Temperature-Sensitive Mutants of Adenovirus Type 12 Defective in Viral DNA Synthesis

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Ten temperature-sensitive (ts) mutants of adenovirus type 12 which produce plaques at 31 but not at 38.5 C have been isolated after mutagenesis with nitrosoguanidine or nitrous acid. The mutants have been classified into six separate complementation groups. DNA-DNA hybridizations have shown that at 38.5 C the ts 401 and 406 mutants of groups B and E, respectively, synthesized less than 10% of the normal level of viral DNA. The two mutants were also defective in the production of late proteins at the nonpermissive temperature, as shown by fluorescent-antibody tests and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Genetic recombination between the ts viruses 401 and 406 has been demonstrated; the recombination frequency for the wild-type virus production was 17.7%. Both mutants induced an increase in thymidine kinase activity at 38.5 C. Moreover, the two viral DNA-defective mutants shut off host DNA synthesis at the restrictive temperature. It is striking that at 38.5 C ts virus 401 transformed two to eight times more hamster cells than the wild-type virus, whereas ts virus 406 transformed at a frequency similar to the wild-type virus.

Temperature-sensitive (ts) mutants of adenovirus types 5 (4, 23, 30, 32, 33), 12 (17, 25; F. Rubenstein, H. S. Ginsberg, and M. K. Dixon, *Abstr. Annu. Meet. Amer. Soc. Microbiol.* 1973, V37, p. 200), 31 (27-29), and CELO virus (10) have been isolated and partially characterized. Relatively few of these mutants are defective in an early function under restrictive, but not under permissive, temperatures during lytic infection (4, 27-29, 32, F. Rubenstein, H. S. Ginsberg, and M. K. Dixon, *Abstr. Annu. Meet. Amer. Soc. Microbiol.* 1973, V37, p. 200). Since only genes which function early in productive infection are expressed in adenovirus-transformed cells (8, 9), the characterization of mutants defective in early functions should be of value in the analysis of the molecular aspects of transformation as well as of virus growth.

We have undertaken to study ts mutants of adenovirus type 12 which are defective in the early events of viral growth. We describe the isolation of ts mutants of two complementation and recombination groups defective in viral DNA synthesis.

MATERIALS AND METHODS

Cells and virus. The growth of primary cultures of human embryonic kidney (HEK) cells and the prepa-

ration of stocks of cloned type 12 human adenovirus (H12) have been described (12).

Infection of cells. Confluent or nearly confluent monolayer HEK cell cultures, containing about 3×10^6 cells in a 60-mm petri dish, were infected with 20 to 30 PFU per cell (12). After adsorption for 1 h at 37 C, the cells were washed twice, covered with 3 ml of Eagle minimal essential medium containing 5% fetal calf serum and 1 mM arginine, and incubated at 31 or 38.5 C in a humidified 5% CO₂-air mixture.

Plaque assay. The plaque method of virus assay has been described (12). This method was used at 31 C with the following modification. After 7 to 8 days, the cells were overlaid with 4 ml of the overlay medium, and 12 to 14 days after infection they were overlaid with 4 ml of the same medium now containing 0.0025% neutral red. Plaques were counted 20 to 22 days after infection.

Mutagenesis: (i) **NG.** HEK cells were infected with cloned wild type (WT) of H12. At 25 h after infection, 3 ml of fresh medium containing 25 μ g of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) per ml was added to each dish for 30 min. The cultures were then washed twice with Hanks balanced salt solution, fed with fresh medium (without NG), and incubated at 31 C for an additional 3 days. The virus was released from infected cells by freezing and thawing three times. The final PFU of NG-treated cultures was 11% of the virus yield obtained from untreated cultures. The infectivity of H12 was not affected by exposure for 1 h to 25 μ g of NG per ml.

(ii) **Nitrous acid (NA).** Cloned WT H12 (8×10^8 PFU/ml) was diluted 1:10 in 0.7 M NaNO₂ in 1 M

sodium acetate buffer (pH 4.6) at room temperature (5). At various times, the reaction was stopped by the addition of 4 volumes of cold 1 M Tris buffer, pH 8.0. The mixture was then further diluted 20-fold with cold Eagle medium containing 5% fetal calf serum. After 15 min, the titer was reduced by almost 4 logs.

Isolation of ts mutants. Mutagenized stocks, diluted to give approximately 20 to 30 plaques per 60-mm petri dish, were plated for plaque production on HEK cell monolayer cultures at 31 C. Isolated plaques were picked with a Pasteur pipette and suspended in 2 ml of Hanks balanced salt solution containing 1% fetal bovine serum. Virus was released from cells by freezing and thawing three to four times. Then 0.1 ml of each plaque isolate was inoculated onto duplicate test tube monolayer HEK cell cultures; one set of cultures was incubated for 15 to 20 days at 31 C, and the second set was incubated for 20 to 30 days at 38.5 C. Virus yields obtained at 31 C from those isolates producing cytopathic effect at 31 but not at 38.5 C were then plaque-assayed at both temperatures. Mutants showing at least a 1,000-fold greater plaqueing efficiency at 31 than at 38.5 C were plaque-purified two more times.

Complementation. HEK cells were mixedly infected with 10 PFU of each of two mutants per cell, or with 20 PFU of each mutant alone per cell. After adsorption for 1 h at 37 C, the cells were washed twice and covered with medium. The cultures were incubated at 38.5 C for 2 to 3 days and then frozen and thawed three times to release virus. The viral yields were assayed by the plaque method at 31 C.

Recombination. HEK cells were infected with 10 PFU of each of two mutants or with 20 PFU of one mutant alone per cell. After adsorption for 1 h at 37 C, the cells were washed twice, covered with medium, and incubated at 31 C for 3 days. Virus was released from infected cells by freezing and thawing three to four times. The viral yields were assayed by the plaque method at 31 C for total infectivity and at 38.5 C to measure formation of the WT recombinants. The WT recombination frequency is expressed as: (the virus titer at 38.5 C/virus titer at 31 C) $\times$ 100. Titration at 38.5 C of yields from self-crossed controls revealed insignificant amounts of virus.

Determination of incorporation of [³H]thymidine into DNA and the acid-soluble fraction. [³Methyl-³H]thymidine (5 μ Ci/ml, 1.9 Ci/mmol) was added to a final concentration of 15 μ Ci in a total volume of 3 ml in each culture dish. Cultures were exposed for 1 h at 38.5 C. The rate of DNA synthesis was determined from the incorporation of the label into acid-insoluble material after extraction of cells with 0.5 N perchloric acid by procedures outlined previously (12). The supernatants resulting from the extraction represented the acid-soluble pool.

Labeling and extraction of cell DNA. H12-infected and mock-infected HEK cell monolayer cultures were grown in the presence of [³H]thymidine (10 μ Ci/ml, 22 Ci/mmol) for 18 to 24 h at the indicated times after infection. The cultures were then washed twice with Tris-buffered saline, and DNA was extracted by procedures described previously (16).

DNA-DNA hybridization. Hybridizations were carried out by the nitrocellulose membrane filter

technique (16). Unlabeled HEK cell DNA or H12 DNA prepared from purified virus (18) was fixed to membrane filters, and ³H-labeled DNA extracted from ts mutant-infected or control cells was added to the filters in amounts given in the text. M. Green kindly furnished some of the unlabeled H12 DNA used.

Immunofluorescent staining for H12 virus antigens. The method for immunofluorescent staining has been described (21). Infected and control cells grown on cover slips were washed with phosphate-buffered saline (pH 7.4), dried, fixed in acetone for 10 min at 4 C, and air-dried. Cells were then incubated with a 1:10 dilution of normal rabbit serum or rabbit antiserum against H12 virus (Grand Island Biological Co., Grand Island, N.Y.) for 1 h at room temperature, washed six times, and incubated for 45 min at room temperature with fluorescein-conjugated goat anti-rabbit immunoglobulin (Microbiological Associates, Inc., Bethesda, Md.) The cover slips were washed six times, mounted in a mixture consisting of 9 parts glycerin and 1 part PBS, and examined with a fluorescence microscope.

Polyacrylamide gel electrophoresis of cell proteins. HEK cells were infected with WT H12 or ts mutants and incubated for 42 h at 38.5 C. The cells were washed twice with Hanks balanced salt solution and incubated for 2 h at 38.5 C in Hanks balanced salt solution with 1% dialyzed fetal calf serum and radioactively reconstituted protein hydrolysate (Schwarz/Mann); hydrolysates of ³H- or ¹⁴C-labeled protein (5 μ Ci/ml) were added to WT and ts mutant-infected cells, respectively. The cells were washed, pooled, lysed in a small volume of 0.01 M Tris (pH 7.5) containing 2% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol, and solubilized by heating at 100 C for 2 min.

The mixed protein fractions from WT and ts mutant-infected cells were coelectrophoresed on SDS-6% polyacrylamide gels (14). Electrophoresis was carried out for 7 h at 7 V per gel (0.6 by 15 cm) containing 6% polyacrylamide, *N,N'*-methylene bisacrylamide as the cross-linking agent, 0.1 M sodium phosphate buffer (pH 7.4), and 0.1% SDS. Radioactivity in solubilized 1-mm gel slices was determined by scintillation spectrometry (13).

To prepare ³H-labeled H12, cells infected with H12 for 20 h were washed and incubated with ³H-labeled protein hydrolysate (10 μ Ci/ml) until 50 h after infection. Virus was then isolated and purified (31). For polyacrylamide electrophoresis, the purified, labeled virus was dissociated with SDS (18).

Transformation of hamster cells. Primary hamster embryo cultures and Eagle enriched medium, as modified by Casto (2), were purchased from BioLabs, Inc., Northbrook, Ill. Cells were removed with 0.2% trypsin, centrifuged, and resuspended in Eagle enriched medium containing 10% heat-inactivated fetal bovine serum and NaHCO₃ (0.11 g/100 ml). For transformation experiments, 3×10^6 cells in 2.8 ml of the above medium were placed in a 60-mm petri dish, and, immediately after plating, 0.2 ml of virus was added. The transformation procedure has been described (1, 2). To determine whether cell killing by virus occurred, after virus adsorbed for 4 h cells were

diluted further to contain 1,000 cells per 3 ml of the above medium, and the cell-survival assay procedure previously described was used (15). Cloning efficiencies of 7% were usually obtained with uninfected cells.

Thymidine kinase assay. The preparation of cell extracts and the procedure for thymidine kinase assay have been described (12). Cell extracts were assayed for enzyme activity using [^3H]thymidine as the substrate. The substrate and the phosphorylated derivatives were then isolated by paper chromatography in an isopropanol- NH_3 solvent. The thymidine kinase activity was expressed as picomoles of thymidine converted to thymidine phosphates per microgram of protein in 30 min. The enzyme reaction was linear with protein concentration and with time under the assay conditions.

RESULTS

Growth of H12 WT in HEK cells. The growth characteristics of H12 in HEK cell cultures incubated at 31 and 38.5 C were examined (Table 1). The eclipse period at 38.5 C was approximately 20 h, and virus synthesis was complete at 50 to 60 h. The growth curve of virus synthesis at 38.5 C was generally similar to that previously studied at 37 C (12). In contrast, at 31 C, synthesis of new infective virus began approximately 30 h after virus adsorption and was complete at 70 to 80 h. Extensive cytopathic changes were evident at the time when virus maturation was virtually complete.

Isolation of ts mutants. The mutagens NG and NA were employed to isolate H12 ts mutants. The virus yield obtained from HEK cells treated with 25 μg of NG per ml of medium was used for the selection of ts mutants. At this concentration, the virus yield was approximately 11% of the yield obtained from un-

TABLE 1. Growth of H12 in HEK cell cultures at 31 and 38.5 C^a

Time after infection(h)	PFU/ml of virus grown ^b	
	38.5 C	31 C
12	8.1×10^4	6.8×10^4
16	7.6×10^4	7.0×10^4
20	9.7×10^4	7.2×10^4
24	7.8×10^4	6.4×10^4
28	3.0×10^4	5.6×10^4
30	7.5×10^3	1.2×10^5
48	1.9×10^3	7.4×10^4
64	3.1×10^2	1.4×10^5
70	2.6×10^2	2.3×10^5
80	ND ^c	2.5×10^5

^a HEK cells were infected with 20 PFU of H12 WT per cell and incubated at 31 or 38.5 C. Samples were removed at the indicated times.

^b The virus yields collected at 31 or 38.5 C were titrated at 37 C with the plaque assay.

^c Not done.

TABLE 2. H12 yields from HEK cells treated with NG^a

Concn of NG ($\mu\text{g}/\text{ml}$)	H12 yield (PFU/ml)	Virus yield from treated relative to untreated cells (%)
0	3.2×10^8	
12.5	7.2×10^7	22.5
25	3.6×10^7	11.3
50	4.5×10^5	0.14
100	$<10^5$	<0.1

^a H12 WT-infected HEK cells were treated with the mutagen NG (nitrosoguanidine). At 25 h postinfection, 3 ml of medium containing various concentrations of NG were added to each culture dish for 30 min. The cultures were then washed twice, fed with fresh medium (without NG), and incubated at 31 C for an additional 3 days. Virus was titrated at 37 C by the plaque assay.

FIG. 1. Nitrous acid (NA) inactivation of H12 WT. Virus was treated with NA and titrated at 37 C, using the plaque assay.

treated cells (Table 2). Treatment of H12 with NA resulted in inactivation of virus with single-hit kinetics (Fig. 1). However, since a two-phase inactivation curve was obtained, it is difficult to be certain that the second phase actually represented single-hit kinetics. Virus inactivated to a level of 5×10^{-4} was used for mutant isolation.

Ten independent ts mutants were isolated which showed a plaquing efficiency of more than 1,000-fold at 31 than at 38.5 C (Table 3). All of the mutants grew well at 31 C, reaching titers of approximately 10^8 to 4×10^8 PFU/ml in 70 to 80 h. The ratio of titers at the permissive to the nonpermissive temperature for these mutant virus yields ranged from approximately $3 \times$

TABLE 3. *Plaque-forming titers of H12 ts mutants at 31 and 38.5 C^a*

Mutant	Mutagen	PFU/ml ^b		
		31 C	38.5 C	38.5 C/31 C
H12 ts 400 ^c	NG	2.4×10^8	5.0×10^1	2.1×10^{-7}
H12 ts 401	NA	9.2×10^7	2.3×10^1	2.5×10^{-7}
H12 ts 402	NG	2.8×10^8	8.8×10^4	3.1×10^{-4}
H12 ts 403	NA	3.4×10^8	9.0×10^4	2.6×10^{-4}
H12 ts 404	NA	2.6×10^8	1.1×10^4	4.2×10^{-5}
H12 ts 405	NA	4.1×10^8	1.5×10^3	3.7×10^{-6}
H12 ts 406	NG	1.3×10^8	1.4×10^1	1.1×10^{-7}
H12 ts 407	NA	2.5×10^8	2.5×10^3	1×10^{-5}
H12 ts 408	NG	1.2×10^8	3.7×10^4	3.1×10^{-4}
H12 ts 409	NG	1.1×10^8	4.5×10^2	4.1×10^{-6}
H12 WT		2.8×10^8	2.9×10^8	1.0

^a Cloned H12 WT was mutagenized by NG (nitrosoguanidine) or NA (nitrous acid), and ts mutants were isolated.

^b Final yield from HEK cells infected with 15 to 20 PFU of each virus per cell and incubated at 31 C for 70 to 80 h. Virus yields were titrated at 31 and 38.5 C.

^c Blocks of mutant numbers have been assigned by J. F. Williams (7).

10^8 to 10^7 . The frequency of mutants in NA-mutagenized virus was 0.74% (5/679); after NG mutagenesis, the frequency was 0.91% (5/550).

Complementation tests. For complementation studies, cells were infected with pairs of mutants or with single mutants at the nonpermissive temperature. As evidenced by the single infection yields, some of the H12 ts mutants exhibited leakiness (Table 4). Nevertheless, it was possible to assign the 10 ts isolates to six separate complementation groups (Table 4). The yields from crosses showing unambiguous complementation were 50 up to 130,000 times higher than the sum of the yields of the two single infections. Those mutant crosses which produced complementation yields ranging from a 0.58- to a 2.6-fold increase over the sums of the two single infections were considered to be negative for complementation. The complementation yields ranged from approximately 4 to 50% of the yield of the WT virus grown under the same conditions. Similar efficient complementation has been obtained in studies with other adenovirus ts mutants (4, 34).

Induction of DNA synthesis. The H12 ts mutants belonging to the six complementation groups were tested for their ability to induce DNA synthesis after infection at the nonpermissive temperature. The rate of incorporation of [³H]thymidine into total DNA was examined at various times after infection at 38.5 C. In previous experiments, infection of HEK cells with H12 WT resulted in an initial drop in the rate of incorporation of thymidine into DNA during the latent period of virus growth, followed by a marked increase in label uptake (16). The observed decrease in label incorporation has

been attributed to a decline in cell DNA synthesis (16). The rise in incorporation rate, on the other hand, has been shown to reflect virus DNA synthesis (16).

With the exception of cells infected with ts 401, 406, and 409 mutants, all of the cultures infected with the remaining mutants under nonpermissive conditions displayed the characteristic patterns of thymidine incorporation into total DNA shown by the WT-infected cells. At 15 h after WT infection, the rate of incorporation per cell of the DNA precursor was only approximately 30% of the control rate; at approximately 40 h, the rate was 10-fold higher than that observed in control cells (Fig. 2). Cultures infected with ts 401, 406, or 409 mutants at 38.5 C also displayed a significant drop in the rate of thymidine incorporation into DNA during the latent period. The subsequent rise in label uptake, however, was considerably less than that shown by WT-infected cells (Fig. 2). At 48 h, the rate of label uptake was from 1.5- to 2-fold higher in cells infected with ts 401 or 406 mutants than that observed in uninfected cells; in ts 409 mutant-infected cells, however, the rate was almost four times higher. Since only the ts 401 and 406 mutants, and possibly 409, appear to be defective in viral DNA synthesis at the nonpermissive temperature (Fig. 2), these three mutants were studied in subsequent experiments.

Inhibition of host DNA synthesis. To determine the extent of inhibition of host DNA synthesis in cells infected with the ts 401, 406, or 409 mutants at the nonpermissive temperature, DNA-DNA hybridization experiments were carried out. Control and the mutant-infected cul-

TABLE 4. Complementation between H12 ts mutants: complementation indexes^a

Mutant	Complementation group ^b									
	A ts 400 ^c	B ts 401	C ts 402	D ts 403	D ts 404	D ts 405	E ts 406	D ts 407	C ts 408	F ts 409
ts 400	(8.9 × 10 ⁴)									
ts 401		120 (2.5 × 10 ²)		280	50	108	330	83	110	870
ts 402			82 630 (1.9 × 10 ⁵)	1,800	120	220	130,000	125	2,050	1,600
ts 403				350 (8.5 × 10 ⁴)	250	470	580	320	0.88	390
ts 404					0.58 (9 × 10 ⁴)	0.92	1,150	1.1	650	460
ts 405						2.6 (4.1 × 10 ⁵)	110	2.3	180	190
ts 406							200	0.90	400	330
ts 407							(2.1 × 10 ⁵)	130 (6.2 × 10 ⁵)	1,200	17,500
ts 408									140 (8.3 × 10 ⁴)	88
ts 409										1,000 (5.5 × 10 ⁵)

^a HEK cells were infected with pairs of mutants each at an input multiplicity of approximately 10, or with single mutants at an input multiplicity of approximately 20. Yields were obtained from cells incubated for 50 h at 38.5 C and assayed at 31 C. Values in parenthesis represent PFU per milliliter of single infections. Complementation indexes: Yield (mutant, + mutant₂) at 38.5 C/(yield mutant₁ at 38.5 C + yield mutant₂ at 38.5 C), where the yields are assayed at 31 C. The values are the means of two experiments.

^b Each mutant has been assigned to the indicated complementation group.

^c Blocks of mutant numbers have been assigned by J. F. Williams (7).

FIG. 2. Rates of incorporation of [^3H]thymidine into total DNA of HEK cells infected with H12 WT or *ts* mutants. Cultures were infected at multiplicities of 20 to 30 and incubated at 38.5 C. A parallel series of cultures were mock-infected. At the indicated times, cultures were exposed to [^3H]thymidine (5 $\mu\text{Ci/ml}$, 1.9 Ci/mmol) for 1 h at 38.5 C, and the radioactivity of acid-insoluble fractions was determined. CPM, counts per min.

tures were given [^3H]thymidine for 18- to 24-h periods at various times after infection; the DNA was then extracted and hybridized with cell DNA (Table 5). Between 20 and 44 h after infection by each of the three mutants at 38.5 C, the proportion of labeled DNA that hybridized with cell DNA decreased variably. The amount of newly synthesized cell DNA was reduced by 70% in *ts* 401-infected cells, and by 93 and 95% in *ts* 406- and 409-infected cells, respectively. At 44 to 68 h, there was an approximate 90 to 95% reduction in label incorporation in all of the *ts* mutant-infected cells incubated at 31 or 38.5 C. The rate of DNA synthesis in uninfected cells also declined somewhat during a period of several days, presumably because the cells were entering or were in the stationary phase of growth. It can be concluded that cell DNA synthesis was shut off to approximately the same extent in HEK cells infected with *ts* 401, 406, or 409 mutants at permissive or nonpermissive temperatures. Additional experiments have revealed that H12 WT also reduced cell DNA synthesis by approximately 90% late in infection at 38.5 C.

Viral DNA synthesis. A comparison was

made of the ability of the *ts* 401, 406, or 409 mutants to induce virus-specific DNA synthesis in cultures infected under permissive (31 C) or restrictive temperatures (38.5 C). Mock infected and the mutant-infected cultures were exposed to [^3H]thymidine for 18- to 24-h intervals at different times. The DNA was extracted at the end of the labeling period and hybridized with H12 WT DNA (Table 6). At the restrictive temperature, the *ts* 401 and 406 mutants synthesized very low amounts of viral DNA. A direct comparison between the extent of hybridization of the labeled viral DNA in the 31 and 38.5 C yields of the *ts* 401 and 406 mutants is not possible because the ratio of filter-bound DNA to the amount of viral DNA present must vary markedly. In fact, if the ratio of bound DNA to labeled DNA of the 31 C yields is increased 10-fold over that used in the hybridization experiments (Table 6), the hybridization efficiency increases. Therefore, the calculation of the fraction of label incorporated into DNA at 38.5 C relative to that incorporated at 31 C (Table 6) gives a minimal figure for the extent of inhibition of viral DNA at 38.5 C. At 68 h, the viral DNA synthesis of *ts* 401 and 406 grown under restrictive conditions was reduced by over 90% of the yield obtained under permissive conditions. The possibility that the higher temperature per se reduced virus DNA synthesis appears to be ruled out by the finding that the WT virus made approximately the same amounts of viral DNA at both 38.5 and 31 C. The *ts* 409 was able to synthesize approximately normal levels of virus DNA at 38.5 C (Table 6). At 31 C most viral DNA was made by 68 h. To ascertain these conclusions, we are now conducting experiments measuring, by reassociation kinetics, the amounts of viral DNA in cells infected with the viral DNA-defective mutants under nonpermissive conditions.

Thymidine incorporation into acid-soluble fractions. Previous results have shown that the rate of incorporation per cell of [^3H]thymidine into the DNA of cells infected with the *ts* 401 or 406 mutants under nonpermissive conditions was inhibited during a portion of the eclipse period; thereafter, the rate of incorporation of the DNA precursor increased somewhat, but this increase was still considerably less than that observed in the WT-infected cells (Fig. 2). The possibility existed that the decreased incorporation of thymidine into the DNA of *ts* 401 and 406 mutants (Table 6) resulted from a defect in thymidine uptake. The effect of infection by these mutants at the nonpermissive temperature on the rate of precursor uptake into the acid-soluble pool was therefore also exam-

TABLE 5. *Synthesis of cell DNA in HEK cell cultures infected with H12 ts 401, 406, or 409 mutants^a*

Time after Infection (h)	Counts per minute of ³ H-labeled DNA hybridized per μ g of input DNA prepared from cells infected with:			
	Uninfected	ts 401	ts 406	ts 409
2 to 20, 38.5 C yield	6,100 ^b ; 14.0%	5,368 (0.88) ^c ; 14.7%	4,636 (0.76); 15.2%	4,392 (0.72); 15.0%
20 to 44, yield	6,403; 16.3%	1,916 (0.30); 13.4%	460 (0.07); 8.4%	307 (0.048); 0.8%
44 to 68, yield	3,200; 14.2%	226 (0.08); 8.8%	312 (0.10); 6.2%	288 (0.09); 0.3%
44 to 68, 31 C yield	3,540; 14.5%	496 (0.14); 0.5%	354 (0.10); 0.4%	424 (0.12); 0.4%

^a HEK cells were infected with H12 ts 401, 406, or 409 mutants at multiplicities of 15 to 20. Cell cultures were then incubated at 31 or 38.5 C. At the indicated times, samples of approximately 3×10^7 infected and mock-infected cells were labeled with 300 μ Ci of [³H]thymidine (22 Ci/mmol). At the end of the labeling period, DNA was extracted. The specific activities (counts per minute per microgram of DNA) of the labeled DNAs extracted from uninfected cells grown at 38.5 C were: 2 to 20 h, 43,541; 20 to 44 h, 39,310; and 44 to 68 h, 22,482. The specific activities of labeled DNAs induced in ts 401-infected cells at 38.5 C were: 2 to 20 h, 36,399; 20 to 44 h, 14,259; and 44 to 68 h, 2,565. The specific activities of DNAs induced in ts 406-infected cells at 38.5 C were: 2 to 20 h, 30,560; 20 to 44 h, 5,458; and 44 to 68 h, 5,032. The specific activities of DNAs induced in ts 409-infected cells at 38.5 C were: 2 to 20 h, 29,360; 20 to 44 h, 24,748; and 44 to 68 h, 77,359. The specific activity for the 31 C DNA yield collected at 44 to 68 h from uninfected cells was 24,390; from ts 401-infected cells, 47,459; from ts 406-infected cells, 46,678; and from ts 409-infected cells, 63, 341. For hybridization experiments, membrane filters containing 9.3 μ g of unlabeled HEK cell DNA were incubated with approximately 1 to 2 μ g of ³H-labeled DNA. From 14 to 16% of the input ³H-labeled DNA from uninfected cells hybridized to the filter.

^b Results are expressed as the counts per minute of ³H-labeled DNA hybridized to the filter per microgram of input DNA. Means of duplicate determinations are given. Background counts were subtracted. The average DNA content per infected cell was similar to that per uninfected cell at all times, except for the 20- to 44-h and the 44- to 68-h yields of ts 409, where the average DNA content per cell was 1.4 and 1.6 times that per control cell, respectively. In addition, the average DNA amount per cell of all 31 C yields was 1.8 to 2.0 times higher than the control. These increases presumably represent virus DNA synthesis (16), and the total DNA found at these times was corrected for virus DNA content. Each value given in the table, therefore, is assumed to be based on cellular DNA alone.

^c Figures in parenthesis indicate fraction of control. Ratio: (counts per minute hybridized per microgram of input DNA extracted from infected cells)/counts per minute hybridized per microgram of DNA from uninfected cells.

ined at various times by observing the incorporation of [³H]thymidine for 1 h into the acid-soluble fraction. The ts 409 mutant was included for comparison. As was previously reported for the WT-infected cells (16), no inhibition of the incorporation of thymidine into the acid-soluble fractions of cells infected with the ts 401, 406, or 409 mutants at the nonpermissive temperature was detected. Furthermore, the incorporation of the DNA precursor into the acid-soluble pool began to increase approximately 15 to 17 h after infection with the three mutants; the rates of incorporation then continued to increase until about 40 to 50 h after infection. At this time, the rate of thymidine incorporation into the acid-soluble fraction was six to eight times greater than that of uninfected cells; similar increases in the incorporation rates were found in the WT-infected cells. At 40 to 50 h, the total radioactivity (counts per minute) of [³H]thymidine incorporated into the acid-soluble fraction per 10^6 cells per h was 8.4×10^3 for the uninfected cells, and 5.1×10^4 to 7.0×10^4 for cells infected with the WT or ts 401, 406, or 409 viruses. The findings suggest

that there was no defect in thymidine uptake in these mutant-infected cells under nonpermissive conditions.

Thymidine kinase activity. A sixfold increase in thymidine kinase activity has been observed in the WT-infected HEK cells (12). The enzyme activity was also measured at different times after infection with the WT or the ts 401, 406, and 409 mutants at 38.5 C. Generally, similar increases in thymidine kinase activity were observed in cells infected with the WT or each of the three mutants (Fig. 3). The enzyme activity began to increase 20 to 30 h after infection, and at 60 h the activity was approximately sixfold higher than that observed in the uninfected cells.

Immunofluorescence test for virion proteins. The anti-H12 rabbit serum was used for indirect fluorescent-antibody staining of cells which were infected with the WT or ts 401, 406, or 409 mutants and incubated at 31 or 38.5 C for various times. Cells infected with the WT or with each of the mutants for 3 days at 31 C exhibited intensive nuclear fluorescence. In contrast, cells infected with ts 401 or 406 for 4 to

TABLE 6. *Synthesis of adenovirus DNA in HEK cell cultures infected with H12 ts 401, 406, or 409 mutants^a*

Time after infection (h)	Hybridization (%) of ³ H-labeled DNA prepared from cells infected with:				
	Uninfected	ts 401	ts 406	ts 409	WT
2-20, 38.5 C yield	0.08 ^b	0.11	0.16	0.61	ND ^d
20-44, 38.5 C yield	ND	0.17 (0.015) ^c	0.60 (0.052)	11.5 (0.95)	ND
44-68, 38.5 C yield	0.12	0.22 (0.015)	0.98 (0.070)	14.7 (0.93)	16.1 (1.1)
20-44, 31 C yield	ND	4.8	9.7	12.1	ND
44-68, 31 C yield	ND	8.3	12.6	15.8	14.7

^a HEK cells were infected with H12 WT or with the ts 401, 406, or 409 mutants at multiplicities of 15 to 20. Portions of cell cultures were incubated at 38.5 or at 31 C. At the indicated times, samples of approximately 3×10^7 infected and mock-infected cells were labeled with 300 μ Ci of [³H]thymidine (22 Ci/mmol). The DNA was extracted at the end of the labeling period. The specific activities (counts per minute per microgram of DNA) of the labeled DNA extracted from uninfected cells at 38.5 C were: 2 to 20 h, 43,541; and 44 to 68 h, 22,482. The specific activities of labeled DNA induced in ts 401-infected cells at 38.5 C were: 2 to 20 h, 36,399; 20 to 44 h, 14,259; and 44 to 68 h, 2,565. The specific activities of DNA induced in ts 406-infected cells at 38.5 C were: 2 to 20 h, 30,560; 20 to 44 h, 5,458; and 44 to 68 h, 5,032. The specific activities of DNA induced in ts 409-infected cells at 38.5 C were: 2 to 20 h, 29,360; 20 to 44 h, 24,748; and 44 to 68 h, 77,359. The specific activity of WT-labeled DNA (44 to 68 h, 38.5 C yield) was 79,550. The specific activities of the 31 C DNA yields from ts 401-infected cells were 19,405 (20 to 44 h) and 47,459 (44 to 68 h); from ts 406-infected cells were 12,942 (20 to 44 h) and 46,678 (44 to 68 h); from ts 409-infected cells were 24,462 (20 to 44 h) and 63,341 (44 to 68 h); and from WT-infected cells, 79,400 (44 to 68 h yield).

^b Results are expressed as the percentage of input ³H-labeled DNA (counts per minute) bound on the filter. For hybridization experiments, membrane filters containing 3.2 μ g of unlabeled H12 WT DNA per filter were incubated with 4 to 6 μ g of ³H-labeled DNA from ts 401- and 406-infected cells (31 or 38.5 C yields), except that 40 μ g of the DNA extracted from ts 401-infected cells (44 to 68 h, 38.5 C yield) were used. The ³H-labeled DNA from uninfected cells was added at a concentration of 4 μ g (2 to 20 h yield) or 40 μ g (44 to 68 h yield). The ³H-labeled DNA from ts 409-infected cells was added at a concentration of 0.04 μ g (20 to 44 h yields, 31 or 38.5 C) or 4 μ g (2 to 20 h yield). For hybridization tests with the 44- to 68-h yields obtained at 31 or 38.5 C from the WT- and ts 409-infected cells, 0.8 μ g of unlabeled H12 DNA was applied to membrane filters, and 0.01 μ g of ³H-labeled DNA extracted from the infected cells was added. Means of duplicate determinations are given. Background counts were subtracted.

^c The figures in parenthesis represent the ratio: (percent hybridization of 38.5 C DNA yields)/(percent hybridization of 31 C DNA yields). The figures were corrected for uninfected cell binding by using a value of 0.10.

^d Not done.

6 days at 38.5 C showed no specific nuclear or cytoplasmic fluorescence; moreover, typical adenovirus cytopathic changes were absent. Cells infected with ts 409, on the other hand, showed granular nuclear fluorescence after infection for 2 to 3 days at 38.5 C.

Polyacrylamide gel electrophoresis. The synthesis of virus and other proteins in cells infected by the viral DNA-defective ts 401 and 406 mutants was investigated further by examining protein extracts using SDS-polyacrylamide gel electrophoresis. Cells were infected with the WT or with the ts 401 (complementation group B) or ts 406 (group E) viruses; in addition, cells were infected with two mutants defective in a late function: ts 409 (group F), or ts 408 (group C). After incubation at 38.5 C for 42 h, hydrolysates of ³H- and ¹⁴C-labeled protein were added for 2 h to the WT- and the mutant-infected cells, respectively. The mixed protein fractions from the WT- and each mutant-infected cells were then coelectrophoresed on SDS-6% polyacrylamide gels (Fig. 4).

Aside from a possible enhancement of production of hexon (designated by the roman numeral II) in cells infected with ts 408, the gel electrophoresis patterns of ³H-labeled protein from the WT-infected cells and ¹⁴C-labeled proteins from ts 408-infected cells were similar (Fig. 4A), indicating that the relative amounts of proteins made were similar. In contrast, cells infected with the ts 401 (Fig. 4B) or 406 mutants (Fig. 4C) synthesized little or no hexon, whereas ts 409 reproducibly made a reduced amount of hexon compared to the WT (Fig. 4D). Although it is difficult to resolve the other viral components, the findings suggest that the synthesis of some of the core polypeptides, particularly component VIII, may also be inhibited in cells infected with the ts 401, 406, or 409 mutants under nonpermissive conditions.

Transformation of hamster cells. The ability of the viral DNA-defective ts 401 and 406 mutants to transform hamster embryo cell cultures at the nonpermissive temperature was compared to the WT transformation induction.

FIG. 3. Thymidine kinase activities of HEK cells infected with H12 WT or *ts* mutants. Cultures were infected at multiplicities of 10 to 20 and incubated at 38.5 C. A parallel series of cultures were treated in the same way, but no virus was added. At the indicated times, cell extracts were prepared and tested for enzyme activity. Results are expressed as picomoles of thymidine phosphorylated per microgram of protein in 30 min at 37 C.

The *ts* 401 and 406 viruses transformed hamster cells at the nonpermissive temperature (Table 7). For the WT-infected cells, the ratio of PFU to the number of transformed foci, designated as transformation efficiency, ranged from approximately 10^6 to 3×10^6 in two different experiments (Table 7). This variability in transformation efficiency may be the result of a difference in the relative proportion of male and female cells in the two different batches of hamster cells used. Female cultures have consistently developed more adenovirus-transformed foci than similarly infected male cultures (20). The WT transformation efficiency obtained (Table 7) agrees well with Casto's study, in which the average ratio of PFU to the number of transformed foci obtained was 1.8×10^6 in different experiments using H12 multiplicities ranging from 5 to 115 (1). The *ts* 406 virus transformed cells with approximately the same efficiency as the WT virus (Table 7). The transformation efficiency of the *ts* 409 mutant defective in a late function was also tested and found to be 1.5- to 1.9-fold higher than for the WT virus (Table 7). On the other hand, the *ts* 401 mutant transformed two to eight times

more cells than the WT under nonpermissive conditions (Table 7).

Recombination between *ts* 401, 406, and 409 mutants. Pairwise crosses between the *ts* 401, 406, and 409 mutants were carried out. The progeny virus yields formed at 31 C were then plaque assayed at 31 C to determine the total yield, and at 38.5 C to obtain the yield of recombinants having the WT phenotype. The frequency of the WT recombinants forming plaques at 38.5 C ranged from 13.6% (*ts* 406 $\times$ *ts* 409), and 16.7% (*ts* 401 $\times$ *ts* 409), to 17.7% (*ts* 401 $\times$ *ts* 406) (Table 8). Self-crossed controls contained 0.01% or less of the WT virus. At least 20 plaques from each cross were picked from the

FIG. 4. Coelectrophoresis on SDS-6% polyacrylamide gels of protein fractions isolated from HEK cells infected with H12 WT or *ts* mutants. Cells were infected at multiplicities of 20 to 30 and incubated at 38.5 C for 42 h. WT- and *ts* mutant-infected cells were labeled with ^3H - and ^{14}C -labeled protein hydrolysates ($5 \mu\text{Ci/ml}$), respectively, for 2 h. The ^3H - and ^{14}C -labeled cell proteins were pooled and dissociated with SDS and mercaptoethanol for 2 min at 100 C. Electrophoresis was carried out for 7 h at 7 V per gel (0.6 by 15 cm) containing 6% polyacrylamide, N,N'-methylene bisacrylamide as the cross-linking agent, 0.1 M sodium phosphate buffer (pH 7.4), and 0.1% SDS. Radioactivity in solubilized 1-mm gel slices was determined by scintillation spectrometry. Purified H12 treated with ^3H -labeled amino acids and dissociated as described yielded 10 labeled bands on SDS-6% polyacrylamide gel electrophoresis, as designated by the roman numerals. The hexon component is designated by the roman numeral II, corresponding to the assignments by Maizel et al. (19). Ordinate: percentage of total counts per minute recovered from the gel. Total recovery was more than 90%. ^{14}C , Solid line; ^3H , dashed line. (A) ^3H -labeled protein from WT-infected cells and ^{14}C -labeled protein from *ts* 408 (complementation group C)-infected cells; (B) ^3H -labeled protein from WT-infected cells and ^{14}C -labeled protein from *ts* 401 (complementation group B)-infected cells; (C) ^3H -labeled protein from WT-infected cells and ^{14}C -labeled protein from *ts* 406 (complementation group E); and (D) ^3H -labeled protein from WT-infected cells and ^{14}C -labeled protein from *ts* 409 (complementation group F)-infected cells.

TABLE 7. Transformation of hamster embryo cells by H12 WT and *ts* mutants^a

Expt no.	Virus	Total PFU added/dish	Virus multiplicity (PFU/cell)	No. transformed foci/dish ^b	Transformation efficiency ^c	Transformation efficiency in mutant relative to WT-infected cultures
1	WT	2.6×10^7	87	26	1.0×10^6	
	ts 401	2.4×10^7	80	54	4.4×10^5	2.3
	ts 406	3.2×10^7	107	33	9.7×10^5	1.0
	ts 409	1.4×10^7	47	21	6.7×10^5	1.5
2	WT	3.0×10^7	100	18	2.7×10^6	
	ts 401	1.8×10^7	60	54	3.3×10^5	8.2
	ts 406	1.2×10^8	40	36	3.3×10^6	0.8
	ts 409	4.1×10^7	137	29	1.4×10^6	1.9

^a Five dishes each were seeded with 3×10^5 cells, and then the indicated PFU were added to each dish. The transformation procedure was then carried out. Cell killing by virus did not occur at the indicated multiplicities, as determined by the cell survival assay.

^b Each figure represents the mean of transformed foci counted on five plates.

^c Transformation efficiency is expressed as the ratio of total PFU added per dish per number of transformed foci counted per dish.

TABLE 8. Recombination with several H12 *ts* mutants^a

Mutant	Recombination of complementation group:		
	B ts 401	E ts 406	F ts 409
ts 401	$(1.7 \times 10^{-9})^b$	17.7	16.7
ts 406		(1.3×10^{-4})	13.6
ts 409			(2.5×10^{-4})

^a HEK cells were infected with pairs of mutants each at an input multiplicity of 10, or with single mutants at an input multiplicity of 20. Yields were obtained from cells incubated for 3 days at 31 C and assayed at both 31 and 38.5 C. The values given represent the percentage of the WT recombinants in the yields expressed as the ratio: [(plaque titer at 38.5 C)/(titer at 31 C)] $\times$ 100.

^b The value in parenthesis represents the reversion frequency expressed as the ratio: (plaque titer at 38.5 C)/(titer at 31 C).

assay plates at 38.5 C and again assayed at 31 and 38.5 C. Approximately equal plating efficiencies were found at both temperatures for all of the plaque isolates. This finding indicates that the isolates were the WT recombinants. Additional recombination experiments with the other *ts* mutants of each complementation group are in progress.

DISCUSSION

The 10 *ts* mutants of type 12 adenovirus isolated have been assigned to six complementation groups. Mutants of two of the complementation groups, B and E, were blocked in viral DNA synthesis at the nonpermissive tem-

perature. These viral DNA-defective mutants both recombine with and complement each other, suggesting that at least two separate functions are essential for viral DNA replication. Phenotypically, the striking defect in these mutants is their markedly reduced ability to make viral DNA under nonpermissive conditions; the *ts* 401 mutant of complementation group B synthesizes less than 2% of the DNA made at 31 C, and *ts* 406 mutant of group E makes less than 7% as much. Adenovirus type 12 *ts* mutants defective in viral DNA synthesis are also defective in the production of late proteins. This is consistent with previous findings obtained with *ts* mutants of other adenovirus types (4, 29).

Even though the DNA-defective mutants studied synthesize very little viral DNA under nonpermissive conditions, cell DNA synthesis is depressed to approximately the same extent as shown by the WT virus. In a study of a DNA-minus *ts* mutant of adenovirus type 5, host DNA synthesis was partially inhibited (4); in another study, a second *ts* mutant formed practically no viral DNA, but depressed host DNA synthesis to the same extent as did the WT virus (32). These data support previous findings that synthesis of adenovirus DNA is not essential to inhibit cell DNA synthesis (6). On the other hand, a study using *ts* mutants of pseudorabies virus have shown that several mutants which are defective in viral DNA synthesis inhibit cell DNA synthesis to a lesser extent than the WT virus. Furthermore, some correlation existed between the ability to inhibit host DNA synthesis and the amount of

viral DNA made (22). It seems possible that the difference noted between the DNA-defective mutants of adenoviruses and pseudorabies virus in their ability to shut off host DNA synthesis may reflect the control of herpesvirus DNA synthesis by a larger number of genes (26).

The ts 401 mutant of complementation group B, which under nonpermissive conditions produces less than 2% of the normal level of viral DNA, transforms hamster cells with a greater efficiency than does the WT virus or the other viral DNA-defective virus studied here. The ts 401 mutant transformed two to eight times more cells than WT virus. In another study, a ts mutant of adenovirus type 12 transformed two to three times more rat cells than did the WT virus (F. Rubenstein, H. S. Ginsburg, and M. K. Dixon, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, V37, p. 200). The reason for this increase in transformation activity is not known. One possibility is that the affected mutant protein is normally a repressor-like product which regulates some event in transformation induction. This event may be a common step in the course of viral DNA synthesis in productively infected cells and that of cell transformation, as has been suggested for papovavirus (11). It is noteworthy that higher transformation induction frequencies were obtained with simian virus 40 or adeno-7-simian virus 40 hybrid viruses after treatment with UV light to the point where little infectivity was left (3, 24). The mechanism of this enhancement effect is unknown.

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