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# DNA synthesis in temperature-sensitive mutants of the cell cycle infected by polyoma virus and adenovirus

(S phase/hamster cells/RNA synthesis/serum stimulation)

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**ABSTRACT** **tsAF8 cells are a temperature-sensitive (ts) mutant of BHK cells that are arrested in $G_1$ at the nonpermissive temperature. When made quiescent by serum restriction, they can be stimulated to enter S phase by 10% serum at 34°C but not at 40.6°C. The same results can be obtained if quiescent cells are infected with polyoma virus or adenovirus 12 instead of serum. However, adenovirus 2 infection stimulates DNA synthesis in tsAF8 cells at both 34°C and 40.6°C. The DNA synthesized after adenovirus 2 infection has been shown to be cellular DNA by CsCl density centrifugation. By density labeling it can be shown that adenovirus 2-induced DNA synthesis is due to semiconservative replication. The difference between adenovirus 2 and polyoma (or serum) is also evident with another ts mutant of BHK cells, ts13 cells. These results open the possibility of identifying the viral or cellular mechanism at the basis of this difference in the induction of host DNA synthesis between adenovirus 2 and polyoma or serum.**

It is generally agreed that the $G_1$ phase of the eukaryotic cell cycle is the critical period for the regulation of cell proliferation (for a review, see ref. 1). Under conditions restrictive for growth, untransformed cell lines tend to accumulate in $G_1$, from which they can be stimulated to re-enter S phase by the application of an appropriate proliferative stimulus (for a review, see refs. 1 and 2). Although many biochemical events have been described in $G_1$ cells (see above reviews), the information is largely descriptive and far from being complete.

An approach to the biochemistry of the $G_1$ period is the combined use of $G_1$ temperature-sensitive (ts) mutants and DNA oncogenic viruses. $G_1$ ts mutants are operationally defined as growth mutants that are arrested at the nonpermissive temperature in the $G_1$ phase of the cell cycle (3). Several such mutants of the mammalian cell cycle have been described, including two lines from BHK cells, tsAF8 (4) and ts13 (5). When such ts mutants are made quiescent by serum deprivation and are subsequently stimulated with 10% serum, they enter the S phase at the permissive temperature but fail to do so at the nonpermissive temperature (4–9).

Certain DNA viruses (especially simian virus 40, polyoma, and adenovirus) stimulate cellular DNA synthesis in resting cells (10–13). In this paper we have asked whether some of these viruses can induce cellular DNA synthesis in quiescent populations of $G_1$ ts mutants kept, after infection, at the nonpermissive temperature. Most of these experiments have been carried out with one of these $G_1$ ts mutants, AF8 cells, and with two DNA viruses, polyoma and adenovirus 2 (Ad2). Polyoma virus was chosen because it induces cellular DNA synthesis in BHK cells (14, 15), from which AF8 cells are derived. Ad2 was chosen because of previous experiments by Laughlin and Strohl (16, 17) showing that Ad2 could induce cellular DNA synthesis in BHK cells in the presence of 0.03 μg of actinomycin D per ml, a concentration of the drug that completely prevented the stimulation of cellular DNA synthesis by serum or adenovirus 12 (Ad12).

## METHODS AND MATERIALS

**Cell Cultures.** tsAF8 cells, originally derived from BHK cells (4), were grown as described (8). In these experiments, the permissive temperature was 34°C and the nonpermissive temperature 40.6°C. BHK cells (the parent cell line) grow normally at 40.6°C.

ts13 cells, also derived from BHK, have been described and characterized as $G_1$ mutants by Talavera and Basilico (5), and they were grown as described (9); permissive temperature was 34°C and nonpermissive temperature 39.5°C.

**Viruses.** Ad2 and Ad12 were grown in HeLa suspension cultures maintained with minimum essential modified suspension medium (Flow Laboratories, Rockville, MD) with 5% fetal calf serum. Virus was purified as described (18), with two cycles of CsCl density gradient centrifugation. The titer was calculated from the optical density [1 OD at 260 nm = $3 \times 10^{10}$ plaque-forming units (PFU)].

Serum-free polyoma was kindly given by Claudio Basilico (New York University), Thomas Benjamin (Harvard University), and Roger Weil (University of Geneva).

**Infection.** Cells were made quiescent by a 48-hr period in 0.5% serum. The medium from quiescent cells was collected and saved. Ad2 and Ad12, diluted in Hanks' balanced salt solution containing 20 mM $MgCl_2$, were allowed to absorb for 1 hr at 37°C (multiplicity of infection = 1000 PFU/cell). The cultures were then re-fed with the "used" (serum-depleted) medium. Polyoma infection was carried out the same way, except that the multiplicity of infection was 400 PFU/cell. Mock-infected cultures were treated identically, except that the virus was omitted from the inoculum.

**Serum Stimulation.** Cells were made quiescent, as described above, and then stimulated with 10% serum (for details, see ref. 7).

**Autoradiography.** Cells were continuously labeled with [$^3$H]thymidine (New England Nuclear, 6.7 Ci/mmol, 0.01 μCi/ml; 1 Ci = $3.7 \times 10^{10}$ becquerels) added immediately after serum stimulation or virus infection, and autoradiographs were made and analyzed as described (19). When incorporated radioactivity was measured by a liquid scintillation counter, we followed the procedure previously published from this laboratory (7).

**Analysis by Equilibrium Sedimentation in Neutral CsCl Gradients.** The total intracellular DNA, labeled for three

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Abbreviations: ts, temperature sensitive; Ad2, adenovirus 2; Ad12, adenovirus 12; PFU, plaque-forming units.

generations with [$^{14}$C]thymidine (0.001 $\mu$Ci/ml), was isolated by lysis of the cells in 0.5% sodium lauroyl sarcosinate and digestion with predigested Pronase B (250 $\mu$g/ml) and subsequently analyzed by equilibrium sedimentation in neutral CsCl density gradients as described (20).

**Immunofluorescence.** T antigen was detected in cells infected with either polyoma virus or Ad2 by the indirect immunofluorescence technique of Pope and Rowe (21). The antisera used are given below.

**Ultraviolet Irradiation of Ad2.** This was carried out exactly as described by Berk and Sharp (22), with a UV source with a maximum peak at 254 nm at a distance of 36 cm for 8 min.

## RESULTS

**Effect of Serum and Polyoma Virus Infection on Proportion of Quiescent tsAF8 Cells Synthesizing DNA.** Quiescent tsAF8 cells were stimulated with 10% serum or infected by polyoma virus (≈400 PFU/cell). After virus adsorption the cells were incubated in conditioned medium, which does not stimulate entry of cells into S (see below). [$^3$H]Thymidine was added to the cultures and the percentage of labeled cells, at various times after serum stimulation or polyoma infection, was determined by autoradiography. Fig. 1 shows the proportion of tsAF8 cells synthesizing DNA. As already described in previous papers (7,8), 10% serum stimulates DNA synthesis in resting tsAF8 cells at 34°C but not at 40.6°C. Fig. 1 shows that polyoma infection, like serum, increases the proportion of cells synthesizing DNA at 34°C but not at 40.6°C. These experiments were repeated with three different batches of polyoma virus from three different sources (see *Methods and Materials*), and they all gave essentially the same results. The proportion of cells synthesizing DNA did not increase in mock-infected cells, as shown in Fig. 1.

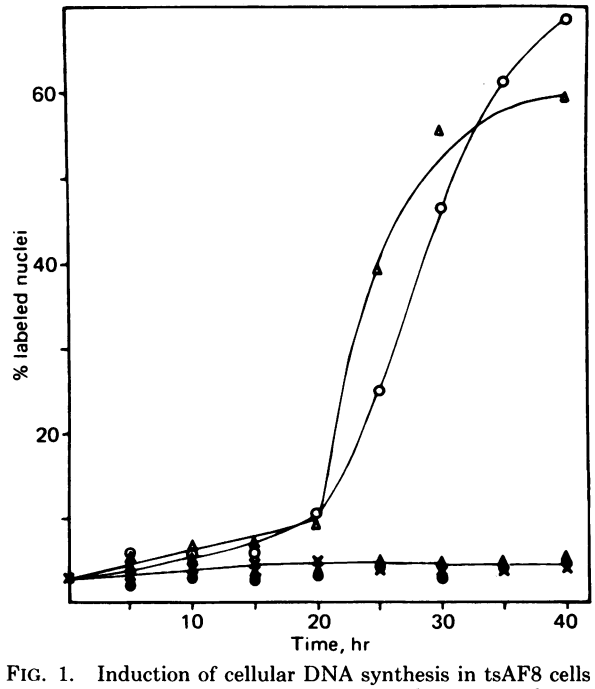


FIG. 1. Induction of cellular DNA synthesis in tsAF8 cells by serum or polyoma virus. tsAF8 cells were made quiescent by serum deprivation for 48 hr. They were then stimulated by 10% serum or infected with polyoma virus at a multiplicity of infection of 400 PFU/cell. The cultures were continuously incubated with [$^3$H]thymidine, and the percentage of labeled nuclei was determined by autoradiography at the times indicated on the abscissa. ○, Cells stimulated with serum at 34°C; ●, cells stimulated with serum at 40.6°C; △, cells infected with polyoma virus at 34°C; ▲, cells infected with polyoma virus at 40.6°C; ×, cells mock-infected at either 34°C or 40.6°C.

Polyoma-infected cells were also tested for the presence of polyoma T antigen by using two different antisera against T antigen, one obtained from Kenneth K. Takemoto (National Institute for Allergy and Infectious Diseases, Bethesda, MD) and the other one from Roger Weil and Hans Türler (Department of Molecular Biology, Geneva, Switzerland). When tested with these antisera by indirect immunofluorescence, about 50% of the tsAF8 cells infected with polyoma were T positive whether at 34°C or 40.6°C. Cells stimulated with 10% serum were 100% T negative.

**Effect of Infection with Ad2 or Ad12 on Proportion of Quiescent tsAF8 Cells Synthesizing DNA.** Quiescent tsAF8 cells were infected with either Ad2 or Ad12 (≈1000 PFU/cell). After infection the cultures were incubated in conditioned medium at 34°C or 40.6°C in the presence of [$^3$H]thymidine. The proportion of tsAF8 cells synthesizing DNA was determined by autoradiography. Fig. 2 shows that infection with Ad12 increases the proportion of DNA synthesizing tsAF8 cells at 34°C but not at 40.6°C, just like serum stimulation or polyoma infection. However, infection with Ad2 gave a strikingly different result. The proportion of tsAF8 cells synthesizing DNA, as determined by autoradiography, increased after Ad2 infection at both 34°C and 40.6°C, as shown in Fig. 2. There was a slight increase in the proportion of cells synthesizing DNA after mock infection at 34°C, whereas mock infection followed by incubation at 40.6°C resulted in no increase whatsoever in the proportion of cells synthesizing DNA.

The difference between Ad2 and Ad12 could be of considerable interest because Ad2 produces a normal lytic cycle in hamster cells whereas the DNA of Ad12 is unable to replicate in BHK cells (23). However, in these experiments we have elected to compare serum stimulation with Ad2 or polyoma infection.

We tested Ad2-infected cells for T antigen by indirect immunofluorescence using an antiserum kindly given to us by Arnold Levine (Princeton University). This is one of the antisera used by Levinson and Levine (24). About 80% of the cells infected with Ad2 became T positive, whether the infection was followed by incubation at 34°C or at 40.6°C. This is in essential

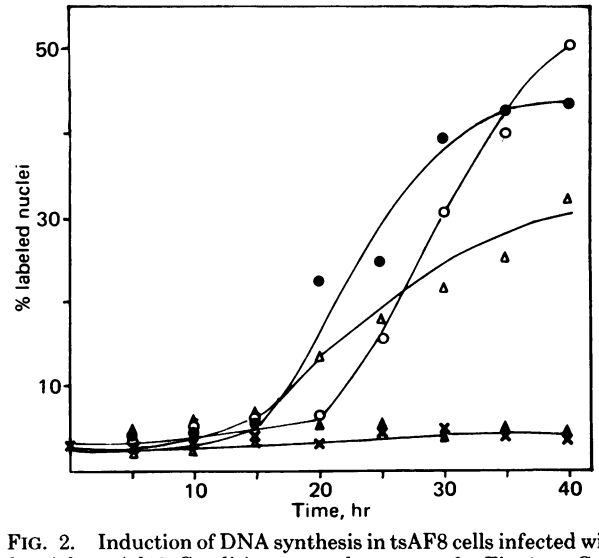


FIG. 2. Induction of DNA synthesis in tsAF8 cells infected with either Ad2 or Ad12. Conditions were the same as for Fig. 1. ○, Cells infected with Ad2 at 34°C; ●, cells infected with Ad2 at 40.6°C; △, cells infected with Ad12 at 34°C; ▲, cells infected with Ad12 at 40.6°C; ×, mock-infected cells.

agreement with the results of Nichimoto *et al.* (25), who found that, although the replication of Ad2 is completely inhibited in tsAF8 cells incubated at the nonpermissive temperature, viral RNA is still synthesized although at lower levels than in AF8 cells infected with Ad2 at the permissive temperature.

**Characterization of DNA Synthesized in tsAF8 Cells Infected with Ad2.** Ad2 DNA replicates in tsAF8 cells at 34°C, but at 40.6°C, the temperature nonpermissive for tsAF8 cells, Ad2 DNA is not replicated (26). It would seem, therefore, that the DNA synthesized by tsAF8 cells after Ad2 infection at 40.6°C ought to be cellular DNA. However, this point was confirmed by a series of experiments described below.

Most of the cellular DNA can be separated from free adenovirus DNA on CsCl gradients due to their different G+C content (27). In these experiments tsAF8 cells were labeled with [$^{14}$C]thymidine and either stimulated by 10% serum at 34°C (Fig. 3*A*), infected with Ad2 and incubated at 34°C (Fig. 3*B*), or infected with Ad2 and incubated at 40.6°C (Fig. 3*C*). After stimulation or infection the cells were exposed to [$^{3}$H]thymidine and the [$^{3}$H]DNA synthesized under these conditions was analyzed by CsCl gradients as described by Strniste and Rall (20). In some of these experiments the buoyant density of Ad2 DNA was determined by adding to the CsCl gradients Ad2 [$^{32}$P]DNA labeled *in vivo*.

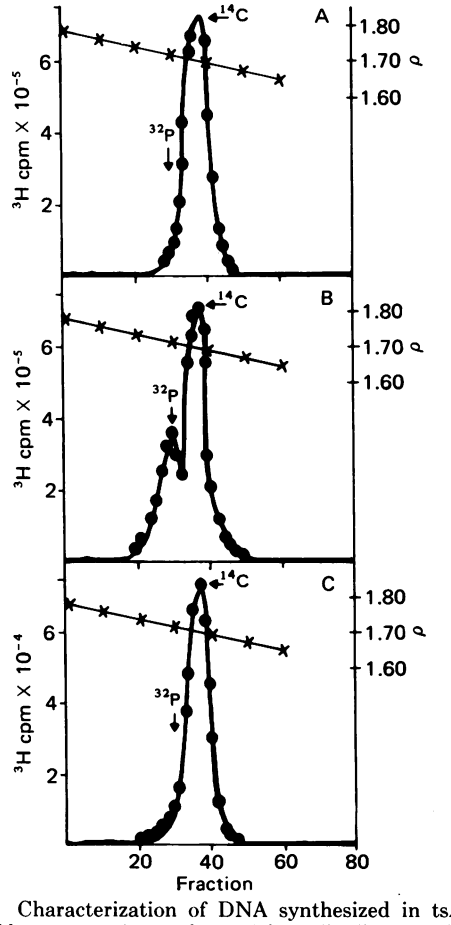


FIG. 3. Characterization of DNA synthesized in tsAF8 cells stimulated by serum or infected with Ad12. All cells were labeled with [$^{14}$C]thymidine, and they were then divided into three groups. One group was stimulated with 10% serum (*A*); the second group was infected with Ad2 at 34°C (*B*); and the third group was infected with Ad2 at 40.6°C (*C*). The stimulated or infected cultures were then incubated with [$^{3}$H]thymidine, and the DNA from the lysates was analyzed on CsCl gradients. Notice that viral DNA (at the left of the main peak) is present only in cells infected with Ad2 at 34°C. $^{14}$C, cellular DNA prelabeled at 34°C; $^{32}$P, viral DNA labeled *in vivo*.

In cells stimulated by 10% serum, only cellular DNA is synthesized and it bands with $^{14}$C-labeled cellular DNA at a buoyant density of 1.700 (Fig. 3*A*). In cells infected with Ad2 at the permissive temperature of 34°C, incorporation of [$^{3}$H]thymidine can be detected both at the density that corresponds to cellular DNA and at the density that corresponds to free viral DNA, 1.714, suggesting therefore that both viral and cellular DNA are synthesized in tsAF8 cells infected with Ad2 at the permissive temperature (Fig. 3*B*). Fig. 3*C* shows the CsCl density gradient of tsAF8 cells infected by Ad2 and subsequently incubated at 40.6°C. Only [$^{3}$H]DNA with the same buoyant density of cellular DNA can be found. No DNA with a buoyant density of Ad2 DNA is synthesized at the nonpermissive temperature, thus confirming the results of Nishimoto *et al.* (26).

After establishing that the DNA synthesized in tsAF8 cells infected with Ad2 at the nonpermissive temperature was cellular, we asked whether this cellular DNA was replicated semiconservatively or was due to repair synthesis; we used the methodology of Cooper and Hanawalt (28), as modified by Sheinin (29). Fig. 4 shows that after serum stimulation at 34°C there is a hybrid peak of DNA containing BrdUrd and $^{3}$H, as well as the original $^{14}$C label, banding at a density of 1.720 g/cm$^3$ compared to the normal, unsubstituted DNA with a density of 1.700 g/cm$^3$. About 33% of the preformed [$^{14}$C]DNA was recovered at this heavier than normal density. After Ad2 infection at 40.6°C, at which no viral DNA is replicated, about

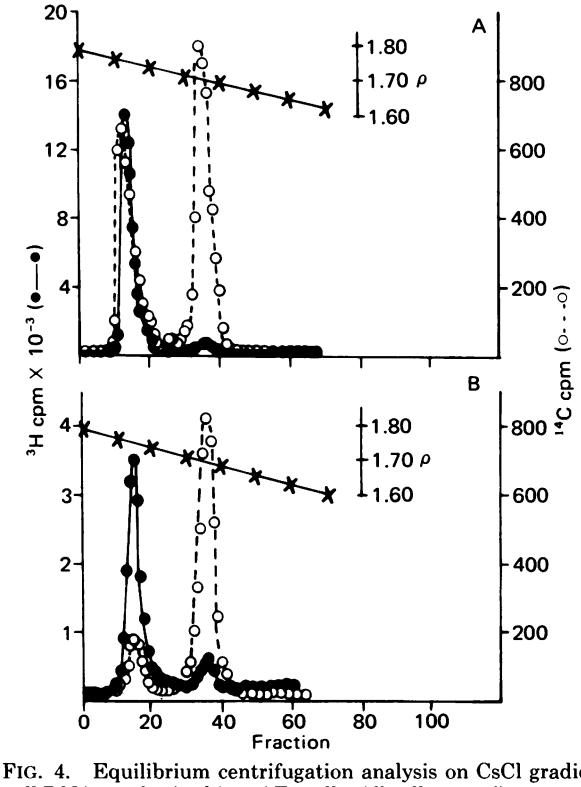


FIG. 4. Equilibrium centrifugation analysis on CsCl gradients of cell DNA synthesized in tsAF8 cells. All cells were first grown at 34°C with [$^{14}$C]thymidine so that the cellular DNA was uniformly labeled. The labeled cells were then made quiescent by 0.5% serum for 48 hr. They were then stimulated by 10% serum at 34°C or infected with Ad2 at 40.6°C. After 24 hr, 16 μM BrdUrd and 1 μM FdUrd were added to the culture for 6 hr. For the last 60 min of the labeling with BrdUrd, 7.5 μCi of [$^{3}$H]thymidine per ml was added. The cells were then harvested and lysed in sodium dodecyl sulfate, and the DNA was analyzed by equilibrium centrifugation in neutral CsCl gradients. ○ - - ○, [$^{14}$C]DNA preformed at 34°C; ●—●, [$^{3}$H]DNA made in the presence of BrdUrd and FdUrd. (*A*) Cells stimulated with 10% serum at 34°C; (*B*) cells infected with Ad2 at 40.6°C.

20% of the preformed [$^{14}$C]DNA banded at the heavier density. The [$^{3}$H]DNA almost entirely banded (more than 90%) with heavier DNA. These results indicate that the DNA synthesized after Ad2 infection at 40.6°C is replicated semiconservatively. The low percentage of DNA shifted to a higher density, even with serum, is due to the short period of labeling with BrdUrd (6 hr), whereas DNA synthesis in stimulated cells is spread over a period of at least 20 hr, as shown in Figs. 1 and 2.

**Effect of UV Irradiation of Ad2 on Its Ability to Stimulate DNA Synthesis in tsAF8 Cells.** Ad2 preparations were irradiated with UV light as described (22) for 8 min, a procedure that effectively inhibits Ad2 transcription and reduces the cytoplasmic concentration of early viral mRNAs to less than 10% of control values. Quiescent tsAF8 cells were then infected with Ad2 or with UV-irradiated Ad2 under exactly the same conditions described above. DNA synthesis in infected and control cultures was measured by the incorporation of [$^{3}$H]-thymidine into acid-precipitable material, which in all of these experiments closely paralleled results obtained by autoradiography. Table 1 shows that UV-irradiated Ad2 does not stimulate DNA synthesis in cultures of tsAF8 cells.

**Effect of Actinomycin D on Ad2-Induced Stimulation of DNA Synthesis.** We repeated, in tsAF8 cells, the experiments described by Laughlin and Strohl (17) on BHK cells. Quiescent tsAF8 cells were stimulated by 10% serum or infected by Ad2 in the presence or absence of actinomycin D. Two concentrations of actinomycin D were used: 0.03 and 0.08 μg/ml. DNA synthesis was measured as in the previous experiments. The results in Table 1 show that tsAF8 cells behave exactly like BHK cells. Actinomycin D (0.08 μg/ml) inhibits entry into S whether the cells are stimulated by serum or by Ad2. However, at 0.03 μg/ml, actinomycin D inhibits serum stimulation but has no effect on Ad2 induction of DNA synthesis.

**Effect of Ad2 Infection on Stimulation of Cellular DNA Synthesis in tsAF8 and ts13 Cells.** Table 2 summarizes our autoradiographic experiments with tsAF8 cells. Two new experiments are described here. In one experiment to reduce the cell death that occurs in tsAF8 at 40.6°C, cells infected with Ad2 were incubated at 40.6°C in 10% serum instead of conditioned medium (tsAF8 cannot enter S with 10% serum alone at the nonpermissive temperature). Almost 80% of the cells were stimulated to synthesize DNA under these conditions. In another experiment, tsAF8 cells were preincubated at 40.6°C for 10 hr before Ad2 infection to give ample time for the ts defect to express itself. Again Ad2 infection was capable of overcoming the ts block of AF8 cells (Table 2).

Table 2 also shows that Ad2 infection can increase the proportion of DNA-synthesizing cells in another ts mutant of BHK cells that is arrested in $G_1$ at the nonpermissive temperature, ts13 cells, which are in a different complementation group of AF8 cells (5). As already reported by Floros *et al.* (9), quiescent ts13 cells stimulated by serum cannot enter S phase at the nonpermissive temperature of 39.5°C. Infection with Ad2 stimulates DNA synthesis in these cells at both permissive and nonpermissive temperatures.

Table 1. Effect of actinomycin D on Ad2 stimulation of DNA synthesis in tsAF8 cells

| Treatment | cpm per culture × $10^{-3}$ |
|---|---|
| Mock-infected | 3 (2–4) |
| 10% serum | 87 (77–98) |
| 10% + Act. D (0.03 μg/ml) | 9 (7–10) |
| 10% + Act. D (0.08 μg/ml) | 1 (0.5–1.5) |
| Ad2 | 33 (30–35) |
| Ad2 + Act. D (0.03 μg/ml) | 27 (21–31) |
| Ad2 + Act. D (0.08 μg/ml) | 0.3 (0.2–0.4) |
| UV-irradiated Ad2 | 0.9 (0.07–0.1) |

Actinomycin D (Act. D) at the concentrations indicated was added immediately after Ad2 infection or serum stimulation. All cultures were kept at 34°C. All other conditions were as described for Fig. 2. Several points (after infection or after stimulation) were taken, but for simplicity we are giving only the results of labeling with [$^{3}$H]thymidine between 28 and 32 hr. Range is given in parentheses.

Table 2. Effect of infection with Ad2 on DNA synthesis in tsAF8 and ts13 cells

| Condition | % labeled cells tsAF8 | % labeled cells ts13 |
|---|---|---|
| 10% serum, at 34°C | 88.9 | 65.7 |
| 10% serum, at 40.6°C | 6.1 | 7.1 |
| Mock infection at 34°C | 8.5 | 5.0 |
| Mock infection at 40.6°C | 2.9 | 7.0 |
| Ad2, at 34°C in conditioned medium | 51.1 | 55.2 |
| Ad2, at 40.6°C in conditioned medium | 46.6 | — |
| Ad2, at 40.6°C in 10% serum | 79.8 | 21.6 |
| Preincubation at 40.6°C for 10 hr, followed by infection at 40.6°C | 69.3 | — |

In all experiments the cells were continuously labeled with [$^{3}$H]-thymidine (0.01 μCi/ml), and the cultures were terminated at 40 hr after serum or infection.

## DISCUSSION

tsAF8 cells are arrested in $G_1$ at the nonpermissive temperature of 40.6°C (4, 7, 8). RNA polymerase II activity decreases in tsAF8 at the nonpermissive temperature with a half-life of 12 hr, whereas RNA polymerase I activity is not affected (7). Collaborative experiments with James Ingles (University of Toronto) have shown that the number of RNA polymerase II molecules (as determined by titration with γ-[$^{3}$H]amanitin) decreases with a half-life of 12 hr in tsAF8 at 40.6°C whereas the same temperature does not affect the number of RNA polymerase II molecules in BHK cells, the parent cell line (unpublished data). tsAF8 cells can therefore be considered as a mutant in which the nonpermissive temperature affects the synthesis, the assembly, or the stability of RNA polymerase II. As mentioned above, Ad2 DNA is replicated in tsAF8 cells at 34°C but not at 40.6°C (ref. 26 and present results). Neither 10% serum nor infection with polyoma or Ad12 can overcome the ts block of the tsAF8 cells. Although DNA synthesis is stimulated at the permissive temperature, none of these procedures induces tsAF8 cells to enter S at 40.6°C. However, Ad2 infection stimulates DNA synthesis in tsAF8 cells at both permissive and nonpermissive temperatures. This was not totally unexpected because Laughlin and Strohl (16, 17) have reported that infection of BHK cells (the parent line of tsAF8) with Ad2 causes these cells to enter S even in the presence of actinomycin D at concentrations (0.03 μg/ml) that inhibit stimulation of DNA synthesis by serum or Ad12 infection. We have confirmed in Ad2-infected tsAF8 cells the results obtained by Laughlin and Strohl (17) with BHK—i.e., actinomycin D (0.03 μg/ml), while preventing serum-stimulated tsAF8 cells from entering S, does not prevent Ad2-induced stimulation of DNA synthesis. It seems therefore that Ad2 has the capacity to induce DNA synthesis in quiescent tsAF8 cells under conditions that ordinarily block their entry into S.

Our results also show that the DNA synthesized in tsAF8 cells infected with Ad2 at the nonpermissive temperature is cellular DNA semiconservatively replicated and that Ad2 infection (but not serum or polyoma virus) can overcome the ts block of another $G_1$ mutant of BHK cells, ts13 cells.

It could be argued that Ad2 infection may cause DNA synthesis in tsAF8 cells at 40.6°C simply because it acts on cellular

targets before the ts function can be expressed. The following findings militate against this interpretation: (*i*) it is unlikely that Ad2 infection can act even more rapidly than serum (at 34°C, 1 hr of stimulation with 10% serum commits most tsAF8 to enter S, and yet at 40.6°C the cells cannot enter S); and (*ii*) even when tsAF8 are preincubated at 40.6°C for 10 hr before infection, Ad2 infection causes them to enter S.

From the point of view of cell cycle regulation, the significance of these findings lies in the observation that, at the temperature nonpermissive for tsAF8, Ad2, but not polyoma, can stimulate DNA synthesis although the infected cells become T-antigen positive for the respective virus in both cases. Clearly, more detailed studies are necessary.

The following working hypothesis is the basis of our future investigations. Polyoma infection stimulates DNA synthesis in hamster cells by a mechanism similar to serum stimulation. On the contrary, Ad2 infection causes cellular DNA synthesis, which is insensitive to actinomycin D (low doses) and to a ts block in $G_1$. We hypothesize that the early proteins of polyoma act on resting cells in a manner analogous to that of simian virus 40 early proteins (30–32), while the early proteins of Ad2 act on a different point in the $G_1$ phase of the tsAF8 cell cycle.

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