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Viral DNA Synthesis in Cells Infected by Temperature-Sensitive Mutants of Simian Virus 40

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Temperature-sensitive mutants of simian virus 40 (SV40) have been classified as those that are blocked prior to viral DNA synthesis at the restrictive temperature, "early" mutants, and those harboring a defect later in the replication cycle, "late" mutants. Mutants of the *A* and *D* complementation groups are early, those of the *B*, *C*, and *BC* groups are late. Our results confirm earlier reports that *A* mutants are defective in a function required for the initiation of each round of viral DNA synthesis. *D* mutants, on the other hand, continue viral DNA replication at the restrictive temperature after preincubation at the permissive temperature. The length of time required for *D* function to be expressed at the permissive temperature—after which infection proceeds unabated on shifting of the cultures to the restrictive temperature—is 10 to 20 h. The viral DNA synthesized in *D* mutants under these conditions progresses in normal fashion through replicative intermediate molecules to mature component I and II DNA molecules.

We previously reported the isolation of 67 temperature-sensitive mutants of simian virus 40 (SV40) and their classification into four complementation groups, *A*, *B*, *C*, and *D* (1). Mutants of the *A* and *B* groups are similar to those previously isolated by Tegtmeyer and his associates (10, 11). In addition to the six mutants of the *C* complementation group which were found, a relatively large group of mutants, 25, failed to complement with mutants of either the *B* or the *C* groups and were therefore classified as *BC* mutants. Several alternative explanations for the existence of this group have been presented (1). It is probable that the class I, class II, and the unassigned mutants of Kimura and Delbecco (5) correspond to our *B*, *C*, and *BC* groups. Similar mutants have also been described by Dubbs et al. (2).

Mutants of the *D* complementation group (which includes *ts101* [8]) behave anomalously in that complementation in CV-1 cells is delayed beyond the time normally required for a single cycle of virus replication. Mutants of this group fail to enhance adenovirus production in monkey kidney cells (M. Jerkofsky, unpublished data). Other mutants which fail to enhance have been described (4, 6).

This paper explores the capacity of these temperature-sensitive mutants to synthesize viral DNA under a variety of conditions.

MATERIALS AND METHODS

Media. Nutrient mixture F-12 (F) or modified Eagle medium (3E) buffered with tricine as previously described were used (1, 7). Fetal calf sera of different concentrations were used to enrich the media and are so indicated, e.g., F-12 medium enriched with 10% serum is referred to as F10, modified Eagle medium enriched with 2% serum is 3E2 or unenriched is 3E0, etc. The depleted medium was 3E5 which was removed from uninfected cell cultures after 4 to 5 days of incubation and refiltered.

Virus and cells. The viral mutants and CV-1 cells used have been described (1, 7). Stocks of the temperature-sensitive mutants were prepared by infecting CV-1 monolayers at low multiplicity of infection (MOI) (approximately 0.01 PFU/cell) at 34 C and allowing the cells to proceed to lysis in 3E5. The infected cultures along with mock-infected controls were frozen and thawed, passed through a 0.22-μm filter (Nalgene filter unit, Sybron Corp., Rochester, N.Y.), and stored at −20 C.

Adsorption of virus. Media were removed from confluent monolayers grown in F10, and the cells were adapted when necessary with 3E5 at 41 C as previously described (1). Next, 0.5 ml of the virus stock was added per 25-cm^2 flask (Falcon tissue culture flask no. 3012, Falcon Plastics, Oxnard, Calif.), giving an MOI of 2 to 5 PFU/cell, and adsorption was allowed to proceed at the appropriate temperature for 2 to 3 h. The virus fluid was aspirated, and the cells were allowed to incubate in the desired media.

For the initial screening of mutants (data in Table 1) the 24 well plates of Linbro Chemical Co., (New

Haven, Conn.) were used. The medium was changed to 3E5 (1 ml per well), after confluence had been reached in F10. Each well then received 10 to 25 μliters of the stock virus, and the plates were immediately incubated for 48 h at 34 or 41 C.

Radioactive labeling. Either [2-^{14}C]thymidine, 55 μCi/μmol or [*methyl*-^{3}H]thymidine, 20 mCi/μmol (New England Nuclear Corp., Boston, Mass.) was used, except in the double-label experiment when the [^{3}H]thymidine was diluted to a final concentration of 1 mCi/μmol.

In the initial screening of cells infected with the different mutants, the media were removed after 48 h, and 0.4 ml of 3E1 containing 0.5 μCi of [^{14}C]thymidine per ml was added per well. The labeling was then allowed to proceed for 20 to 24 h. In the experiments in which the rate of viral DNA synthesis was to be followed as a function of time, the unlabeled media from infected cultures were removed at the appropriate times and 2 ml of 3E0 with 0.5 μCi of [^{14}C]thymidine per ml were added for 1 h at the indicated temperature. In the shift-up experiments in which labeling was carried out for 10-min intervals after a temperature shift from 34 to 41 C or for 20-min intervals after a shift from 41 to 34 C, the labeling was performed by removing the old media and adding 2 ml of prewarmed 3E0 containing 2 μCi of [^{14}C]thymidine per ml. In the experiments in which the viral DNA was to be subjected to electrophoresis, the labeling was carried out with 2 ml of [^{3}H]thymidine in 3E0 (100 μCi/ml) for 10 min at 41 C or for 20 min at 34 C. In the double-label experiment the infected cultures were incubated for 20 min with 2 ml of 3E0 containing 25 μCi of [^{14}C]thymidine at 34 C. This medium was then removed, and 3 ml of 3E0 medium enriched with 0.1 mM unlabeled thymidine prewarmed to 41 C was added. After 50 min at 41 C, 1.5 ml of 3E0 with [^{3}H]thymidine at 300 μCi/ml was added to each flask, and the incubation was stopped after an additional 10 min of incubation at 41 C.

Temperature control. In all experiments involving rapid temperature shifts, the medium to be used was prewarmed to the appropriate temperature and the flasks were tightly sealed and immersed in thermostated water baths.

DNA extraction. After the radioactive media had been removed, the cultures were washed 2 to 3 times with excess phosphate-buffered saline (7) and then extracted by the method of Hirt (3). Each well of the Linbro plates received 0.4 ml of the EDTA-sodium dodecyl sulfate (SDS) solution, whereas the flasks received 1.0 ml each. The extracts were brought to 1 M in NaCl by the addition of 5 M salt. After overnight incubation at 4 C, 100-μliter samples of the supernatant material from the wells of the Linbro plates were carefully removed and directly precipitated as described below. In all experiments but those described in Table 1, the supernatant solutions were separated from the precipitates by centrifugation as described by Hirt (3). Samples to be analyzed by electrophoresis were dialyzed against 10 mM EDTA, 10 mM Tris-hydrochloride, final pH of 7.5.

Preparation of samples for counting. The supernatant solutions of the Hirt extraction procedure were precipitated with an equal volume of 10% cold trichloroacetic acid. The precipitates of the extraction procedure were dissolved in 3 ml of water and were precipitated with an equal volume of 10% trichloroacetic acid. The trichloroacetic acid precipitates were collected on GFC glass fiber filters, and washed extensively with 5% trichloroacetic acid followed by washes with 6 ml of cold 0.6 M HCl and 6 ml of cold 95% ethanol. The filters were dried, placed into scintillation vials containing 5 or 10 ml of Liquiflor (New England Nuclear Corp.), and counted in a Nuclear-Chicago Mark III scintillation counter.

The sample gels which had been subjected to electrophoresis were removed from the glass tubes and sliced into approximately 25 fractions. Each slice was allowed to incubate in triton-toluene for 24 h before counting.

RESULTS

Viral DNA replication of the temperature-sensitive mutants. Each of the mutants were tested for their ability to synthesize viral DNA at the restrictive temperature by using a slight modification of the procedure of Hirt (see Materials and Methods). The results in Table 1 demonstrate that *A* and *D* mutants are "early" (incapable of viral DNA replication at 40 C), whereas *B*, *C*, and *BC* mutants are "late" (capable of viral DNA replication at 40 C). In addition, it appears that *C* mutants make relatively less viral DNA at 40 than 33 C, whereas the wild-type, *B* and *BC* mutants make relatively more DNA at 40 than at 33 C. Representative mutants of each group were then retested by the standard procedure of Hirt (3) with similar results (Table 2), except that the distinction between wild-type, *B* and *C* mutants was less apparent.

All of the mutant virions, with the exception of the *D* class, induced host DNA replication as effectively as wild-type virions at the restrictive temperature (Table 2). Although the stimulation of host DNA synthesis was reduced in all of the *D* mutants, all but *D202* did induce some host DNA replication after 24 h at the restrictive temperature.

To document further the inability of *A* and *D* mutants to synthesize viral DNA at the restrictive temperature and the inability of *D202* to induce host DNA synthesis, a series of cultures infected with various mutants alone, or in combination, were incubated at the restrictive temperature in 3E2 medium and pulsed with radioactive thymidine at varying times postinfection. Some of the results are illustrated in Fig. 1 and 2. It can be seen that *A207*, *A209*, and *D202* do not synthesize appreciable amounts of viral DNA at the restrictive temperature at any

TABLE 1. *Viral DNA synthesis in temperature-sensitive mutants of SV40*

Mutant	Expt 1		Expt 2		Expt 3		Expt 4		Expt 5		Expt 6		Expt 7		Expt 8	
	40 C[a]	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C
Wild type	2,710	1,116	2,514	445	2,465	607	2,067	918	2,322	860			3,015	2,380		
A30			147	380	200	1,412	120	2,266	270	1,715						
A207			122	581	80	1,895										
A209			2	110	56	1,490										
A239													110	4,175		
A241													156	3,493		
A255													100	2,201		
A276															67	1,062
B201	1,120	1,510														
B204			3,842	521												
B205			7,912	1,800												
B212					5,127	1,701										
B213							4,571	2,623								
B215							3,103	1,892								
B218							2,603	1,725								
B220							4,407	2,571								
B221							1,920	2,077								
B227							3,537	3,390								
B228							2,610	1,310								
B231											2,044	1,873				
B232											2,602	1,755				
B233											2,201	1,875				
B237											1,414	1,900				
B242													5,430	5,553		
B246													2,501	2,310		
B253													3,697	3,028		
B262													3,766	4,940		
B264													3,230	3,451		
B265													3,280	4,081		
B267													4,445	3,874		
B269															3,593	3,042
B271															3,193	1,907
B273															1,935	1,332
C219							908	2,058								
C240											937	3,104				
C244											885	1,896				
C259											1,004	2,395				
C260											975	3,103				
C261											1,011	3,665				
BC206			2,414	497												
BC208			2,834	297												
BC210			546	80												
BC211					5,000	3,096										
BC214							3,655	1,780								
BC216							2,792	1,726								
BC217							3,208	2,030								
BC223									1,533	2,226	2,948	3,217				
BC226							2,556	2,045								
BC229									2,773	1,233						
BC230											2,371	2,953				
BC245													3,725	3,925		
BC247													4,301	3,996		
BC248													3,650	3,382		
BC249													1,613	694	1,614	393
BC250													4,173	4,921		
BC251													1,181	2,264		
BC254													461	167	1,092	275
BC256													861	3,839		
BC257													1,769	1,681		
BC258													1,387	4,462		
BC268															2,453	2,381
BC272															940	842
BC274															2,007	408
D101	510	1,850														
D202	30	830			46	677									7	1,841

TABLE 1—*Continued*

Mutant	Expt 1		Expt 2		Expt 3		Expt 4		Expt 5		Expt 6		Expt 7		Expt 8	
	40 C[a]	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C	40 C	33 C
D222							205	2,452	184	1,520			545	4,008		
D238											94	2,474				
D263													757	3,698		
D270															46	722
D275															227	1,827

[a] After incubation for 48 h at 40 or 33 C, cultures were pulse labeled with [^{14}C]thymidine at the respective temperatures, and the supernatant fractions of the modified Hirt procedure prepared and precipitated with trichloroacetic acid as indicated in Materials and Methods. The values listed represent counts per minute in the infected minus mock-infected controls. All mocks had 100 to 200 counts/min.

TABLE 2. *Host and viral DNA synthesis 24 h after infection of CV-1 cells at permissive and restrictive temperatures with selected temperature-sensitive mutants*

Mutant	Counts/min incorporated at 40 C		Counts/min incorporated at 34 C	
	Host[a]	Viral	Host	Viral
A30	24506 (3.6)	770	29053 (5.6)	39878
A207	18068 (2.9)	98	18010 (3.8)	11182
A209	18053 (2.9)	230	17692 (3.8)	11733
A255	8991 (1.9)	0	25160 (5.0)	5372
B201	34262 (4.6)	27375	13213 (3.1)	40512
B253	25166 (3.7)	11244	35648 (6.6)	30750
B269	37214 (4.9)	12486	41243 (7.5)	14352
C219	18374 (2.9)	5917	38242 (7.0)	14900
C240	23956 (3.5)	4297	10060 (2.6)	15075
C260	26724 (3.8)	5113	23856 (4.8)	16815
D202	0 (1.0)	10	13030 (3.1)	16140
D222	3612 (1.4)	37	25141 (5.0)	29358
D238	3173 (1.3)	0	29323 (5.6)	12447
D263	11180 (2.2)	0	19738 (4.1)	11222
D270	6002 (1.6)	3	22830 (4.6)	5470
D275	2101 (1.2)	0	15617 (3.5)	16113
Wild type	19592 (3.1)	21254	30567 (5.8)	41410

[a] Monolayers in 25-cm^2 flasks were infected with virus at an MOI of 2 to 5. After 2 to 3 h of adsorption at the appropriate temperature, 3EO medium was added and cultures were incubated at the appropriate temperature for 24 h. The cultures were then labeled (see Materials and Methods) for 1 h at 34 or 40 C and extracted by the Hirt procedure. Host refers to the trichloroacetic acid-precipitable counts per minute in the salt precipitates and viral to the trichloroacetic acid-precipitable counts per minute in the supernatant fractions. The values for the mock-infected controls have been subtracted. The mock-infected cultures at 40 C gave values (counts/min) of: host, 9,480; viral, 496; and at 34 C: host, 6,360; viral, 470. The values in parenthesis are the ratios of mutant to mock counts per minute (without the subtraction of mock values) so that 1.0 represents no stimulation.

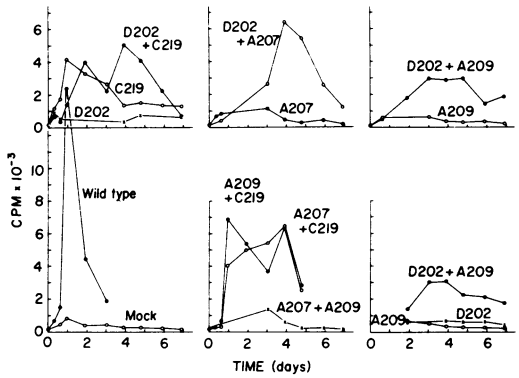

FIG. 1. *Viral DNA synthesis at 40 C in cells infected with the temperature-sensitive mutants alone or in combination. Confluent monolayers of CV-1 cells were infected with virus at an MOI of 2 to 5 as described in Materials and Methods and were incubated at 40 C. At the times indicated, the cultures were pulse labeled at 40 C with [^{14}C]thymidine, and the supernatant fractions of the unmodified Hirt extraction procedure (3) were precipitated and counted. The cultures in the lower right-hand figure were shifted to 34 C for 2 h prior to the pulse labeling at 34 C.*

time after infection, whereas *C219* does. *D202* does not induce host DNA synthesis at the restrictive temperature (Fig. 2), although *A*, *C*, and *B* (not shown) mutants do.

Interestingly, when cells are coinfected with *D202* and mutants of the *A*, *B* (not shown), or *C* classes, viral DNA synthesis proceeds with unusual kinetics (Fig. 1). The previous observation that complementation could not be observed with *D* mutants at 72 h postinfection correlates with the observation that viral DNA synthesis is only just beginning to reach its maximum rate at 72 h when *A207* or *A209* coinfect a *D202*-infected culture. However, because *A* mutants do not synthesize viral DNA at 41 C, the activity observed presumably reflects normal *A* function supplied by the *D* mutant. As described below, *A209* fully recovers function at the permissive temperature after at least 1.5 h at the restrictive temperature. Therefore, to

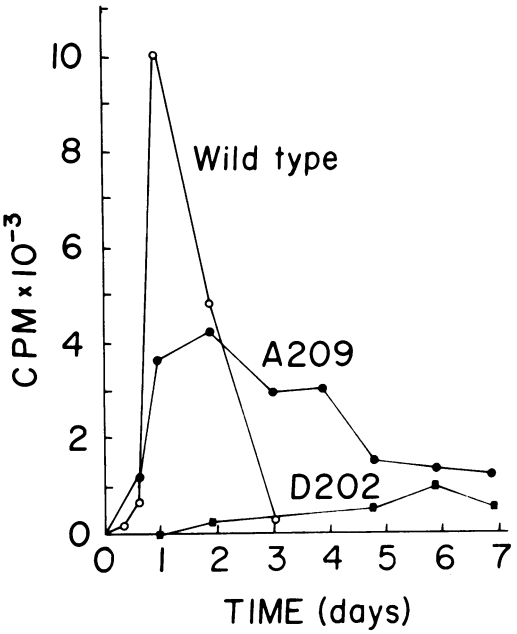

FIG. 2. *Host DNA synthesis in mutant-infected cultures. The precipitates from the Hirt extraction procedure (3) obtained from the experiment illustrated in Fig. 1 were treated as described in Materials and Methods.*

determine if the synthesis of the mutant *A* function of *A209* (which cannot be expressed at the restrictive temperature) followed a different time course, coinfected cultures were also shifted to the permissive temperature for labeling (compare upper and lower right-hand graphs of Fig. 1). Again, maximal DNA synthesis did not appear until after nearly 72 h postinfection. The kinetics of viral DNA synthesis on coinfection of *D202* and wild type are indistinguishable from wild type alone. An extensive analysis of the progeny of complementation experiments will be the subject of a future communication (Chou and Martin, unpublished data).

Establishment and maintenance of viral DNA replication. Tegtmeyer (9) demonstrated that *A* mutants are defective in the maintenance of viral DNA replication. If infection is allowed to proceed at the permissive temperature for 72 h and the cultures are then shifted to the restrictive temperature, the rate of viral DNA synthesis rapidly drops. Figure 3 corroborates those findings with a number of new *A* mutants. *A209* shows the most dramatic response, viral DNA synthesis being completely halted after only 30 min at 41 C. Similar results were obtained when BSC-1 cells were used instead of CV-1 cells. Returning these cultures to the permissive temperature after 90 min at 41 C results in a rapid resumption of viral DNA synthesis.

Cells infected with *D* mutants for 72 h at the permissive temperature continue to synthesize viral DNA when shifted to the restrictive temperature (Fig. 3). To ensure that the viral DNA synthesis observed after "shift-up" of the *D* mutants represented normal viral DNA replication, representative mutants of each class were incubated for 72 h at the permissive temperature, then shifted to 41 C and immediately pulsed for 10 min with radioactive thymidine. Supernatant material from the Hirt extraction procedure was subjected to agarose gel electrophoresis (9). The ratio of replicative intermediate to mature viral DNA (component I and II) was the same in *D* mutants as in wild type and one representative each of the two late mutant classes (Fig. 4, left-hand side). Only mutants of the *A* group showed any deviation, and this, as

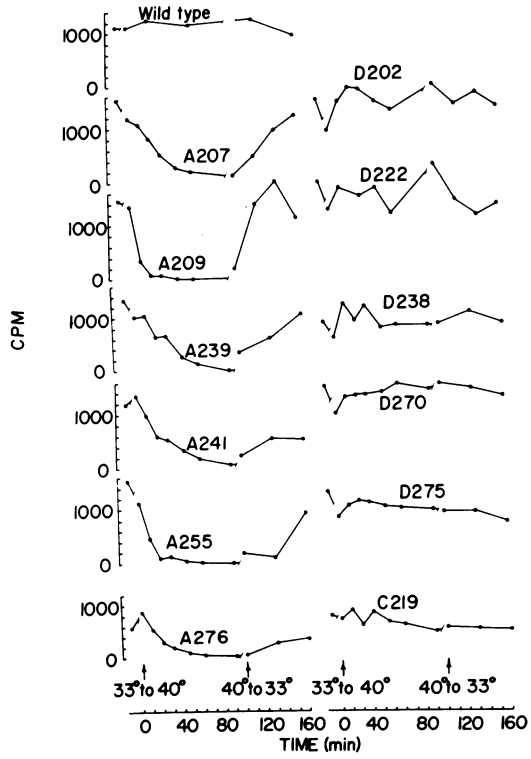

FIG. 3. *Viral DNA synthesis in temperature-sensitive mutants after temperature shifts. Infected cultures incubated for 72 h at 34 C were pulse labeled for 20 min. Parallel cultures were then shifted to 41 C and pulse labeled for 10 min from 0 to 10, 10 to 20 min, etc., after the shift. Additional cultures were shifted back to 34 C after 90 min at 41 C and pulse labeled for 20 min at 34 C at the times indicated.*

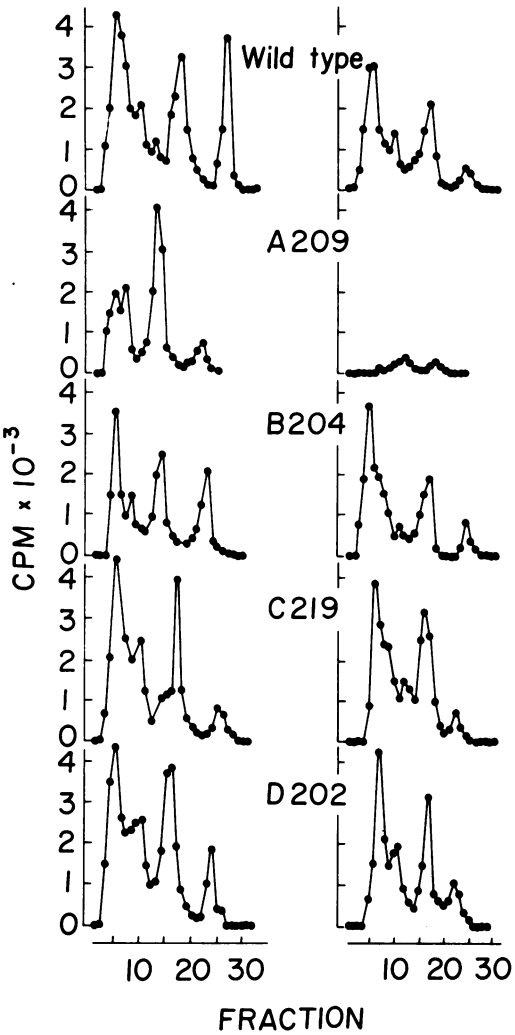

FIG. 4. *Electrophoresis of pulse-labeled viral DNA. Mutant-infected cultures were pulse labeled as described from 0 to 10 min after shift to 40 C (left-hand figures) or from 50 to 60 min after the shift (right-hand figures), and the supernatant fractions were subjected to electrophoresis according to the method of Tegtmeyer (9). The most rapidly migrating species is component I supercoiled viral DNA, the second peak corresponds to component II viral DNA, and the third peak corresponds to replicative intermediate forms.*

expected, was a reduction in the relative amount of replicative intermediate molecules as compared to complete viral DNA molecules. When the pulse labeling was delayed to 50 min (Fig. 4, right-hand side) after the shift to the restrictive temperature, the pattern remained the same except for the *A* mutants, which failed to incorporate any thymidine.

To determine whether replication initiated at 33 C is completed after shifting to 41 C, cultures of various mutants were incubated at the permissive temperature for 72 h. Cultures were then pulse labeled for 20 min prior to the temperature shift. Immediately upon shifting the infected cultures to 41 C, the media was changed and a "chase" with cold 1 mM thymidine was allowed to proceed for 1 h at 41 C. In all cases (Fig. 5), including the *A* mutants, the radioactivity in the replicative intermediates was completely chased into mature DNA forms. These results support the suggestion of Tegtmeyer (9) that *A* mutants are blocked at the initiation of viral DNA synthesis, but not in the elongation of replicating chains.

The chase conditions of the preceding experiment were such (1 mM thymidine) as to minimize the initiation of new viral DNA replication even with wild-type virions. These conditions were chosen so that the fate of the pulse-labeled replicative intermediate molecules of *A209* synthesized prior to the chase and shift could be followed carefully. As the results clearly show, all of the replicative intermediate molecules mature to component I and II after 60 min at the restrictive temperature. To insure that some artifact was not introduced by the high level of cold thymidine during the chase, the experiment was repeated with 0.1 mM thymidine, adding an additional labeling period with a second radioactive isotope from 50 to 60 min after the shift. Replicative intermediate molecules continued to be formed normally in the wild-type control after 50 min at 41 C, whereas *A209* had ceased all synthesis (Fig. 6). By this time all of the replicative intermediate molecules labeled prior to the shift had been converted to mature forms in both wild-type and *A209*-infected cultures.

The observations (i) that viral DNA synthesis in *D* mutants does not occur at the restrictive temperature (ii) but that after 72 h at the permissive temperature followed by shift-up it continues unabated, (iii) that replicative intermediate molecules are synthesized normally, and (iv) that replicative intermediate molecules mature to component I and II molecules suggest that the *D* function is required for the establishment of the viral DNA replication system, but not for its maintenance.

Localization of the time when D function is required. It is apparent from the above experiments that the block in viral replication with *D* mutants precedes viral DNA synthesis. In a previous investigation of *D101* it was demonstrated that adsorption and penetration proceeded normally at the nonpermissive temperature (8).

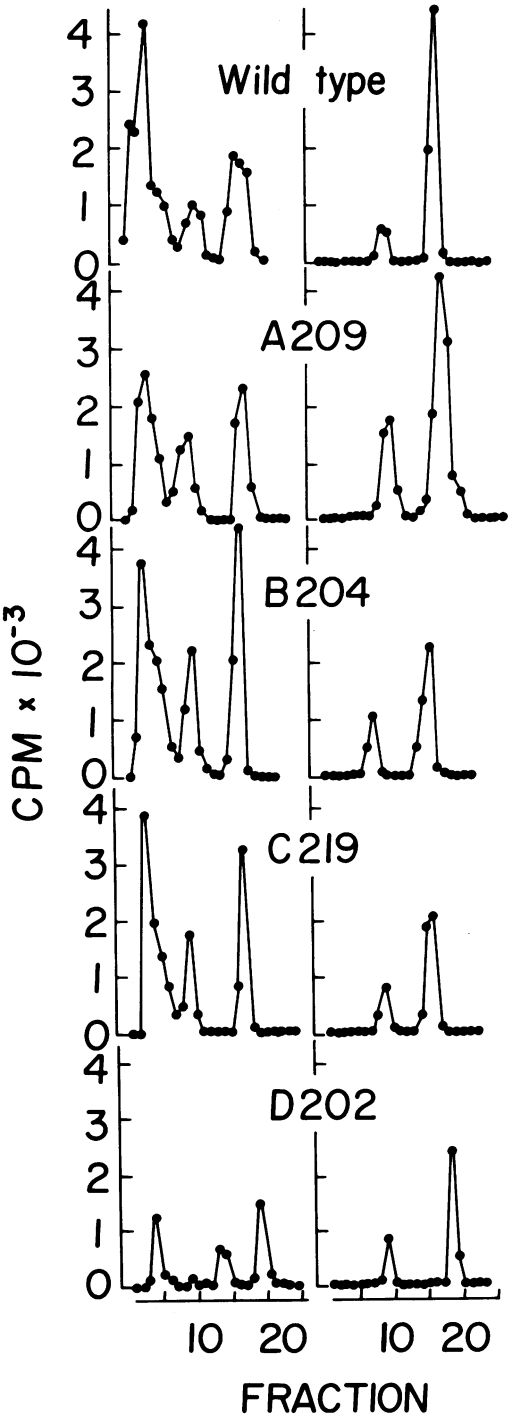

FIG. 5. *Electrophoresis of viral DNA after pulse labeling and chase. Infected cultures were labeled for 20 min at 34 C after 72 h of incubation at 34 C (left-hand figures). The cultures were then changed to medium containing 1 mM thymidine and incubated at 41 C for 1 h (right-hand figures). The supernatant fractions of the Hirt extraction procedure were treated as in Fig. 4.*

To localize more precisely the point in time when *D* function is required for replication, a series of cultures were incubated in depleted media at the permissive temperature for varying lengths of time. The cultures were then shifted to the restrictive temperature, and incubation was allowed to proceed until 46 h postinfection. At this time the cultures were pulsed with radioactive thymidine to estimate the rate of viral DNA synthesis. This assay is thus an estimate of the capacity of the mutant to replicate viral DNA subsequently at the restrictive temperature, as a function of incubation time at the permissive temperature. As a control, the rate of viral DNA synthesis at the permissive temperature was also determined.

The capacity of *D* mutants to synthesize viral DNA increases dramatically between 10 and 20

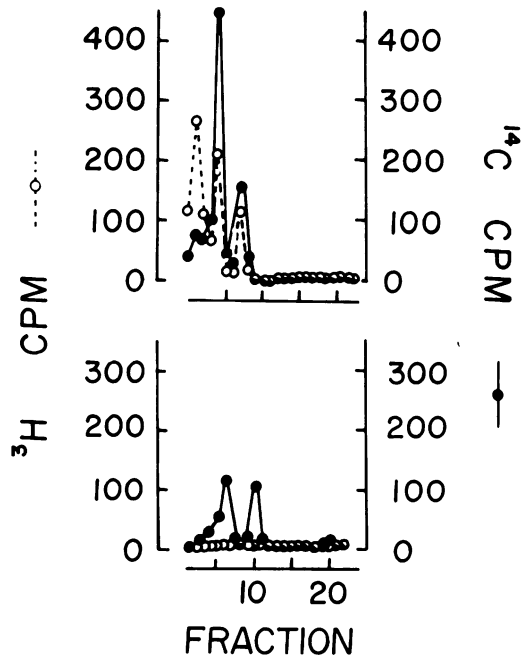

FIG. 6. *Electrophoresis of wild-type and A209 viral DNA in a pulse-chase-pulse experiment. Wild-type- (upper figure) and A209- (lower figure) infected cultures were incubated for 72 h at 34 C. They were then labeled with [^{14}C]thymidine for 20 min (right-hand scale), shifted to fresh medium containing 0.1 mM thymidine at 41 C, and incubated 50 min, at which point [^{3}H]thymidine (left-hand scale) was added for 10 min. The supernatant fraction of the Hirt extraction procedure was subjected to electrophoresis as in Fig. 4.*

h postinfection at the permissive temperature (Fig. 7). The "tightest" *D* mutant, *D202*, is incapable of any subsequent viral DNA synthesis prior to 10 h of incubation at 34 C, whereas the "leakier" mutants, *D222* and *D270*, synthesize some viral DNA even when the entire incubation is carried out at 41 C (the zero time points on the graphs). However, even these latter mutants show a significant increase in their ability to synthesize viral DNA when the infection is allowed to proceed for 10 to 20 h prior to the temperature shift. Late mutants analyzed by the same type of shift experiment show a maximal capacity for subsequent viral DNA synthesis even when the total infection is carried out at the restrictive temperature. The control with mutant *A209* is of little significance because *A* mutants rapidly lose the capacity to synthesize viral DNA at the restrictive temperature.

The permissive temperature controls indicate that the increased capacity for subsequent synthesis after 20 h of incubation at the permissive temperature occurs at a point prior to any detectable viral DNA synthesis. Viral DNA synthesis does not start before 25 to 30 h at 34 C. Thus, *D* function is required prior to DNA synthesis.

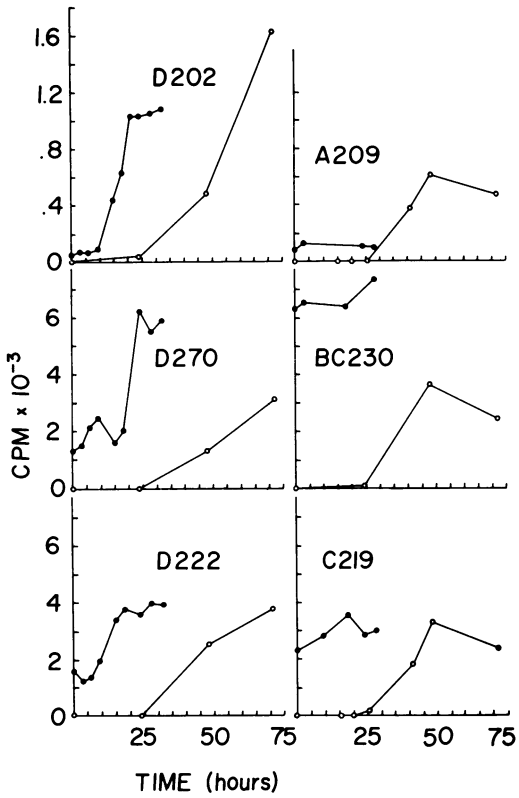

FIG. 7. *Capacity to synthesize viral DNA after shift up and viral DNA synthesis at the permissive temperature. Cultures were infected and incubated at 34 C. At the indicated times they were either pulse labeled for 1 h (open circles) or shifted to 41 C. All of the cultures shifted to 41 C were pulse labeled for 1 h at 46 h postinfection (closed circles).*

DISCUSSION

The results clearly demonstrate that *A* and *D* mutants are blocked prior to viral DNA replication, whereas *B*, *C*, and *BC* mutants are defective at a later stage of the growth cycle. *D202*, the tightest of the *D* mutants, is also blocked in the induction of host DNA replication. In complementation experiments between *D202* and *A* mutants, the delayed complementation previously observed (1) is correlated with a delay in the onset of viral DNA synthesis. The cause for the delayed viral DNA synthesis, however, remains unresolved.

A mutants appear defective in their ability to initiate new rounds of viral DNA synthesis in agreement with the observations of Tegtmeyer (9). This is shown by the fact that upon shifting an *A* mutant to the restrictive temperature after 3 days of infection at the permissive temperature: (i) the rate of DNA replication rapidly drops to zero; (ii) the DNA labeled upon a short pulse after shift-up is relatively deficient in replicative intermediate molecules; and (iii) replicative intermediate molecules formed prior to shift-up can be converted to mature component I and II molecules.

D mutants are blocked in a function required prior to viral DNA synthesis. The behavior of the *D* mutants in temperature shift experiments is in marked contrast to that of the *A* mutants. After 3 days of infection at the permissive temperature, a shift to the restrictive temperature does not result in a drop in the rate of viral DNA synthesis in *D* mutants. Moreover, to the extent studied, it appears that the DNA synthesized after the shift contains the usual proportion of SV40 replicative intermediate molecules which are converted to mature molecules in the normal fashion.

Little viral DNA synthesis is subsequently observed when cultures infected with *D* mutants are shifted to the restrictive temperature after up to 10 h of incubation at the permissive temperature. However, after approximately 20 h of incubation at the permissive temperature, at a time when no detectable viral DNA synthesis has started, infected cultures have passed the point where *D* function is required. Cultures

now shifted to the restrictive temperature are capable of subsequently synthesizing viral DNA at a maximal rate.

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