Selection and Preliminary Characterization of Temperature-Sensitive Mutants of Type 5 Adenovirus¹

MARCIA J. ENSINGER AND HAROLD S. GINSBERG

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received for publication 25 April 1972

Eight temperature-sensitive (ts) mutants that replicate normally at 32 C but poorly, if at all, at 39.5 C have been isolated from mutagenized stocks of a wild-type strain of type 5 adenovirus. Three mutagens were employed: nitrous acid, hydroxylamine, and nitrosoguanidine. Ts mutants were isolated from mutagenized viral stocks with frequencies between 0.01 and 0.1%. All eight mutants had reversion frequencies of 10^{-5} or less. Complementation experiments in doubly infected cultures at the nonpermissive temperature separated the mutants into three nonoverlapping complementation groups. Complementation yields ranged from a 2.3- to a 3,000-fold increase over the sums of the yields from the two singly infected controls. Genetic recombination was also demonstrated; approximate recombination frequencies ranged from 0.1 to 15%. Preliminary biochemical and immunological characterization of the mutants indicated that: (i) the single mutant in complementation group I did not replicate its deoxyribonucleic acid (DNA) or synthesize late proteins at the nonpermissive temperature but did inhibit host DNA synthesis to 25% of an uninfected control; (ii) the four group II mutants replicated viral DNA, shut off host DNA synthesis, synthesized penton base and fiber, but did not synthesize immunologically detectable hexon; the three mutants in complementation group III synthesized viral DNA, shut off host DNA synthesis, and made immunologically reactive capsid proteins (hexon, penton base, and fiber).

The structural components of the adenovirus virion have been identified (18, 28, 29), and biochemical analysis of adenovirus-infected cells has provided information on the sequence of events in the multiplication cycle of the virus (15, 16). Some of the most interesting aspects of adenovirus replication, however, have proved refractory to biochemical analysis. For example, the set sequence of events in infected cells suggests that there exist precise regulatory mechanisms that govern the biosynthesis of viral subunits and the selective inhibition of the synthesis of host deoxyribonucleic acid (DNA), ribonucleic acid, and protein. As yet, however, very little is known about how the virus regulates its own replication or the biosynthesis of the host macromolecules.

Conditional-lethal mutants of bacteriophages (4, 9, 10) have proved to be valuable tools for analyzing viral replication, and the more recent discoveries of temperature-sensitive (ts) mutants of animal viruses (2, 5, 11) suggest that these too

will be valuable for investigating the regulation of virus multiplication. The use of ts mutants seemed to be particularly applicable to investigate adenovirus multiplication since genetic markers were already available in the form of the viral structural components and the events in the viral replication cycle. This communication describes the isolation and preliminary genetic and biochemical characterization of eight ts mutants of type 5 adenovirus. A similar approach used in other laboratories has yielded ts mutants from CELO virus (22), and types 31 (34), 5 (38), and 12 (27) adenoviruses.

MATERIALS AND METHODS

Virus. The prototype strain of type 5 adenovirus used in this laboratory was found to plaque with half the efficiency at 39.5 C as at 37 C. We therefore sought a strain which would plaque equally well at these two temperatures. The desired virus designated as the wild-type (WT) strain was obtained after 10 serial passages in KB cell suspension cultures at 39.5 C, followed by two cycles of plaque purification at 39.5 C; all of the ts mutants described below were isolated from it. The efficiency of plating of WT virus

¹ A preliminary report of this investigation was presented at the annual meeting of the American Society for Microbiology, 1971 (Bacteriol. Proc., p. 223, 1971).

at 32 C was variable, ranging from 50 to 100% of the number of plaques obtained at 37 or 39.5 C.

Tissue culture and cell propagation. Suspension cultures of KB cells were grown in Eagle minimal essential medium supplemented with 10% calf or human serum (1, 7, 8). The cells were suspended in the same medium supplemented with 5% horse serum before infection. Viral stocks were prepared either in suspension culture (26) or in monolayers under maintenance medium (17), hereafter called infecting fluid (IF).

Temperature of incubation and temperature control. The permissive temperature selected was 32 C, and 39.5 C was the nonpermissive temperature. Suspension cultures were incubated in water baths under a 5% CO₂ in air atmosphere; the temperature was controlled to 0.1 C by a Braun Thermomix circulating thermoregulator. For the incubation of plates and bottles, a Hotpack CO₂ incubator with a fan was used at 39.5 ± 0.3 C and a NapCo water-jacketed CO₂ incubator at 32 C ± 0.5 C.

Infectivity assays. (i) *Plaque assay.* The previously reported 37 C plaque assay (25) was used with the following modifications. The plates were fed on the seventh day after infection with 3.5 ml of the overlay medium and stained on the 12th day with 2.5 ml of the overlay medium containing 0.014% neutral red; plaques were counted on the 13th and 14th days. At 32 and 39.5 C, the plates were infected and fed as described for the 37 C assay. At 39.5 C the stain was added on day 10, and the plaques counted on days 11 and 12. At 32 C the plates were stained on day 16 and counted until the titer became stable (usually by day 18 or 19).

(ii) *Fluorescent focus assay.* Infectious virus was also quantitated by an indirect immunofluorescence assay as described previously (14). The 37 and 39.5 C assay plates were incubated for 24 to 28 hr after adsorption before they were fixed with methanol and stained. An incubation period of 36 to 40 hr was required for virus assayed at 32 C.

Preparation of antisera. The immunization of rabbits with purified type 5 adenovirus, purified hexon, and purified fiber has been reported elsewhere (36, 37).

Mutagenesis. (i) *Nitrous acid.* The WT virus (2×10^{11} plaque-forming units [PFU]/ml) was diluted 1:10 in Hanks balanced salt solution (BSS) without NaHCO₃ and mixed with an equal volume of 0.2 M NaNO₂ in 0.2 M sodium acetate buffer, pH 4.0, at room temperature. Samples were removed at various times and diluted 1:5 in IF to which sufficient 0.1 N NaOH had been added to raise the pH to 7.5 to stop the reaction. The samples were dialyzed overnight against BSS. Residual infectivity was determined by the fluorescent focus assay. After 45 min the titer was reduced at least 5 logs.

(ii) *Hydroxylamine (HA).* WT virus was diluted 1:1 in 2 M hydroxylamine-hydrochloride in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0). The reaction mixture was incubated at 37 C. Samples were removed at various times, diluted 1:5 in IF, dialyzed overnight against BSS, and titrated by the fluorescent focus assay.

After 26 hr, the titer had decreased approximately 5 logs.

(iii) *Nitrosoguanidine (NG).* Monolayers of KB cells in 250-ml Falcon flasks were infected with 100 PFU/cell of the WT virus and overlaid with 25 ml of IF; the cultures were incubated at 32 C. At 6 hr postinfection (PI), 25 µg/ml NG was added. At 60 hr PI, the cells were removed from the flask and concentrated by centrifugation to 5 ml, and the virus was released by freezing and thawing six times. The NG-treated cultures yielded about half the quantity of virus obtained from cultures grown without the mutagen.

Isolation of ts mutants. The mutagenized stocks were screened for the presence of ts mutants either directly after mutagenesis (HA and NG) or after one passage in KB cell monolayers at 32 C when nitrous acid was employed. A two-step selection procedure was employed. The first step was an adaption of the plaque-enlargement technique used to isolate mutants of T4 (9). The mutagenized virus was diluted to give 20 to 40 plaques per plate and inoculated onto KB cell monolayers as described for the plaque assay. The plates were incubated at 32 C and stained with neutral red 14 days after infection. They were reincubated at 32 C for 24 to 48 hr until small plaques appeared. The circumference of each plaque was carefully outlined, and the plates were shifted to 39.5 C for an additional 24 to 48 hr. Plaques that failed to enlarge when shifted to the nonpermissive temperature were picked with a pasteur pipette and suspended in 2 ml of IF. Virus was released by freezing and thawing six times, and the resultant stocks were used for further testing.

The second step of the selection procedure assayed the ability of the potential mutants to produce complete adenovirus cytopathic effects (CPE) at 39.5 C in monolayers of KB cells infected with a low multiplicity (less than 0.1 PFU/cell): complete CPE would be produced only if infectious virus was made as a result of the initial virus-cell interaction. KB cell monolayers were infected in duplicate with <0.01 PFU of the potential ts mutants per cell and incubated for 3 to 5 days at 39.5 C or 4 to 6 days at 32 C. Those viruses producing CPE at 32 but not at 39.5 C were then plaque-assayed at both temperatures as a final test of mutation. Mutants were plaque-purified two more times before a final working stock was prepared. Several parallel stocks of each mutant were made, and the one with the lowest number of revertants was used.

Complementation. Suspension cultures of KB cells (10^6 cells/ml) were infected with 15 PFU of each of two ts mutants per cell in pairwise crosses; control cultures were infected similarly with 15 PFU of each mutant alone per cell. After 2 hr of incubation at 39.5 C, unadsorbed virus was removed by centrifugation at $800 \times g$ for 15 min, and the cells were suspended to a final concentration of 1.5×10^6 cells/ml in fresh, prewarmed medium. Samples were removed at various times and titrated at 39.5 C by using the fluorescent focus assay.

Recombination. Monolayers of KB cells in 30-ml Falcon flasks were infected with 15 PFU of each of two mutants or of one mutant alone per cell and incu-

bated at 32 C for 65 hr. The cells were harvested, virus was released by freezing and thawing, and the viral titer was assayed at 32 and 39.5 C by the plaque assay. Recombination frequencies were expressed as: two-times yield titrated at 39.5 C/yield titrated at 32 C $\times$ 100 (9). The yields from the controls singly infected with the mutants titrated at 39.5 C proved to be so low that they were insignificant in the calculations.

Preparation of cellular extracts from cells infected at the nonpermissive temperature. Suspension cultures of KB cells were infected with 20 PFU/cell and incubated for 36 hr at 39.5 C. The cells were harvested by centrifugation, washed twice with PBS, and suspended in 0.01 volume of 0.01 M sodium phosphate buffer, pH 7.0. The cells were lysed by freezing and thawing, and the cell debris removed by centrifugation. The supernatant fluid contained the soluble virus-specific antigens.

Immunodiffusion. Cordis disposable agarose immunodiffusion plates (IDF cell 1) were employed. Each well held 50 μ liters. Antiserum to purified type 5 adenovirus was placed in the center well, whereas the cellular extracts from cells infected with the mutants at 39.5 C as well as WT extracts, purified hexon, and purified fiber were placed in the peripheral wells. The plates were kept at room temperature for 1 to 3 days until the bands were well developed.

Purification of type 5 adenovirus antigens. The purification of type 5 adenovirus antigens has been described previously (26, 37).

Radioactive labeling of DNA in cells infected with the mutants at 39.5 C. Suspension cultures of KB cells (10^6 cells/ml) were infected with 200 PFU/cell and incubated at 39.5 C for 2 hr. Unadsorbed virus was removed by centrifugation, and the cells were suspended in fresh prewarmed medium at a concentration of 2×10^5 per ml. At 10 hr PI, 2 μ Ci of 3 H-thymidine (3 H-methyl, 27.8 mCi/mg, New England Nuclear) per ml was added; the cells were harvested by centrifugation 3 hr later. Just prior to the pulse, a sample was removed from each culture and reincubated for an additional 20 hr at 39.5 C as a check on reversion. The sodium dodecyl sulfate, Pronase, phenol method for extracting DNA from infected KB cells has been described elsewhere (14).

Isopycnic gradient centrifugation of DNA. The DNA samples were diluted to a concentration of 10 μ g/ml in 0.01 M ethylenediaminetetraacetic acid, 0.01 M tris(hydroxymethyl)aminomethane - hydrochloride, pH 8.1. A 0.1 ml fraction of the unknown DNA plus 0.1 ml of the 10 μ g/ml marker DNA (14 C-thymidine-labeled DNA extracted either from uninfected KB cells or from purified virions [14]) were added to 4.7 ml of a CsCl solution (specific gravity, 1.7 g/ml) prepared in the same buffer. Centrifugation was carried out in a Spinco no. 40 fixed-angle rotor for 72 hr at 35,000 rev/min at 20 C. Four-drop fractions were collected from the bottom of the tube directly onto Whatman no. 3 filter paper discs (2.3 cm). The discs were oven dried, and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer by using 5 ml of a solution of 0.5 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene and 6.0 g of 2,5-diphenyloxazole per liter of toluene.

Chemical determination of DNA. DNA was quantitated by the Burton modification of the diphenylamine reaction (3) with herring sperm DNA as the standard.

RESULTS

Growth characteristics of the WT strain at 32, 37, and 39.5 C. The one-step growth curves of the WT strain in suspension cultures of KB cells at the permissive and nonpermissive temperatures, as well as at 37 C, are shown in Fig. 1. The final yield per cell was approximately the same at all three temperatures. The viral multiplication at 39.5 C was slightly accelerated when compared to the replication at 37 C: the eclipse period ended at 9 to 10 hr PI instead of 11 to 12 hr, and the maximal yield was reached about 10 hr earlier. At 32 C, however, the replication cycle was considerably slower; the eclipse period was about 22 hr, and the maximal yield was not attained until approximately 60 hr PI.

Isolation of ts mutants. Eight independent ts mutants, which replicate at 32 C but not at 39.5 C, have been isolated by using the selection techniques described above. All three mutagens were effective in inducing ts mutants (Table 1). Approximately 1% of the plaques originally plated

FIG. 1. The initial cycle of multiplication of type 5 adenovirus at 32, 37, and 39.5 C. Suspension cultures of KB cells were infected with 100 PFU WT type 5 adenovirus per cell and incubated at one of three temperatures: 32, 37, or 39.5 C. Two hr PI, the unadsorbed virus was removed by centrifugation, and the cells resuspended in fresh prewarmed medium. Samples were taken at various times and assayed for infectious virus at 37 C by using the fluorescent focus assay.

TABLE 1. *Growth of ts mutants of type 5 adenovirus at 32 and 39.5 C^a*

Mutant	Mutagen	Titer (PFU/ml) ^b		Reversion frequency ^c	Fluorescent focus units/cell ^d	
		32 C	39.5 C		6 Hr, 39.5 C	36 Hr, 39.5 C
ts 14	NA	5.2×10^{10}	$<1.7 \times 10^{5e}$	$<3.3 \times 10^{-6}$	0.041	0.020
ts 16	NA	7.2×10^{10}	$<1.7 \times 10^5$	$<2.4 \times 10^{-6}$	0.025	0.016
ts 215	HA	5.0×10^{10}	$<1.7 \times 10^5$	$<3.4 \times 10^{-6}$	0.031	0.025
ts 225	HA	4.1×10^{10}	1.5×10^5	3.7×10^{-6}	0.058	0.061
ts 228	HA	7.4×10^{10}	$<1.7 \times 10^5$	$<2.3 \times 10^{-6}$	0.040	0.010
ts 35	NG	3.6×10^{10}	$<1.7 \times 10^5$	$<4.7 \times 10^{-6}$	0.037	0.037
ts 38	NG	1.4×10^{11}	1.3×10^5	9.3×10^{-6}	0.027	0.13
ts 332	HA	2.0×10^{10}	$<1.7 \times 10^4$	$<8.5 \times 10^{-7}$	0.065	0.031
WT		1.2×10^{11}	1.5×10^{11}	1.25	0.045	4,400

^a Abbreviations: NA, nitrous acid; HA, hydroxylamine; and NG, nitrosoguanidine.

^b Final yield from suspension cultures of KB cells infected at 32 C with 15 PFU/cell. Virus was harvested at 65 hr PI and titrated at 32 and 39.5 C by using the plaque assay.

^c Reversion frequency: yield titrated at 39.5 C/yield titrated at 32 C.

^d Final yield from suspension cultures of KB cells infected at 39.5 with 15 PFU/cell. Samples were taken at 6 and 36 hr PI and titrated at 32 C by using the fluorescent focus assay.

^e Maximum possible value. See text.

failed to increase in size when sifted to the non-permissive temperature. Of these, 1 to 10% did not produce complete adenovirus CPE in monolayers infected with low viral multiplicities at 39.5 C. The overall frequency for the isolation of ts mutants from mutagenized viral stocks was therefore 0.01 to 0.1% by using the procedures described. Four thousand plaques from the unmutagenized WT stock and from the prototype strain of type 5 adenovirus were submitted to the same selection procedure to screen for spontaneous mutants, but none were found.

All the mutants obtained had reversion frequencies of 10^{-5} or less (Table 1). The numbers given are approximate and varied from experiment to experiment. In most cases, only the maximum possible value for the titer at 39.5 C could be determined because of the cytotoxic effect of the inoculum when low dilutions were plated; the cell sheets on these cultures did not take up any of the stain although cells infected with the next highest dilution had intact monolayers and no plaques.

A second problem was encountered in determining the reversion frequencies of three mutants, ts 228, ts 35, and ts 38. On plates infected at 39.5 C with more than 10 to 100 PFU/plate based on the titer at 32 C, numerous small plaque-like areas of cell killing appeared at the same time as normal plaques, but the small "plaques" never reached normal size. When these small plaque-like areas were picked and assayed at 32 C, they were found to contain 1 to 2 logs less infectious virus than in normal plaques; about 25% did not contain detectable virus. However, when the

yields of these "plaques" were assayed at 39.5 C, only small plaque-like areas again appeared, indicating that although virus had been produced at 39.5 C, its genotype was ts. It is unlikely that these small plaque-like areas were caused by WT revertants because these mutants failed to produce infectious virus in suspension cultures at the non-permissive temperature (Table 1). Ishibashi (22) observed similar plaque-like areas on monolayers infected at the nonpermissive temperature with some of the ts mutants of CELO virus, an avian adenovirus. He attributed them to leakiness or cell death owing to an abortive infection.

Viral yields in monolayers or suspension cultures infected with the mutants at the permissive temperature were normal or nearly normal when compared to WT yields (Table 1). In contrast, the mutants replicated poorly, if at all, in suspension cultures infected at the nonpermissive temperature (Table 1). For comparison, the residual titer at 6 hr is also given (Table 1); only one mutant, ts 38, produced any virus above the background eclipse value, and even this amount was only 2.5×10^{-5} of the final WT yield.

Complementation. Complementation studies in doubly infected suspension cultures at the non-permissive temperature were undertaken to group the mutants according to affected cistrons. Figure 2 shows the kinetics of viral production in one cross between ts 225 and ts 332 as well as in cultures infected singly with each mutant or the WT virus. Very little virus was produced in the two controls singly-infected with the mutants. Table 2 summarizes the results from all possible crosses. On the basis of these results, the mutants can be

FIG. 2. Kinetics of complementation between two *ts* mutants of type 5 adenovirus. Suspension cultures of KB cells were infected with 15 PFU of each of the 2 mutants, *ts* 225 and *ts* 332 per cell; control cultures were infected with 15 PFU of each of the mutants per cell, separately or with the WT virus. Samples were taken at the indicated times and titrated at 32°C by using the fluorescent focus assay.

divided into three complementation groups: group I, *ts* 225; group II, *ts* 14, 16, 215, and 332; and group III, *ts* 228, 35, and 38. The complementation yields ranged from a 2.3- to a 3,000-fold increase over the sums of the two single infections. The highest yield, from the cross between *ts* 225 and *ts* 332, was 7% of the WT yield. There was one ambiguous result, in the cross between *ts* 225 and *ts* 14. The complementation yield (0.19 fluorescent focus units [FFU]/cell) was only 2.3-fold greater than the sum of the two single infections; this increase, however, was reproducible. In the cross between *ts* 35 and *ts* 38, although the yield from the mixed infection was 0.13 FFU/cell, this was actually less than the sum of the yields of the two single infections (0.167 FFU/cell).

Recombination. It was thought that the *ts* mutants would provide an ideal system in which to study recombination between animal viruses. Problems such as heteropolyploidy, which often occurs in viruses that bud from the cytoplasmic membrane (Newcastle disease virus, 6, 19), and genotypic mixing or reassortment, which has been demonstrated in viruses having segmented

genomes (influenza virus, 21, 33; reovirus, 12), are not encountered with adenoviruses. The one hazard to the analysis was the background of small plaque-like areas on plates infected with the mutants in complementation group III. However, it was possible to distinguish between these small plaques and normal plaques in almost all cases.

Monolayers of KB cells were doubly infected at 32°C, and the final yields measured at 32 and 39.5°C by using the plaque assay. When the assay plates incubated at 39.5°C were stained, however, the small plaque-like areas appeared in great numbers, even on plates from crosses not involving the three mutants known to produce these small plaques. They were found on all the 39.5°C plaque assay plates from crosses between mutants in different complementation groups and from all crosses involving group III mutants. They were never found in crosses within group II or in controls singly infected with group I or group II mutants. This distribution suggests that complementation was occurring on the assay plate at 39.5°C. A discussion of this and other possibilities is presented below.

An attempt was made to determine recombination frequencies by counting only normal-sized plaques on those plates that also had the small plaque-like areas. Although the results are only approximate at best, a few general statements can be made. Recombination frequencies ranged from 0.1% to 15% and were generally higher for inter-complementation group crosses than for intra-group crosses. The results were fairly reproducible; duplicates seldom varied more than twofold when done simultaneously or in different experiments. Fifteen normal-sized plaques were picked from three representative inter-group crosses and were found to be true WT recombinants based on the criterion of having equal plating efficiencies at the permissive and nonpermissive temperatures. They were picked from plates inoculated with sufficiently high dilutions to rule out the possibility that they were revertants. The occurrence of true recombination was also indicated by the crosses between group II mutants in which the problem of small plaques did not exist. The recombination frequencies for these crosses are given in Table 3. The yields assayed at 39.5°C were 100- to 450-fold higher than the sums of the two single infections.

Immunological detection of the synthesis of viral capsid antigens by the mutants at the nonpermissive temperature. The structural components of the adenovirus virion have been well characterized, both immunologically and biochemically. The capsid consists of three types of multimeric subunits (18): the 240 hexons form the faces and edges of the icosahedron; the penton base and fiber joined together form the 12 corner units of

the capsid; and the pentons. In addition, there are from two to four internal proteins associated with the DNA (24, 31, 32). The ability of the ts mutants to synthesize the three capsid subunits

was tested under permissive and nonpermissive conditions by using immunofluorescence and immunodiffusion techniques. The results are summarized in Table 4 and Fig. 3.

TABLE 2. *Complementation between ts mutants of type 5 adenovirus*

I ^a	ts 225	[0.061]							
II	ts 14	(+) 0.19 ^b	[0.020] ^c						
	ts 16	(+) 1.6	(-) 0.017	[0.016]					
	ts 215	(+) 0.58	(-) 0.013	(-) 0.010	[0.025]				
	ts 332	(+) 270	(-) 0.018	(-) 0.007	(-) 0.024	[0.031]			
	ts 228	(+) 37	(+) 22	(+) 11	(+) 34	(+) 107	[0.010]		
III	ts 35	(+) 73	(+) 35	(+) 30	(+) 61	(+) 170	(-) 0.030	[0.037]	
	ts 38	(+) 32	(+) 13	(+) 9	(+) 35	(+) 51	(-) 0.034	(-) 0.13	[0.13]
	ts 225	ts 14	ts 16	ts 215	ts 332	ts 228	ts 35	ts 38	
	I	II					III		

^a Roman numerals refer to the number of the complementation group to which the mutant has been assigned.

^b Suspension cultures of KB cells were doubly infected at 39.5 C with 15 PFU of each of two mutants per cell, and the final yield was assayed at 32 C as described in the legend to Fig. 2. The final yields are expressed as fluorescent focus units (FFU)/cell.

^c Yield from cultures infected with 15 PFU of only one mutant per cell and assayed at 32 C. Numbers are expressed as FFU/cell. Yield from a culture infected with the WT virus at 39.5 C was 4.4×10^8 FFU/cell.

TABLE 3. *Recombination between complementation group II mutants*

ts 14	$(<6.8 \times 10^{-6})^a$			
ts 16	0.459 ^b	$(<4.5 \times 10^{-6})$		
ts 215	3.47	2.35	$(<1.2 \times 10^{-5})$	
ts 332	0.129	0.127	1.28	$(<6.3 \times 10^{-6})$
	ts 14	ts 16	ts 215	ts 332

^a Reversion frequency: yield titrated at 39.5 C/yield titrated at 32 C. Monolayers of KB cells were singly infected at 32 C with 15 PFU/cell. The 65-hr yield of virus was titrated by the plaque assay at 32 and 39.5 C.

^b Recombination frequency: (two-times yield titrated at 39.5 C/yield titrated at 32 C) $\times 100$. Monolayers of KB cells were doubly infected with 15 PFU of each of two ts mutants per cell at 32 C. The 65-hr yield was titrated by the plaque assay at 32 and 39.5 C.

TABLE 4. *Immunofluorescence in KB cell monolayers infected with ts mutants of type 5 adenovirus at 39.5 C^a*

Group	Mutant	Antisera prepared against:		
		Purified hexon	Purified fiber	Purified virus
I	ts 225	—	—	—
II	ts 14	—	±	±
	ts 16	—	+	±
	ts 215	—	+	±
	ts 332	±	+	±
III	ts 228	+	+	+
	ts 35	++	+	+
	ts 38	++	+	+
None	WT	+++	+++	+++

^a After 26 hr of incubation, the plates were fixed with methanol and stained with rabbit antisera prepared against purified type 5 adenovirus, purified hexon, or purified fiber. The indirect stain was fluorescein-conjugated goat anti-rabbit antiserum.

Antisera prepared against purified type 5 adenovirus, purified hexon, and purified fiber were used to stain monolayers of KB cells infected with the mutants as described for the fluorescent focus assay (Table 4). Immunofluorescence showed that at 39.5 C cells infected with the group I mutant, ts 225, failed to stain with any of the antisera; group II-infected cells stained with type 5 antiserum and fiber antiserum but not with hexon antiserum, with the exception of ts 332 which stained very faintly with the hexon antiserum; group III-infected cells stained with all three antisera.

To confirm the results obtained in the immunofluorescence studies, we tested protein extracts prepared from suspension cultures of KB cells infected with the mutants at the nonpermissive temperature for the presence of virus-specific antigens by using the Ouchterlony immunodiffusion technique (30). Typical patterns for representatives of the three complementation groups are shown in Fig. 3. The WT extract contained two major precipitin bands. The heavier band was the hexon, indicated by its reaction of identity with the band from the well containing purified hexon. This band was not detected in either the group I or group II extracts. The second band was probably penton (penton base and fiber) based on its reaction of partial identity with the fiber band. It was present in the WT extracts and in all of the mutant extracts except ts 225. It was also seen in the same extracts when the antiserum

used was prepared against purified fiber or type 2 adenovirus, indicating that it contained both fiber and penton base determinants. A third precipitin band was sometimes seen in the WT extracts, which was probably detected free fiber. However, it was at the same position as the hexon band and was therefore difficult to see. These results confirmed the immunofluorescence experiments. Mutant ts 225 did not synthesize viral capsid antigens; group II mutants synthesized penton base and fiber but not hexon; and group III mutants synthesized hexon, penton base, and fiber. It should be emphasized that both of these techniques measure the synthesis of immunologically reactive proteins which could still be biologically defective, and they do not detect improperly made proteins that are immunologically unreactive.

DNA synthesis in cells infected by the mutants at the nonpermissive temperature. It has been

FIG. 3. *Immunodiffusion of protein extracts from cells infected with ts mutants at 39.5 C. Suspension cultures were infected with 20 PFU/cell. The cells were harvested at 36 hr PI, lysed by freezing and thawing six times, and cleared of cellular debris by centrifugation. Antiserum to purified type 5 adenovirus was placed in the center well of each plate, and the cell extracts from mutant-infected cells, WT extracts (WT), purified hexon (H), and purified fiber (F) were placed in the peripheral wells. The presence of an air bubble in the right hand WT well of the ts 215 plate prevented the extract from diffusing completely into the well. The presence or absence of hexon is best seen in the upper mutant extract wells. For example, two WT extracts were neighbors of the ts 14 mutant extract in the lower well; there was sufficient hexon in the two WT extracts to permit the hexon bands to meet, which gave the appearance that ts 14 (lower well) also contained hexon.*

shown that viral DNA replication is a necessary prerequisite for the synthesis of viral capsid antigens (13). One could therefore predict that the mutants in groups II and III would replicate their DNA normally at 39.5 C, whereas ts 225 might be a DNA-minus mutant. To test this hypothesis, the incorporation of ^3H -thymidine into DNA was examined in suspension cultures of infected KB cells at 39.5 C from 10 to 13 hr PI. Advantage was taken of the fact that type 5 adenovirus DNA has a buoyant density in CsCl of 1.718, whereas that of KB cell DNA is 1.702, enabling one to separate them by CsCl equilibrium density gradient centrifugation (20). The sedimentation patterns of the pulse-labeled DNA from the infected cultures are shown in Fig. 4. It is apparent that

host and viral DNA were readily separated by this technique (Fig. 4D), and that pulse-labeled WT DNA cosedimented with the ^{14}C viral DNA marker (Fig. 4C); the absence of radioactivity in the lighter region of the gradient indicated that host DNA synthesis had been shut off by 10 hr after infection. The pulse-labeled DNA from the cultures infected with ts 215 (Fig. 4A) as well as ts 14 and ts 38 (not shown) displayed sedimentation patterns indistinguishable from the WT pattern. Viral DNA synthesis and the shut-off of host DNA synthesis proceeded normally in cultures infected with these mutants at the nonpermissive temperature. Ts 225, however, did not synthesize any detectable viral DNA during the 3 hr of the pulse (Fig. 4B). The marker DNA in

FIG. 4. CsCl gradient analysis of DNA synthesis in KB cells infected with ts mutants at 39.5 C. Suspension cultures were infected with 200 PFU/cell. ^3H -thymidine (2 $\mu\text{Ci}/\text{ml}$) was added at 10 hr PI; the cells were harvested, and the DNA was extracted 13 hr PI. A 1- μg amount of the ^3H -pulse-labeled DNA was mixed with 1 μg of a ^{14}C -thymidine-labeled DNA marker extracted from either purified virus or uninfected KB cells. The mixture was centrifuged to equilibrium in CsCl for 72 hr at 35,000 rev/min. Four-drop fractions were collected from the bottom of the gradient. A, ^3H -labeled DNA extracted from cells infected with ts 215 and ^{14}C -labeled DNA from purified virus; B, ^3H -labeled DNA from ts 225-infected cells and ^{14}C -labeled DNA from uninfected KB cells (the arrow indicates the position at which viral DNA would sediment in this gradient); C, ^3H -labeled DNA from WT-infected cells and ^{14}C -labeled DNA from purified virus; D, ^3H -labeled DNA from uninfected KB cells and ^{14}C -labeled DNA from purified virus; $\circ$, ^3H -thymidine, counts/min; $\bullet$, ^{14}C -thymidine, counts/min.

this gradient was KB cell DNA, and the arrow indicates the position in which viral DNA would sediment in the gradient. After infection with ts 225 (DNA-minus mutant), host DNA synthesis was inhibited to about 25 to 35% of the DNA synthesized during the same time period in the uninfected control culture (Fig. 4D). To determine whether viral DNA synthesis was absent or merely delayed, a similar culture infected with ts 225 was labeled with ^3H -thymidine from 13 to 19 hr PI; the results were identical to those with the 10 to 13 hr pulse.

High multiplicities of inactivated type 5 adenovirus have been shown to inhibit host DNA synthesis in the absence of viral DNA replication (H. S. Ginsberg, *unpublished data*). To establish that the partial inhibition of host DNA synthesis by ts 225 at 39.5 C was not merely the result of the inoculum, the experiment was repeated using three different multiplicities of infection (10, 50, and 200 PFU/cell). An earlier pulse period from 8 to 10 hr PI was chosen to ensure that the inhibition of host DNA synthesis observed in the WT control would be the result of early DNA shut-off and not the result of late antigen synthesis (16). The results summarized in Table 5 indicated that multiplicities as low as 10 PFU of ts 225 per cell had an appreciable effect on host DNA synthesis at the nonpermissive temperature.

DISCUSSION

We have isolated eight ts mutants of type 5 adenovirus that replicate normally at 32 C but poorly or not at all at 39.5 C. Table 6 presents a summary of their properties. Although the two-step selection procedure employed permitted screening of a large number of plaques at one time, it is possible that this procedure did not detect some mutants. For example, ts plaques

that have not appeared by the time the plates are shifted to the nonpermissive temperature would be missed, and the CPE test would eliminate leaky mutants. In addition, some highly cytotoxic mutants may produce plaques that enlarge when shifted to the nonpermissive temperature. These features of the selection procedure may explain the relatively low frequencies with which mutants were isolated (0.01 to 0.1%) when compared to other groups who have isolated ts mutants of adenoviruses by using the same mutagens. Williams et al. (38) isolated ts mutants of type 5

TABLE 5. *Effect of viral multiplicity on the inhibition of host DNA synthesis in cells infected with ts 225 at 39.5 C*

Virus ^a	Multiplicity (PFU/cell)	Counts/min in host DNA ^b	% Control ^c
ts 225	10	8,033	44.5
ts 225	50	6,726	37.9
ts 225	200	5,142	28.5
Wt	10	4,641	25.7
Wt	50	3,607	20.0
Wt	200	3,297	18.3
None		18,037	100

^a Suspension cultures of KB cells were infected at 39.5 C with varying multiplicities of ts 225 or WT virus. ^3H -thymidine (2 $\mu\text{Ci}/\text{ml}$) was added from 8 to 10 hr PI. DNA was extracted and centrifuged to equilibrium in CsCl as described in the legend to Fig. 4.

^b Counts per minute of ^3H -thymidine incorporated into the host peak when 1 μg of labeled DNA was placed on each gradient.

^c Counts per min in host peak/counts per min in host peak from uninfected culture $\times 100$. In both cases, 1 μg of labeled DNA was applied to each gradient.

TABLE 6. *Properties of ts mutants of type 5 adenovirus^a*

Mutant	Mutagen	Complementation group	Antigens produced at 39.5 C				DNA synthesis at 39.5 C
			Immunofluorescence		Immunodiffusion		
			Hexon	Fiber	Hexon	Penton	
ts 225	HA	I	—	—	—	—	—
ts 14	NA	II	—	+	—	+	+
ts 16	NA	II	—	+	—	+	NT
ts 215	HA	II	—	+	—	+	+
ts 332	HA	II	±	+	—	+	NT
ts 228	HA	III	+	+	+	+	NT
ts 35	NG	III	+	+	+	+	NT
ts 38	NG	III	+	+	+	+	+
WT			+	+	+	+	+

^a Abbreviations: HA, hydroxylamine; NA, nitrous acid; NG, nitrosoguanidine; and NT, not tested.

adenovirus with reported frequencies of 8.4, 9.6, and 0.55% depending on the mutagen; and Ishibashi (22) reported isolating mutants of CELO virus with frequencies between 0.5 and 3.0%. Both groups randomly selected plaques from plates infected with mutagenized virus at the permissive temperature and tested them for temperature sensitivity.

The eight mutants described may be divided into three complementation groups (Tables 2 and 6). Only the complementation cross between ts 225 and ts 14 gave a low viral yield, which could be the result of a double mutation in one or both of these mutants. Ts 14, however, is WT for the biochemical defect of ts 225 (DNA replication), and ts 225 complements the other group II mutants with normal to high efficiency (Table 2). It is possible that both of these strains have a second identical ts defect that has not yet been identified.

Recombination between ts mutants of type 5 adenovirus described above has proved to be more complex than was originally anticipated. True recombination between adenoviruses clearly occurs since the crosses between the mutants of group II unambiguously yielded WT viruses (Table 4) and the 15 large plaque isolates from the 39.5 C assay plates from three of the intercomplementation group crosses had WT genotypes. The small plaque-like areas seen in the intercomplementation group crosses and in the single infections with the group III mutants did not yield WT viruses. In the single infections with group III mutants, they could be due to leakiness or abortive infections. The latter explanation appears possible for the following reasons. Under nonpermissive conditions, the mutants replicate viral DNA, produce all capsid proteins, and block biosynthesis of host DNA; and the small plaque-like areas are noted in monolayer cultures, but infectious WT virions are not formed in cell suspensions. Hence, viral DNA and even defective virions may have sufficient limited infectivity and transmission to neighboring cells in order to form these "pseudo-plaques."

In the recombination experiments, however, their appearance only in intercomplementation group crosses suggests that they may be the result of complementation on the assay plate. A preliminary reconstruction experiment was performed to test this hypothesis. Monolayers of KB cells were infected with either ts 225, ts 16, or an artificial mixture of the two in equal proportions. The plates were incubated at 39.5 C, fixed, and stained with hexon antiserum as described for the fluorescent focus assay. Neither of the cell cultures infected with a single mutant stained with hexon antiserum at 39.5 C (Table 3).

There was, however, a 50-fold increase in the number of cells staining with the hexon antiserum in the doubly infected monolayers when compared to those infected with only one mutant. Ishikawa and Di Mayorca (23) extensively studied one recombination cross between two polyoma virus ts mutants from different complementation groups. When the mutants were propagated together at the permissive temperature and the yield titrated under nonpermissive conditions, only 10 of 67 plaques appearing at the nonpermissive temperature were found to have a WT genotype. They also performed a reconstruction experiment in which plaque assay plates were infected with an artificial mixture containing the same numbers of the two mutants present in the yield from the doubly infected culture. This mixture had almost the same number of plaques as the true cross when titrated at the nonpermissive temperature, but none of these (0/67) had a WT genotype when picked and tested for temperature sensitivity. A more detailed study of a few intercomplementation group crosses is underway in our laboratory to investigate this question. It is possible that to determine recombination frequencies unambiguously it will be necessary to characterize the genotype of each plaque appearing at 39.5 C by picking and replating each at both temperatures. It will also be necessary to determine whether or not the noninfectious particles found in adenovirus mutant preparations are able to participate in genotypic or phenotypic interactions with other mutants.

Preliminary biochemical analyses have shown that mutants belonging to the same complementation group appear to have the same biochemical defect(s). The single group I mutant does not replicate its DNA at the nonpermissive temperature. Group II mutants replicate their DNA and synthesize pentons, which consist of penton base and fiber proteins, but do not make immunologically reactive hexon protein. Group III mutants replicate their DNA and synthesize all capsid proteins, at least as determined by immunological techniques. It should be noted that the amount of hexon synthesized by group III mutants appears to be reduced, although it has not yet been adequately quantitated (Fig. 3 and Table 4). A more complete analysis of these mutants is being carried out, including identification of the viral proteins by polyacrylamide gel electrophoresis.

Only one of the mutants obtained, ts 225, is an early mutant based on the criterion of DNA replication at the nonpermissive temperature. Although viral DNA is not made, host DNA synthesis is partially inhibited, even at low

input multiplicities. These data confirm the finding that adenovirus DNA replication is not necessary for the shut-off of host DNA synthesis (15). KB cells were infected with type 5 adenovirus in the presence of 10^{-6} M 5-fluoro-2-deoxyuridine (FUdR); when the FUdR block was reversed 10 hr after infection, viral DNA synthesis began immediately, whereas host DNA synthesis was not resumed (15).

The type 5 adenovirus genome has a molecular weight of 23×10^6 , which is sufficient to code for approximately 25 to 50 proteins (35). Hence, the three complementation groups described represent only a small fraction of the number of possible cistrons. Seven additional mutants, which are as yet uncharacterized, have been isolated. It is anticipated that the isolation of these and other mutants will increase the variety of genes represented and thus also increase their usefulness in studying the regulation of adenovirus replication.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-0362 and training grant AI-203, from the National Institute of Allergy and Infectious Diseases. It was also under the sponsorship of the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board, and was supported by the U.S. Army Medical Research and Development Command, Department of the Army, under research contracts DA-49-193-MD-2131 and DADA 17-70-C-0121.

LITERATURE CITED

- Bello, L. J., and H. S. Ginsberg. 1967. Inhibition of host protein synthesis in type 5 adenovirus-infected cells. *J. Virol.* 1:843-850.
- Burge, B. W., and E. R. Pfefferkorn. 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. *Virology* 30:204-213.
- Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric determination of deoxyribonucleic acid. *Biochem. J.* 62:315-322.
- Campbell, A. 1961. Sensitive mutants of bacteriophage λ . *Virology* 14:22-32.
- Cooper, P. D. 1969. The genetic analysis of poliovirus, p. 177-218. *In* H. B. Levy (ed.), *The biochemistry of viruses*. Marcel Dekker, New York.
- Dahlberg, J. E., and E. H. Simon. 1969. Physical and genetic studies of NDV: evidence for multiploid particles. *Virology* 38:666-678.
- Eagle, H. 1955. Propagation in a fluid medium of a human epidermoid carcinoma strain KB. *Proc. Soc. Exp. Biol. Med.* 89:362-364.
- Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* 130:432-437.
- Edgar, R. S., and I. Lielausis. 1964. Temperature-sensitive mutants of bacteriophage T4D: their isolation and genetic characterization. *Genetics* 49:649-662.
- Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy De La Tour, E. Chevalley, R. S. Edgar, M. Susman, G. T. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional-lethal mutants of bacteriophage T4D. *Cold Spring Harbor Symp. Quant. Biol.* 28:375-392.
- Fenner, F. 1969. Conditional-lethal mutants of animal viruses. *Curr. Top. Micro. Immunol.* 48:1-28.
- Fields, B. N., and W. K. Joklik. 1969. Isolation and preliminary genetic and biochemical characterization of temperature-sensitive mutants of reovirus. *Virology* 37:335-342.
- Flanagan, J. F., and H. S. Ginsberg. 1962. Synthesis of virus-specific polymers in adenovirus-infected cells: effects of 5-fluorodeoxyuridine. *J. Exp. Med.* 116:141-157.
- Friedman, M. P., M. J. Lyons, and H. S. Ginsberg. 1970. Biochemical consequences of type 2 adenovirus and simian virus 40 double infections of African green monkey kidney cells. *J. Virol.* 5:586-597.
- Ginsberg, H. S. 1969. The biochemistry of adenovirus infection, p. 329-359. *In* H. B. Levy (ed.), *The biochemistry of viruses*. Marcel Dekker, New York.
- Ginsberg, H. S., L. J. Belle, and A. J. Levine. 1967. Control of biosynthesis of host macromolecules in cells infected with adenovirus, p. 547-572. *In* J. S. Colter and W. Paranchych (ed.), *Symposium on the molecular biology of viruses*. Academic Press Inc., New York.
- Ginsberg, H. S., E. Gold, and W. S. Jordan, Jr. 1955. Tryptose phosphate broth as a supplementary factor for the maintenance of HeLa cell culture. *Proc. Soc. Exp. Biol. Med.* 89:66-71.
- Ginsberg, H. S., H. G. Pereira, R. C. Valentine, and W. C. Wilcox. 1966. A proposed terminology for the adenovirus antigens and virion morphological subunits. *Virology* 28:782-783.
- Granoff, A. 1962. Heterozygosis and phenotypic mixing with Newcastle disease virus. *Cold Spring Harbor Symp. Quant. Biol.* 27:319-326.
- Green, M. 1962. Studies on the biosynthesis of viral DNA. *Cold Spring Harbor Symp. Quant. Biol.* 27:219-233.
- Hirst, G. K. 1962. Genetic recombination with Newcastle disease virus, polioviruses, and influenza. *Cold Spring Harbor Symp. Quant. Biol.* 27:303-309.
- Ishibashi, M. 1971. Temperature-sensitive conditional-lethal mutants of an avian adenovirus (CELO). I. Isolation and characterization. *Virology* 45:42-52.
- Ishikawa, A., and G. Di Mayorca. 1970. Recombination between two temperature-sensitive mutants of polyoma virus, p. 294-299. *In* L. G. Sylvestri (ed.), *Proceedings of the second Lepetit colloquium on the biology of oncogenic viruses*. North Holland/American Elsevier, Inc., New York.
- Laver, W. G. 1970. Isolation of an arginine-rich protein from particles of adenovirus type 2. *Virology* 41:488-500.
- Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. *J. Virol.* 1:851-867.
- Levine, A. J., and H. S. Ginsberg. 1967. Mechanism by which fiber antigen inhibits multiplication of type 5 adenovirus. *J. Virol.* 1:747-757.
- Lundholm, U., and W. Doerfler. 1971. Temperature sensitive mutants of human adenovirus type 12. *Virology* 45:827-829.
- Maizel, J. V., Jr., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus. I. Evidence for multiple components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36:115-125.
- Maizel, J. V., Jr., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus. II. Soluble proteins, cores, top components, and the structure of the virion. *Virology* 36:126-136.
- Ouchterlony, O. 1958. Diffusion-in-gel methods for immunological analysis. *Progr. Allerg.* 5:1-178.
- Prage, L., U. Pettersson, and L. Philipson. 1968. Internal basic proteins in adenovirus. *Virology* 36:508-511.
- Russell, W. C., K. McIntosh, and J. J. Skehel. 1971. The preparation and properties of adenoviruses cores. *J. Gen. Virol.* 11:35-46.
- Simpson, R. W., and G. K. Hirst. 1968. Temperature-sensitive

- mutants of influenza A virus: isolation of mutants and preliminary observations on genetic recombination and complementation. *Virology* 35:41-49.
34. Suzuki, E., and H. Shimojo. 1971. A temperature-sensitive mutant of adenovirus 31, defective in viral DNA replication. *Virology* 43:488-494.
35. van der Eb, A. J., and L. W. van Kesteren. 1966. Structure and molecular weight of the DNA of adenovirus type 5. *Biochim. Biophys. Acta* 129:441-444.
36. Velicer, L. F., and H. S. Ginsberg. 1968. Cytoplasmic synthesis of type 5 adenovirus capsid proteins. *Proc. Nat. Acad. Sci. U.S.A.* 61:1264-1271.
37. Velicer, L. F., and H. S. Ginsberg. 1970. Synthesis, transport, and morphogenesis of type 5 adenovirus capsid proteins. *J. Virol.* 5:338-352.
38. Williams, J. F., M. Gharpure, S. Ustacelebi, and S. McDonald. 1971. Isolation of temperature-sensitive mutants of adenovirus type 5. *J. Gen. Virol.* 11:95-101.