistrons for lytic replication of the virus.

ACKNOWLEDGMENT

We thank Peter Tegtmeyer for providing us with his mutants.

ADDENDUM IN PROOF

Since this paper was submitted a C mutant similar to the ones described in this paper has been reported, D. R. Dobbs, M. Rachmeler, and S. Kitt, 1974, Recombination between temperature-sensitive mutants of Simian Virus 40. Virology **57**:161-174.

LITERATURE CITED

1. Khoury, G., and M. A. Martin. 1972. Comparison of SV40 transcription *in vivo* and *in vitro*. Nature N. Biol. **238**:4-7.
2. Kimura, G., and R. Dulbecco. 1972. Isolation and characterization of temperature-sensitive mutants of simian virus 40. Virology **49**:394-403.
3. Kit, S., S. Tokuno, K. Nakajima, D. Trkula, and D. R. Dubbs. 1970. Temperature-sensitive simian virus 40 mutant defective in a late function. J. Virol. **6**:286-294.
4. Lindstrom, D. M., and R. Dulbecco. 1972. Strand orientation of SV40 transcription in productively infected cells. Proc. Nat. Acad. Sci. U.S.A. **69**:1517-1520.
5. Martin, R. G. 1969. Control of gene expression, p. 181-216. *In* H. L. Roman (ed), Annual review of genetics, vol. 3. Annual Reviews, Inc., Palo Alto, Calif.
6. Robb, J. A., and R. G. Martin. 1970. Genetic analysis of simian virus 40. I. Description of microtitration and replica plating techniques for virus. Virology **41**:751-760.
7. Robb, J. A., and R. G. Martin. 1972. Genetic analysis of simian virus 40. III. Characterization of a temperature-sensitive mutant blocked at an early stage of productive infection in monkey cells. J. Virol. **9**:956-968.
8. Robb, J. A., P. Tegtmeyer, R. G. Martin, and S. Kit. 1972. Proposal for a uniform nomenclature for simian virus 40 mutants. J. Virol. **9**:562-563.
9. Sambrook, J., P. A. Sharp, and W. Keller. 1972. Transcription of SV40 DNA and hybridization of the separated strands to RNA extracted from lytically infected and transformed cells. J. Mol. Biol. **70**:57-71.
10. Tegtmeyer, P. 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591-598.
11. Tegtmeyer, P., C. Dohan, and C. Reznikoff. 1970. Inactivating and mutagenic effects of nitrosoguanidine on simian virus 40. Proc. Nat. Acad. Sci. U.S.A. **66**:745-752.
12. Tegtmeyer, P., and H. L. Ozer. 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. **8**:516-524.