Genetic Analysis of Adenovirus Type 2

II. Preliminary Phenotypic Characterization of Temperature-Sensitive Mutants

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The properties of temperature-sensitive mutants of adenovirus type 2 representing 12 complementation groups were studied. All mutants were normal with respect to adsorption as measured by viral inclusion formation and viral DNA synthesis as shown by velocity sedimentation in alkaline sucrose gradients. One mutant, however, formed viral inclusions of altered morphology at the nonpermissive temperature. The synthesis of the major capsid proteins was examined by immunodiffusion. On this basis, the complementation groups could be arranged as follows: (i) one group was negative for all three proteins; (ii) three groups failed to synthesize penton bases; (iii) eight groups were positive for hexons, pentons, and fibers. The assembly of virus particles at 39 C was examined by equilibrium sedimentation in CsCl; three groups were found defective, whereas two of the penton-negative groups were positive for virion production. Tests of the thermolability of virions at 50 C revealed eight groups labile whereas the remainder were insensitive to heat inactivation. None of five mutants inoculated in newborn rats induced tumors, although three of them were capable of *in vitro* transformation.

In a previous communication we have reported the isolation of temperature-sensitive (ts) mutants of adenovirus type 2 (2). These mutants were classed into 12 complementation groups and arranged in a linear sequence on a tentative genetic map by recombination analysis. In this report we examine some of the functional characteristics of these mutants.

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MATERIALS AND METHODS

Virus and cells. Human adenovirus type 2 (Ad 2) designated as the wild type (WT) was propagated in KB cells and titrated by the plaque assay on KB cells or by the infected cell unit assay on HEP-2 cells, as described previously (2, 19). The isolation of the ts mutants and their preliminary genetic characterization have also been described previously (2). In the interest of abiding by uniform nomenclature, the mutant numbers published in the preceding paper which are greater than 99 have been revised as follows: ts 241 is ts 1; ts 496 is ts 2; ts 235 is ts 3; ts 500 is ts 4; ts 458 is ts 5; ts 155 is ts 6; ts 517 is ts 7; ts 166 is ts 8; ts 186 is ts 9; ts 231 is ts 11; ts 469 is ts 12. Henceforth the mutants shall be referred to by this new nomenclature, in conformity with other adenovi-

rus laboratories (7). The KB cells were obtained from Flow Laboratories (Rockville, Md.) and were never used beyond the sixth passage. Both HEP-2 and KB cells were cultured in Dulbecco-modified, Eagle minimal essential medium containing 10% calf serum. Virus-infected cells were cultured in minimal essential medium supplemented with 2.5% calf serum and 0.4 mM arginine.

DNA synthesis. Confluent monolayers of KB cells grown in 50-mm diameter Falcon petri dishes were inoculated with ts mutants at a multiplicity of input of 5 PFU/cell. After a 50-min adsorption period at 33 C, the cells were washed three times with Tris-buffered saline (pH 7.4). Fresh medium was then added and the infected cultures were incubated at 33 and 39 C, then labeled with 5 μ Ci of [3 H]thymidine per ml (40 to 60 Ci/mM) 24 and 16 h later, respectively. At 40 h postinfection, the medium of the cultures incubated at 39 C was removed, and the cells were washed with Tris-buffered saline, scraped off with a rubber policeman, and resuspended in cold Tris-buffered saline. The cultures incubated at 33 C received the same treatment 65 h postinfection. In some experiments the cells were labeled for 1 h only and either lysed immediately or further incubated in medium without [3 H]thymidine. Linear, 5 to 20% sucrose gradients (in 1.0 M NaCl, 0.01 M EDTA, and 0.3 M NaOH, pH 12.9) were prepared in polyallomer centrifuge tubes essentially as described by Doerfler (5). A lysing layer (0.20 ml) consisting of 0.5 M NaOH and 0.01 M EDTA was carefully applied on top of the preformed gradients. Samples of about

10^4 cells (contained in 0.05 ml) were mixed with [^{14}C]thymidine-labeled marker virus and gently pipetted onto the lysing layer. The cells were allowed to lyse for 1 h at room temperature or overnight at 4 C. The preparations were then centrifuged in the B60 International ultracentrifuge using the SB-405 rotor, at 60,000 rpm for 84 min at 4 C. At the end of the run the gradients were collected dropwise on Whatman (GF-81) fiber glass filter disks from the bottom of the tubes. The filters were washed with ice cold 5% trichloroacetic acid solution followed by ethanol, and then dried and counted in a toluene based scintillation fluid.

Immunodiffusion. Double diffusion in agar gels was carried out by a modification of the microtechnique of Crowle (4) and also by the standard Ouchterlony macrotechnique using the Gelman (Gelman Instruments, Ann Arbor, Mich.) apparatus (14). After development for 1 to 2 days, the slides were soaked in 0.15 M NaCl for 1 to 3 days, then dried at a distance of 25 cm under a 250-W infrared lamp. To prevent shrinkage during drying, the wells of the macrotechnique gels were filled with water. The precipitin lines were stained with amido black (1 g of stain in 450 ml of 1 M acetic acid, 450 ml of 0.1 M sodium acetate, and 100 ml of glycerol).

Antisera. Whole virion antiserum was prepared by immunizing rabbits with virions which were purified by two extractions with fluorocarbon (Freon 113, Dupont Co.) followed by two cycles of centrifugations to equilibrium in CsCl. Hexon, penton, and fiber were purified by chromatography of the top components from CsCl gradients on DEAE-cellulose (9). The purity of these proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1) and by immunodiffusion against whole virion antiserum. Antisera against these proteins was also prepared by immunizing rabbits. The rabbit antisera were rechecked by immunodiffusion.

Virus assembly. KB cell monolayers were infected with virus, incubated at 33 C, and labeled at 24 h or incubated at 39 C and labeled at 18 h after infection with $1\ \mu\text{Ci}$ of ^3H -labeled amino acids mixture per ml (International Chemical and Nuclear Corporation, Irvine, Calif.). The label was left on the cells until the cells were harvested in Tris-buffered saline 2 and 3 days after infection at 39 and 33 C, respectively, lysed, and extracted with trichlorotrifluoroethane (Freon 113, Dupont of Canada, Ltd.). This material was gently pipetted on top of a CsCl solution ($1.40\ \text{g}/\text{cm}^3$) and centrifuged to equilibrium overnight. The tubes were photographed; then fractions were collected through the bottom. The density of the CsCl was determined by refractometry whereas the radioactivity was determined as 5% cold trichloroacetic acid-precipitable counts in toluene based scintillation fluid. The negatives of the photographs were scanned at 540 nm in a Gilford spectrophotometer equipped with a linear scanning device.

A second series of KB cell cultures were infected with virus and incubated at 33, 39, and 40.5 C, then labeled with $2\ \mu\text{Ci}$ of [^3H]thymidine per ml (60 Ci/nM, New England Nuclear) at 6, 4, and 3 h after infection, respectively. The label was left on the cells

until the end of the infection period at 3 days (33 C) and 2 days (39 and 40.5 C) postinfection. The virus was extracted with Freon as above and treated with $50\ \mu\text{g}$ of pancreatic DNase I per ml (Calbiochem) at 37 C for 30 min, and then 0.1 ml was layered on top of a preformed CsCl gradient (1.2 to $1.5\ \text{g}/\text{cm}^3$) and centrifuged at 60,000 rpm for 1 h in rotor SB-405 of an International B60 ultracentrifuge. The fractions were treated as described above.

RESULTS

Adsorption and inclusion body formation.

The efficiency of adsorption of the mutants was tested by the formation of inclusion bodies (15, 19, 20) at the permissive and nonpermissive temperatures. Single cover slips (1-cm diameter) with a confluent monolayer of HEp-2 cells were placed in separate petri dishes and preincubated at 33 or 39 C, and then $25\ \mu\text{l}$ of prewarmed virus at several dilutions was added to corresponding cover slips and allowed to adsorb at 33 or 39 C. After 1 h, unadsorbed virus was rinsed off, medium prewarmed to 33 C was added, and all cover slips were incubated at 33 C for 3 days. At this time they were fixed and stained, and the number of virus-induced inclusion bodies was counted as described previously (19). Since several dilutions of virus were tested, it was possible to choose a dilution which produced between 0.1 to 1% inclusion bodies (19), and to read the cover slips corresponding to this dilution at the two adsorption temperatures. The ratio of inclusion bodies at 33 C to inclusion bodies at 39 C did not significantly deviate from unity for the 15 mutants tested (belonging to the 12 complementation groups). As all the mutants appeared normal for adsorption, the actual data are not shown.

In a second experiment we wished to determine whether the mutants were capable of forming inclusion bodies at the nonpermissive temperature. The experiment was essentially conducted as above, except that adsorption was at 33 C and incubation at both 33 and 39 C.

As in the adsorption experiment, the ratio of inclusion bodies at 33 C to inclusion bodies at 39 C was not significantly different from unity. With the exception of ts 3, the morphology of the inclusions was also independent of temperature. Although at 33 C the ts 3-infected cells are indistinguishable from the WT, incubation at 39 C causes the cells to appear crenated.

It was concluded that these ts mutants were not defective in adsorption and inclusion body formation.

Viral DNA synthesis. The ability of the mutants to synthesize DNA at the nonpermissive temperature was determined by velocity

sedimentation of [^3H]thymidine-labeled infected cell DNA in alkaline sucrose gradients. Under the conditions employed, viral DNA from infected cells cosedimented with purified Ad 2 marker DNA, whereas cellular DNA was pelleted. Figure 1 shows typical profiles obtained for ts 53, 74, 6, 8, 9, 11, 3, 1, 5, 2, 4, 7, and WT. Similar results were obtained when using the following modifications of the basic procedure: (i) the labeling period with [^3H]thymidine was reduced to 1 h and the cells were lysed immediately or after a 24-h chase; (ii) the alkaline lysis period was extended to overnight at 4 C; (iii) the temperature of incubation was raised to 40.5 C. In all cases there was always somewhat more DNA synthesized at the nonpermissive than at the permissive temperature. Reversion can be ruled out since we are dealing with a single growth cycle and the rate of reversion was never greater than 10^{-3} (2). Although leakiness cannot be ruled out, it appears highly improbable that DNA synthesis during a 1-h labeling period should be appreciably affected by it. From this result it may be concluded that these mutants are not defective in viral DNA synthesis.

Synthesis of viral capsid antigens. Using the standard macroimmunodiffusion technique, three distinct precipitin lines were obtained with infected cell lysates and antiserum prepared against highly purified complete virus

FIG. 1. Viral DNA synthesis. Alkaline sucrose gradient velocity sedimentation of [^3H]thymidine-labeled DNA from KB cells infected with ts mutants. This profile is representative of all the ts mutants and WT tested. The arrow indicates the position of [^{14}C]thymidine-labeled Ad 2 marker DNA. Symbols: Δ , [^3H]thymidine counts/min labeled at 33 C; $\blacktriangle$, [^3H]thymidine counts/min labeled at 39 C.

(Fig. 2). A fourth faint line was also present sometimes. By means of the microtechnique, two to three additional, fainter precipitin lines can also be detected. The three major precipitin lines were identified by means of monospecific antisera to hexons and pentons (penton base and fiber). At the present time we do not know the nature of the extra precipitin lines. The ability of the ts mutants to synthesize immunologically cross-reactive material of the three major capsid antigens (hexon, penton, and fiber) was tested under permissive and nonpermissive conditions by both these techniques. The results are summarized in Table 1. It can be seen that ts 3 fails to synthesize any of the capsid antigens; ts 11, ts 4, and ts 7 representing three distinct complementation groups produced normal precipitin lines corresponding to hexon and fiber antigens but reduced precipitin lines corresponding to pentons, whereas the remaining mutants representing eight complementation groups appear to synthesize normal levels of the antigens.

Virus assembly. The immunodiffusion experiments described above have demonstrated that some ts mutants were defective in the synthesis of one or more capsid antigens at the nonpermissive temperature, whereas the majority had apparently synthesized a pattern of antigens indistinguishable from the WT virus. Since the synthesis of cross-reactive material is no guarantee of the functional integrity of the antigen, nor the absence of cross-reactive material for the absence of virions, the problem of the assembly of virus particles was examined by the following experiments. KB cell cultures were singly infected with mutants or WT at 33 and 39 C and labeled with ^3H -labeled amino acids, and the infected cell extracts were centrifuged to equilibrium in CsCl . The virus bands were photographed in the tubes, and then fractions were collected and measured for density and radioactivity. Figure 3 shows the radioactivity profiles and the densitometric tracings of the photographs of three mutants and the WT. The profiles show the sharp virus band in the 1.34 density region and a broader band of material which includes empty virions, virus capsids (top components), and probably some cellular proteins around the 1.30 density region (3, 8, 17). The disproportionate virus band in the densitometric tracings is due to the strong diffraction of visible light by virions. Of the mutants tested, ts 53, 74, 6, 8, 9, and 1 had profiles identical to WT; ts 2, 7, and 11 produced a normal amount of virus but a reduced quantity of top components; ts 4 and 5 produced

FIG. 2. Double diffusion in agar. Center well contains rabbit antiserum against Ad 2 and peripheral wells extracts from cells infected with virus as follows: 1 and 4, ts 1 at 39 C; 2, WT at 39 C; 3 and 6, ts 1 at 33 C; 5, WT at 33 C.

no virus but an apparently normal amount of top components; and ts 3 produced neither virus nor top components. Since ts 3 was apparently defective in the production of capsid cross-reactive material, it is satisfying to note the drastic reduction of both virions and top components at the nonpermissive temperature. Although ts 4, 11, and 7 were defective in the production of pentons, only ts 4 appeared also to be defective in virion production. The defects in ts 5 and 2 may involve a minor virion component or a nonstructural virus function. The defects in mutants which were indistinguishable from the WT will need to be investigated by other methods.

To test some of the propositions enumerated above, the experiment was repeated with some modifications. Although the above experiment dealt with changes detected on the basis of following the proteins, the present one was designed to study the assembly of nucleic acid-containing particles. Virus was labeled with [^3H]thymidine at 33, 39, and 40.5 C and purified as described above. The radioactivity profiles are shown in Fig. 4. Under these conditions

ts 1, 2, 6, 8, 9, 53, and 74 were identical to WT as expected; ts 3 and 4 remained unchanged whereas ts 5, 11, and 7 (panel B) showed a considerable drop in virion assembly or maintenance at 40.5 C. This result appears to be consistent with the notion of leakiness in ts 11 and 7.

Heat inactivation. Figure 5 gives the results of experiments testing the thermolability of mutant and WT virus particles at 50 C. The slope of the inactivation curve varied depending on the state of purification and the suspension medium. To have uniform conditions, crude virus samples were diluted with the same buffer and all viruses were tested at the same time. The mutants appeared to cluster in three groups: those indistinguishable from WT (ts 2, 4, 7, 8); those inactivated 10- to 50-fold faster than WT (ts 1, 3, 5, 6, 9, 11); and those inactivated 1,000-fold faster than WT (ts 53, 74). To check if the survivors contained revertants, some plaques from the 32-min exposure were picked and replaques at 33 and 39 C; all were shown to be ts mutants. This result suggests that mutants of eight complementa-

TABLE 1. Immunodiffusion of lysates of cells infected with ts mutants at 39 C^a

Complementation group	Mutant	Virus capsid antigens		
		Hexon	Fiber	Penton
J	ts 3	—	—	—
E	ts 11	(+) (±)	(+) (±)	(±)
F	ts 4			
C	ts 7			
B	ts 53	(+) (+)	(+) (+)	(+) (+)
H	ts 74			
H	ts 26			
G	ts 6			
L	ts 8			
A	ts 9			
D	ts 1			
D	ts 12			
I	ts 5			
K	ts 2			
Unassigned	ts 10			

^aThis represents a qualitative summary of several experiments using both the macro- and microimmunodiffusion methods with complete virus antiserum and monospecific antiserum to hexon and penton antigens.

tion groups (A, B, D, E, G, H, I, J) may have defects in genes for structural components of the virion.

Tumorigenesis. WT Ad 2 has been shown to be oncogenic indirectly by the transplantation of in vitro transformed cells (6). To our knowledge there exists no recent report to induce tumors by direct inoculation of the virus into newborn rodents. To fill this gap, and more importantly to test the oncogenicity of ts mutants, newborn rats (Sprague-Dawley, Madison, Wis.) were inoculated with WT and 5 ts mutants. At about 6 to 8 weeks of age, and also 1 week later, each rat received intramuscular injections of 2.5 mg of cortisone acetate as an immunosuppressive. The rats were palpated weekly for the presence of tumors up until a maximum of 14 weeks of age. Although the rat's body temperature of 38.2 C closely approaches the nonpermissive temperature, in this experiment neither the WT nor the ts mutants induced tumors at the site of inoculation (Table 2). Preliminary results of transformation experiments using primary cultures of rat embryo cells indicate that WT and ts 1, 3, and 4 are capable of transformation at either 33 or 39 C. The reason for the failure of in vivo transformation remains unclear at present.

DISCUSSION

The present study showed that Ad 2 ts mutants representing 12 complementation

groups were all defective in late functions. Table 3 presents a summary of the properties of the mutants studied. All of the mutants adsorbed normally, synthesized viral DNA, and produced a cytologically recognizable intranuclear inclusion body. The morphology of the inclusion of ts 3 at the nonpermissive temperature was atypical. This was also the only mutant which failed to produce hexon, penton, and fiber antigens as evidenced by immunodiffusion. Working with Ad 31 ts mutants, Suzuki et al. (18) have observed similar alterations in inclusion body morphology, accompanied by a reduction in the accumulation of the capsid antigens. The drastically altered phenotype of ts 3 however is not likely due to a multiple mutational event as evidenced by the complementation, recombination, and reversion tests (2).

Immunodiffusion analysis also revealed three complementation groups which showed reduced accumulation of the penton antigen, whereas

FIG. 3. Radioactivity and absorbance profiles of cesium chloride equilibrium gradients of extracts of cells infected in the presence of ³H-labeled amino acids with WT and ts mutants at the permissive and nonpermissive temperatures. A and C, WT, ts 53, 74, 6, 8, 9, 1; B and D, ts 4 and 5; E and G, ts 11, 2, 7; F and H, ts 3. —, 33 C; ---, 39 C.

FIG. 4. Radioactivity profiles of cesium chloride equilibrium gradients of extracts of cells infected in the presence of [^3H]thymidine with WT and *ts* mutants at the permissive and nonpermissive temperatures. Panel A: *ts* 1, 2, 6, 8, 9, 53, 74, and WT. Panel B: *ts* 5, 7, 11. Panel C: *ts* 4. Panel D: *ts* 3. Symbols: ●, 33 C; ■, 39 C; ▲, 40.5 C.

normal levels of hexon and fiber antigen were present. The precipitin line corresponding to the penton cross-reacts with that of the fiber and has been shown to contain the entire penton capsid, consisting of penton base and fiber (9). Since the fiber antigen is present in each of the three mutants, and current data suggests that the penton base may consist of more than one copy of a single molecular weight class of polypeptide (10), the existence of three complementation groups appears to be an intriguing finding. Two of these complementation groups may in fact be involved with regulatory aspects affecting the synthesis, processing, or metabolism of penton bases. Alternatively, we may be dealing with intracistronic complementation, or multiple-step mutants. The first possibility is unlikely as the complementation indices were

greater than 10 for the combinations *ts* 11 $\times$ *ts* 4 or *ts* 11 $\times$ *ts* 7 and, though that between *ts* 4 $\times$ *ts* 7 was only 2, these mutants mapped distantly by recombination. The genetic evidence and the high rate of reversion (2) also argue against the second possibility. Russell et al. (12, 13) have demonstrated six complementation groups which affect hexon synthesis, and three complementation groups which affect fiber synthesis, whereas only one group affected penton synthesis.

Of the three mutants defective in penton synthesis, only the least leaky one, *ts* 4, was also

FIG. 5. Heat inactivation of virions of *ts* mutants. Crude samples of virus grown at 33 C were sonically treated to disperse possible aggregates, and diluted 1:10 in Tris-buffered saline, pH 7.4. Samples were exposed to 50 C for the indicated times, then cooled rapidly in an ice bath. Survival was assayed by plaque formation at 33 C.

TABLE 2. Attempts to induce tumors in rats with WT and *ts* mutants^a

Virus	Virus dose (PFU/rat)	No. of baby rats injected	Number of weeks tumor free
WT	10^7 – 10^8	25	13–14
<i>ts</i> 1	10^7	27	9
<i>ts</i> 6	10^7	8	8
<i>ts</i> 4	10^6	8	11
<i>ts</i> 3	10^7	5	10
<i>ts</i> 74	10^6	15	10

^a Newborn litters were injected subcutaneously with 0.1 ml of virus within 24 h of birth and checked for tumors weekly. At about 6 to 8 weeks of age and 1 week later, each rat received two intramuscular injections of 2.5 mg of cortisone.

TABLE 3. *Properties of ts mutants of type 2 adenovirus^a*

Mutant	DNA synthesis (39 C)	Inclusion body formation (39 C)	Antigens produced (39 C)			Virion assembly (39 C or 40 C)	Thermosensitivity (50 C)	In vivo tumor formation	In vitro transformation (39 C)
			Hexon	Fiber	Penton				
H2tsJ 3	+	+	-	-	-	-	+	-	+
H2tsE 11	+	+	+	+	±	±	+	NT	NT
H2tsF 4	+	+	+	+	±	-	-	-	+
H2tsC 7	+	+	+	+	±	±	-	NT	NT
H2tsB 53	+	+	+	+	+	+	+	NT	NT
H2tsH 74	+	+	+	+	+	+	+	-	NT
H2tsH 26	NT	+	+	+	+	NT	NT	NT	NT
H2tsG 6	+	+	+	+	+	+	+	-	NT
H2tsL 8	+	+	+	+	+	+	-	NT	NT
H2tsA 9	+	+	+	+	+	+	+	NT	NT
H2tsD 1	+	+	+	+	+	+	+	-	+
H2tsD 12	NT	+	+	+	+	NT	NT	NT	NT
H2tsI 5	+	+	+	+	+	±	+	NT	NT
H2tsK 2	+	+	+	+	+	+	-	NT	NT
H2ts 10	NT	+	+	+	+	NT	NT	NT	NT
WT	+	+	+	+	+	+	-	-	+

^a Abbreviations: NT, not tested; H2, human type 2 adenovirus; J, E, F, etc., denoted the complementation group.

defective in virion assembly at 39 C. The two leaky mutants, ts 11 and ts 7, only showed reduced assembly at 40.5 C.

The assembly of virions in the absence of adequate numbers of pentons is not as surprising in view of the experiments of Prage et al. (11) which showed that virions lacking pentons and even peripentonal hexons are stable in CsCl gradients and can be visualized by means of the electron microscope. Further phenotypic separation of these mutants was obtained by the demonstration of the thermolability of only one of these three mutants. Increased thermolability of the mutant virions has been taken to suggest the defectiveness of a structural component or a protein implicated in the determination of the structure of the virion. Unaltered lability however, fails to indicate if the ts lesion is in a structural or a nonstructural protein. By this criterion, ts 3 and 5 are two further complementation groups which fail to produce virions and are probably defective in a structural protein. In total, eight complementation groups showed increased thermosensitivity, thus suggesting eight cistrons specifying proteins implicated in the architecture of the virion. The remaining four complementation groups failed to show increased thermolability. According to current data (1), Ad 2 may synthesize up to 14 molecular weight classes of structural proteins and perhaps seven nonstructural proteins. Assuming that these proteins are indeed coded for by Ad 2, the total molecular weight of 920,000 of these 21 proteins accounts for 80% of the coding

capacity of the virus. The 12 complementation groups discussed in this paper may represent about half of the total genetic complement of Ad 2.

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