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Temperature-Sensitive Cell Mutations that Inhibit Adenovirus 2 Replication

(ts BHK cell line/host functions and viral growth)

TAKEHARU NISHIMOTO, HESCHEL J. RASKAS, AND CLAUDIO BASILICO*

Departments of Pathology and Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110; and
* Department of Pathology, New York University School of Medicine, New York, N. Y. 10016

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ABSTRACT **Five temperature-sensitive growth mutants of the hamster cell line BHK-21 were tested for the ability to support adenovirus 2 multiplication at 39° and 33°. Wild-type BHK-21 and mutants ts 422E and ts BCH yielded comparable amounts of virus at 33° and 39°, whereas in three other mutants, ts T22, ts T23, and ts AF8, virus production at 39° was reduced to about 1% of that at 33°. Virus yield in the three mutants was not reduced because of a delay in virus production; for all cells tested maximal virus yield at 39° was obtained by 40–50 hr after infection. Normal yields of infectious virus were not obtained from ts AF8 even with a very high multiplicity of infection. In contrast, the virus yield from ts T22 and ts T23 was multiplicity-dependent. Shiftup experiments demonstrated that in ts AF8, a cell cycle mutant which at 39° becomes arrested in G1, virus multiplication was thermosensitive for the first 40 hr of infection. In ts T22 and ts T23, the thermosensitivity was only for the first 3–4 hr of the infection. In all three mutants viral DNA synthesis was reduced by at least 95% at the higher temperature. The cell function specified by the ts AF8 mutation seems to be required for the early period of adenovirus 2 replication, after virus entry into the cell but before the onset of viral DNA replication.**

We present here an initial report of experiments designed to determine the role of the host cell in regulating the multiplication of an oncogenic DNA virus. For these studies human adenovirus 2 was used together with a set of conditional lethal, temperature-sensitive (ts) hamster cell mutants isolated by Meiss and Basilico (1). The five independent ts mutants used for our study grow at 33° but not at 39°. We have examined the possibility that the ts lesion affects a cellular function necessary for virus replication. Of the five mutants tested, all support virus replication at temperatures permissive for cell growth. However, three of the cell mutants do not support virus replication at 39°.

MATERIALS AND METHODS

The parental Syrian hamster cell line (BHK-21) and the five temperature-sensitive mutants (ts AF8, ts 422E, ts T23, ts T22, and ts BCH) have been described (1). All hamster cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (K. C. Biological), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Adenovirus 2 stocks were prepared by growth in suspension cultures of human KB cells (2). Virus was purified by CsCl density gradient centrifugation (ref. 2; Craig, Zimmer, and Raskas, in preparation), diluted 10-fold, and stored in 10 mM Tris·HCl (pH 8.1), 0.15 M NaCl, 0.1% serum albumin, and 50% glycerol. Infectious virus was determined by plaque assay on monolayers of KB cells (3).

Growth of adenovirus 2 in the various cell lines was assayed in the following manner: Cells were seeded onto 60-mm petri dishes at a concentration of 1 to 2 × 10^5 cells per dish. The cultures were then incubated for 2 days at 33° or 39° as indicated. For virus adsorption, virus stocks were diluted in modified Eagle's medium supplemented with 2% fetal calf serum. After virus adsorption for 2 hr at the indicated temperature, the inoculum was removed and the cell sheets were washed twice with 5 ml of modified Eagle's medium without serum. The cultures were incubated with 5 ml of modified Eagle's medium for 2 more hr to permit penetration of adsorbed virus. The medium was then replaced with modified Eagle's medium supplemented with 5% pooled human serum containing antibodies to adenovirus 2, and the cultures were incubated for an additional hour to inactivate any remaining extracellular virus. The medium containing human serum was then removed, the monolayers were washed two more times with 5 ml of modified Eagle's medium without serum, and 5 ml of complete medium were added. For virus growth curves, infected cells were incubated for various periods at the indicated temperature and the cultures frozen without removal of the medium. At a later time, we determined the plaque-forming units (PFU) in each culture after thawing the samples, scraping the cells from the dish, and disrupting the cells by three cycles of freezing and thawing followed by sonication.

To label DNA synthesized by infected and mock-infected cells, we incubated cultures with [^{3}H]thymidine (20 μCi/ml, 6.7 Ci/mmol) for 3-hr intervals. Labeling was initiated by replacing the medium with fresh medium containing [^{3}H]-thymidine. At the end of the labeling period, the cultures were washed three times with phosphate-buffered saline (pH 7.2) (4). To purify labeled DNA, monolayers were lysed by incubation for 30 min at 37° with 1.0 ml of 10 mM Tris·HCl (pH 7.1), 10 mM EDTA, 0.6% sodium dodecyl sulfate (Na-DodSO$_4$), and 500 μg of preincubated Pronase B (5). Three extractions were performed with an equal volume of phenol saturated with 50 mM Tris·HCl (pH 8.0), 5 mM EDTA. Phenol remaining in the aqueous phase was removed by a single extraction with an equal volume of ether, and the ether was evaporated by bubbling air through the solution. The DNA was then dialyzed against 0.1 × SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate). Virion DNA was extracted from virus particles purified by CsCl centrifugation (6). DNA·DNA hybridization was performed essentially by the method of Pina and Green (7). Viral DNA immobilized on

Abbreviations: ts, temperature-sensitive; PFU, plaque-forming units; NaDodSO$_4$, sodium dodecyl sulfate.

6.5-mm nitrocellulose filters (Schleicher and Schuell, Type B6) was incubated with sonicated radioactive DNA in a total volume of 150 μl of 2× SSC, 0.1% $NaDodSO_4$. Hybridization mixtures were incubated for 24 hr at 66°. The membranes were then washed four times with 3 mM Tris·HCl (pH 7.4) and dried; radioactivity was determined in a liquid scintillation counter.

RESULTS

Human cells are the natural host for productive replication of human adenoviruses (8). However, adenovirus 2 can replicate in hamster cells (5), thus allowing the study described here. The parental hamster cell line (wt BHK) and the ts mutants all grow at about the same rate at 33°, a rate significantly slower than at 37°. However the time required for complete cessation of cell division after a shift to 39° is variable (1); ts T23, for example, continues to increase in cell number for 2–3 days at 39°, whereas other mutants show no increase in cell number after the first 10–15 hr.

Adenovirus Multiplication in Cell Mutants. Each cell line was freshly seeded, incubated at 33° for 1 day, and then shifted to 39°. The cultures were maintained at 39° for 20 hr to maximize the temperature effect, and then infected with 1000 PFU per cell of adenovirus 2. A high multiplicity of infection is required because adsorption of adenovirus 2 to hamster cells is 10-fold less than to human cells (refs. 9 and 10; unpublished results of T. Nishimoto). Three days after infection most cells became round and detached from the plate. At this time the cultures were harvested and assayed for infectious virus. Of the five mutants tested, three (ts AF8, ts T23, and ts T22) had a significantly reduced capacity to support virus replication at 39° (Table 1). Mutants ts 422E (Table 1) and ts BCH (see Table 2) yielded virus in essentially the same amounts as wt BHK.

As a control, virus growth was determined in the same cell lines at 33° (Table 1). At 33°, virus yield in all cells tested was reduced and delayed, with progeny virus not appearing until 70–80 hr after infection. This temperature effect could be overcome by infecting at 33° and then shifting to 37° after 24 hr. Under these conditions all three mutants that failed to replicate virus at 39° yielded virus upon infection at 33°; at this temperature virus production in ts AF8, ts T22, and ts T23 was about 100-fold higher than at the nonpermissive temperature.

TABLE 1. *Multiplication of adenovirus 2 in temperature-sensitive BHK mutants at 33° and 39°*

Cell line	Average virus yield/cell	
	High temperature	Low temperature
wt BHK	110	240
ts AF8	0.29	53
ts T23	1.6	89
ts T22	1.3	300
ts 422E	130	850

Cultures were seeded at 33° as described in *Materials and Methods;* 1 day later a set of plates was transferred to 39°, and 20 hr later the cultures were infected with 1000 PFU per cell of adenovirus 2. The cultures were harvested after 4 days and assayed for infectious virus by plaque formation. For assays of virus production at low temperature, cultures were incubated at 33° 2 days after seeding; 24 hr after infection the cultures were shifted to 37°, harvested 3 days later, and assayed for virus production.

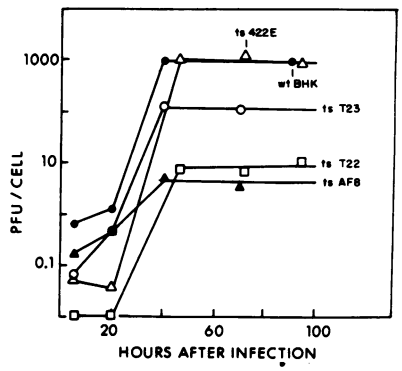

FIG. 1. Replication of adenovirus 2 in temperature-sensitive BHK cells at 39°. Cultures were seeded at 33°, shifted to 39° 1 day later, and after 20 hr infected with adenovirus 2 at the following multiplicities (PFU per cell): BHK (●), 1000; ts T23 (○), 3000; ts T22 (□), 1400; ts AF8 (▲), 2500; ts 422E (△), 1000. At the indicated times, cultures were harvested and assayed for infectious virus (see *Materials and Methods*).

Analysis of the time course of virus production in cultures infected at 39° demonstrated that the reduced virus yield in ts AF8, ts T23, and ts T22 did not represent delayed production of virus (Fig. 1). By 40 hr after infection all the infected cultures attained a maximal level of virus production. In this particular experiment the yield of virus from ts T23 was reduced to only one-tenth the yield of BHK-21 and ts 422E.

TABLE 2. *Effect of multiplicity of infection on adenovirus 2 replication in BHK mutants at 39°*

Cell	Multiplicity of infection (MOI)	Free virus at 3 hr (PFU/dish)	Final virus yield (PFU/dish)	Average virus yield/cell	Increased yield at high MOI
wt BHK	10^3	4×10^8	3.9×10^7	59	2.2×
	10^4	7.4×10^4	8.7×10^7	130	
ts AF8	10^3	3.7×10^4	4×10^4	0.07	7.7×
	10^4	5.4×10^4	2.8×10^5	0.54	
ts T23	10^3	1×10^8	2.0×10^6	0.87	60×
	10^4	8×10^8	1.2×10^7	52	
ts T22	10^3	$<1 \times 10^8$	9×10^4	0.37	170×
	10^4	1.6×10^4	1.5×10^7	63	
ts 422E	10^3	2×10^8	2.3×10^7	160	2.1×
	10^4	1.1×10^4	4.6×10^7	330	
ts BCH	10^3	2×10^8	1×10^8	208	1.6×
	10^4	1.8×10^4	1.6×10^8	330	

Cultures of parental BHK and five mutant cell lines were grown at 33°, shifted to 39°, and 20 hr later infected with adenovirus 2 at the indicated multiplicity. After virus adsorption and treatment with human serum (*Materials and Methods*), the free virus at the beginning of the infection (3 hr) was determined. The average virus yield per cell was calculated from the final virus yield per dish and the number of cells per dish. For this calculation the free virus at 3 hr was not subtracted from the final virus yield. The number of cells per plate for each cell line was the following: wt BHK, 6.6×10^5; ts AF8, 5.2×10^5; ts T23, 2.3×10^5; ts T22, 2.4×10^5; ts 422E, 1.4×10^5; and ts BCH, 4.8×10^5.

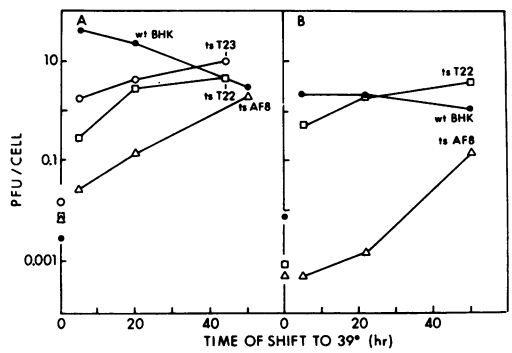

FIG. 2. Effect of shift to 39° on replication of adenovirus 2 in temperature-sensitive BHK cells. Two days after cultures were seeded at 33°, wt BHK (●) and the mutants ts T23 (○), ts T22 (□), and ts AF8 (△) were infected with 1000 PFU per cell of adenovirus 2. At the indicated times cultures were shifted to 39°, and 40 hr after the temperature shift, they were harvested and assayed for virus production. For one set of cultures (A) the infections were performed in the presence of 15% fetal calf serum; in the other set (B) the infections were performed in medium lacking serum. Time points at 0 hr represent the amount of free virus in the cultures after adsorption and incubation with human serum.

Since ts T23 had been infected with a multiplicity of 3000 PFU per cell, as compared to 1000 PFU per cell used for other studies (Table 1), the dependence of virus yield on multiplicity of infection was determined.

In cultures infected at 39° the yield of virus from ts T22 and ts T23 was shown to be multiplicity-dependent (Table 2). Parental BHK and the five ts mutants were infected with either 10^3 or 10^4 PFU per cell. Since only 1–3% of adenovirus 2 adsorbs to BHK-21 (unpublished results), an inoculum of 10^4 PFU represents at most 100 to 300 infectious virus particles adsorbing to each cell. Virus yield from the parental line and the two mutants that support virus replication at 39°, ts 422E and ts BCH, was not greatly affected by a 10-fold increase in the multiplicity of infection. In contrast, a 10-fold increase in the multiplicity of infection stimulated virus yield 60-fold in ts T23 and 170-fold in ts T22. These increased levels of virus production are comparable to those attained in the parental cell line. The higher multiplicity of infection increased the final yield in ts AF8 about 8-fold, but this yield was still only 0.5 PFU per cell, as compared to about 100 PFU per cell in wt BHK cells at 39° or in ts AF8 at 33°.

Virus Yield in Cultures Shifted to 39° at Various Times After Infection. Shiftup experiments were performed to determine what stage of viral replication is thermosensitive in cell lines ts AF8, ts T22, and ts T23. Cultures were infected at 33°, shifted to 39° at various times, and then harvested 40 hr after shiftup (Fig. 2A). Different results were obtained with ts AF8 and with the two mutants, ts T22 and ts T23, which, as seen in the preceding section, exhibited leakiness at high multiplicity; ts AF8 did not give a virus yield comparable to that of parental BHK-21 unless the shiftup was performed later than 20 hr after infection. Infection of ts T22 and ts T23 gave reasonably high yields of virus even when the cells were shifted to 39° within the first few hours after infection, suggesting that the block in adenovirus 2 replication in these cells occurs at a very early stage in the replicative cycle.

Identical shiftup experiments were also performed in serum-free medium. When infections were performed in the absence of serum, virus yield was decreased by about a factor of 10 for all cells infected, but more distinct differences were seen with the three mutants as compared to the infections performed with serum (Fig. 2B). Shifting ts T22 to 39° at early times gave nearly the same virus yield as wt BHK; ts AF8 shifted to 39° at 20 hr gave a final virus yield that was about 10^{-4} of that of BHK-21. A shiftup at 50 hr increased the yield 1000-fold. Thus, the effect of the ts AF8 mutation on virus replication seems to occur after virus adsorption. Apparently the cell function(s) affected by the ts AF8 mutation is required in the interval 20–50 hr after infection.

TABLE 3. *Viral DNA synthesis in temperature-sensitive BHK mutants infected at 39°*

Cell line	Average virus yield (PFU/cell)	Percent DNA hybridizing: DNA labeled 20–23 hr	Percent DNA hybridizing: DNA labeled 26–29 hr
wt BHK	28	9.4	7.6
ts T23	0.01	0.24	0.82
ts T22	0.1	0.14	0.80
ts AF8	0.06	0.37	1.1
TS 422E	170	9.4	26.1

Cultures were grown at 33°, shifted to 39°, and infected 20 hr later with 1000 PFU/cell of adenovirus 2. At the indicated times the medium was removed and replaced with fresh medium containing 2 μCi/ml of [^{3}H]thymidine. The labeled DNA was purified, and the specific activity estimated assuming that one A_{260} unit equals 50 μg of DNA. The specific activities (cpm/μg) of the samples labeled at 20 hr and 26 hr, respectively, were the following: BHK, 24,600 and 15,500; ts T23, 27,700 and 24,100; ts T22, 12,200 and 17,400; ts AF8 15,000 and 22,900; and ts 422E, 19,500 and 29,400. The following amounts of [^{3}H]DNA (cpm) were hybridized to 3 μg of adenovirus 2 DNA: BHK, 71,374 and 26,970; ts T23, 48,280 and 37,140; ts T22, 28,860 and 34,102; ts AF8, 34,720 and 33,834; and ts 422E, 48,302 and 73,536. As a control, a culture of mock-infected BHK cells was labeled for 3 hr; of 49,575 input cpm, the amount bound to adenovirus DNA after hybridization was 71 cpm above background or 0.14%. Virus yield for this infection was determined in samples harvested at 40 hr.

Viral DNA Synthesis in Mutant Cells Infected at 39°. To determine whether the block in viral multiplication occurring in some ts mutants affected the level of viral DNA synthesis, cultures were infected at 39° and exposed to [^{3}H]thymidine; the labeled DNA was hybridized to adenovirus 2 DNA. Hybridization of DNA labeled 20–23 or 26–29 hr after infection showed that viral DNA replication in ts T23, ts T22, and ts AF8 was drastically reduced compared to wt BHK (Table 3).

Since the shiftup experiments (Fig. 2) had established that the temperature-sensitive period in virus replication in ts AF8 was between 20 and 50 hr after infection, the time of viral DNA synthesis (5), viral DNA replication in ts AF8 was analyzed in more detail (Fig. 3A). Cultures of ts AF8 were infected at 33°; half of the plates were maintained at 33° while the remainder were shifted to 39° 3 hr after infection. At various times after infection the two sets of cultures were labeled with [^{3}H]thymidine for 3-hr intervals. Total DNA was then purified and hybridized to adenovirus 2 DNA. As a control, viral DNA synthesis was also followed in wt BHK cells in-

cubated at either 33° or 39°. In infected wt BHK cells incubated at 39°, extensive viral DNA replication began before 20 hr; viral DNA synthesis did not begin until 40 hr in identical cultures incubated at 33°. This result is consistent with the delay in virus production observed at 33° mentioned earlier (Table 1).

The experiments with ts AF8 demonstrated the inhibition of viral DNA replication in this cell line at 39° as compared to 33°. At 75 hr after infection, 19% of the DNA labeled at 33° hybridized to viral DNA, whereas only 1% of the DNA from the 39° infection annealed to viral DNA. A comparison can also be made of viral DNA replication in wt BHK and ts AF8 when both were incubated at 39°. At 30 hr after infection, 16% of the DNA synthesized in wt BHK annealed to viral DNA as compared to less than 0.5% of the labeled DNA from AF8.

Shiftup experiments were performed to determine the incubation time at 33° required for infected ts AF8 cultures to escape inhibition of viral DNA replication (Fig. 3B). As already shown (Fig. 3A), transferring cultures to 39° at 3 hr after infection prevented viral DNA replication; viral DNA synthesis was still reduced 75–85% in cultures shifted as late as 33 hr after infection. When the shiftup was performed at 50 hr or later, viral DNA replication approached that of cultures maintained throughout at 33°.

DISCUSSION

The use of cell mutants to identify host cell functions necessary for virus replication has been exploited in bacteriophage systems, enabling the identification of two bacterial functions that are required for replication of phage lambda (11, 12). The present study suggests that a similar approach may be successful in identifying and characterizing host activities that influence the course of animal virus infection.

Five independently isolated temperature-sensitive hamster cell mutants have been tested for the ability to support adenovirus 2 replication at 39°. In two of the mutants, ts 422E and ts BCH, viral replication was comparable at 33° and 39°. At 39° ribosomal RNA processing is altered in ts 422E, leading to a block in the production of 28S ribosomal RNA (13). The observation that this cell function is not essential for adenovirus 2 replication was expected, for ribosomal RNA synthesis is inhibited in adenovirus-infected cells (14). In three other mutants, ts T22, ts T23, and ts AF8, virus replication was greatly reduced at 39°. Since adenovirus 2 replicates in all three of these mutants at lower temperatures, the cell mutation that inhibits growth at 39° must prevent, directly or indirectly, the replication of the virus.

The three mutants form two distinct groups: ts T22 and ts T23 are thermosensitive only at the very early stages of virus replication (Fig. 2), and virus production in these cells is multiplicity-dependent (Table 2). These observations suggest that at 39°, virus adsorption, penetration, or uncoating may be defective in these two mutants. This may suggest that the T22 and T23 mutations affect the structure or functionality of the cell surface at 39°. This hypothesis is also supported by the finding that these cells tend to round up and detach from the plate at the nonpermissive temperature, and also show a sharp drop in their intracellular concentration of cyclic AMP (Burstin and Basilico, unpublished). However, further work is clearly necessary before any conclusion on the nature of these two mutations can be reached.

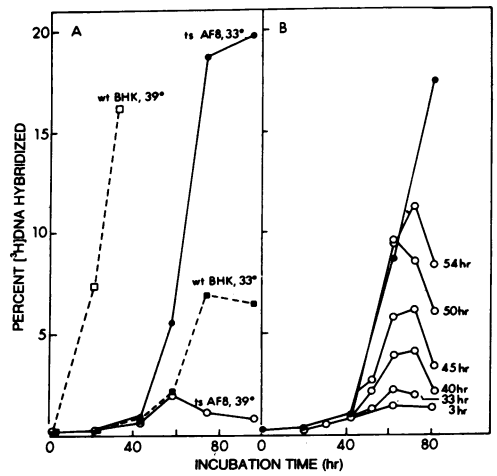

FIG. 3. Time course of viral DNA synthesis in wt BHK and ts AF8 at 33° and 39°. Cultures of ts AF8 (○,●) and wt BHK (□,■) were grown at 33° for 2 days and then infected with 1000 PFU per cell of adenovirus 2. For the experiment of panel A, one set of plates of each culture was shifted to 39° (open symbols) at 3 hr after infection and labeled with [³H]thymidine (40 μCi/ml) for 3 hr at the indicated times. Another set of plates was maintained and labeled at 33° (filled symbols). The percent viral DNA in the [³H]DNA was determined by hybridization to 3 μg of adenovirus 2 DNA. The values obtained were plotted as the midpoint of the labeling period. In a separate experiment (panel B) cultures of ts AF8 were shifted to 39° at various times after infection (open circles, ○); viral DNA synthesis was analyzed by labeling with [³H]thymidine and subsequent hybridization of [³H]DNA. The time of shift to 39° is shown in the figure. The filled circles (●) represent viral DNA synthesis in a culture maintained at 33° throughout the infection.

In the ts AF8 mutant, viral multiplication is thermosensitive for the first 40 hr after infection, throughout the period of viral DNA replication (Fig. 2). Also, viral DNA replication at 39° in ts AF8 is greatly reduced as compared to wt BHK cells (Fig. 3). Thus, in ts AF8, viral replication is inhibited after virus entry into the cells but before viral DNA accumulation begins. This inhibition of adenovirus 2 replication in ts AF8 is similar to the block in phage lambda DNA synthesis in bacterial cells containing the *groP* mutation (11).

A recent study has shown that ts AF8 is a cell cycle mutant (15). When shifted to 39°, cellular DNA synthesis is reduced. This reduction in DNA synthesis is caused by the accumulation of individual cells in the G1 phase of the cell cycle; cells that have passed a certain critical point in G1 are able to enter and complete the period of DNA synthesis. The inhibition of adenovirus DNA replication in ts AF8 seems to occur more rapidly than the inhibition of cell DNA replication. When infected ts AF8 cells are shifted to 39° 3 hr after infection, viral DNA synthesis at 20 hr is one-fifteenth of that in similarly treated wt BHK cells (Fig. 3). In contrast, such extensive reduction in cellular DNA synthesis in ts AF8 is not reached until about 40 hr after shiftup (15). More important, when synchronized AF8 cells are shifted to 39° at the end of the G1 phase of the cycle, they enter and complete S phase normally. However, the rate of viral DNA synthesis in infected ts AF8 cells depends on the time of temperature shift to 39° (Fig. 3B), even if the cells are shifted to 39° after viral DNA synthesis has begun. Because we have studied a cell population rather than individual infected cells, and the time

course of infection is not very synchronous, we cannot now distinguish between limited viral DNA synthesis in each cell or the alternative model, a fraction of cells that have no viral DNA synthesis.

Our results show conclusively that a cell function that is required for progression through the G1 phase of the cell cycle is also required stringently for adenovirus DNA replication. A previous study (16) demonstrated that, in HeLa cells, the time from infection to the onset of adenovirus 2 viral DNA replication was the same when synchronized cells were infected in either the S or G1 phase of the cycle. This is consistent with the conclusion that the effect of the ts AF8 mutation on viral DNA synthesis cannot be ascribed to the fact that these cells are arrested in G1. In fact, as discussed above, inhibition of adenovirus DNA synthesis in ts AF8 at 39° can be demonstrated before the time at which the cells become uniformly arrested in G1. Therefore, it seems likely that in normal cells the gene product affected by the ts AF8 mutation may be present and functional throughout the cycle, but is necessary to prepare for *cell* DNA replication only at some specific time in G1. Alternatively, virus infection may activate the synthesis or functionality of the ts AF8 protein.

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