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Isolation and Characterization of Temperature-Sensitive Mutants of Adenovirus Type 7

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Fifty temperature-sensitive mutants, which replicate at 32°C but not at 39.5°C, were isolated after mutagenesis of the vaccine strain of adenovirus type 7 with hydroxylamine (mutation frequency of 9.0%) or nitrous acid (mutation frequency of 3.8%). Intratypic complementation analyses separated 46 of these mutants into seven groups. Intertypic complementation tests with temperature-sensitive mutants of adenovirus type 5 showed that the mutant in complementation group A failed to complement H5ts125 (a DNA-binding protein mutant), that mutants in group B and C did not complement adenovirus type 5 hexon mutants, and that none of the mutants was defective in fiber production. Further phenotypic characterization showed that at the nonpermissive temperature the mutant in group A failed to make immunologically reactive DNA-binding protein, mutants in groups B and C were defective in transport of trimeric hexons to the nucleus, mutants in groups D, E, and F assembled empty capsids, and mutants in group G assembled DNA-containing capsids as well as empty capsids. The mutants of the complementation groups were physically mapped by marker rescue, and the mutations were localized between the following map coordinates: groups B and C between 50.4 and 60.2 map units (m.u.), groups D and E between 29.6 and 36.7 m.u., and group G between 36.7 and 42.0 m.u. or 44.0 and 47.0 m.u. The mutant in group A proved to be a double mutant.

Temperature-sensitive (*ts*) mutants of several serotypes of human adenoviruses have been isolated (2, 7, 8, 18, 22, 24, 25, 31, 32, 35–37, 42), and their study has given valuable insight into the organization of the viral genome and the regulation of the viral replication cycle. However, only a few of the more than 20 virus-encoded proteins have been linked firmly to a conditionally lethal, *ts* phenotype. Moreover, serotypes 3, 4, 7, 8, and 11, which are most commonly associated with disease in humans, are represented in these studies by a single *ts* mutant, "Ad7 (19)" (8). To determine whether the limited number of genes altered in the relatively large number of adenovirus mutants already isolated was peculiar to the particular viruses predominantly studied (adenovirus type 2 [Ad2], Ad5, and Ad12), this investigation was initiated using a virus from another subgroup. Ad7 was selected since it is an important etiologic agent of respiratory and ocular diseases and thus is one of the viruses contained in the live virus vaccine employed mainly in the Armed Forces (40). Therefore, it was possible that this study could provide not only mutants important for the study of regulation of viral replication but also a virus that may serve as an even better vaccine strain than that presently employed.

This report describes the isolation and preliminary characterization of 50 conditionally lethal *ts* mutants of the vaccine strain of Ad7. Forty-eight mutants have been assigned to seven complementation groups. The physical location of the *ts* lesions of 14 mutants, selected from each of the complementation groups, has been determined using the marker rescue technique (11).

MATERIALS AND METHODS

Cells and virus. Suspension cultures of KB cells were grown in Eagle minimal essential medium (GIBCO Laboratories) supplemented with 5% human or 10% calf serum. Before infection, cells were suspended in minimal medium containing 5% calf serum. Monolayer cultures of KB and of gpt^+ KB cell lines 8, 16, and 18 (1) were grown in Dulbecco modified minimal essential medium (GIBCO Laboratories) supplemented with 10% calf serum. Cloned 293 cells, obtained from E. Frost, were grown as monolayer cultures in Dulbecco modified minimal essential medium supplemented with 10% calf serum. Human foreskin cells (HF cells), kindly supplied by D. L. Engelhardt, and primary human embryonic cells (HEK cells, Flow Laboratories) were grown as monolayers in Dulbecco modified minimal essential medium supplemented with 10% fetal bovine serum. All monolayer cultures were maintained after infection in infecting fluid (13) or Dulbecco modified minimal essential medium containing 3% chicken serum.

The Ad7 virus employed as wild-type (Wt) virus was isolated from an oral vaccine tablet (Wyeth Laboratories) by a single passage in HEK cells. It was plaque purified two times at 38.8°C in HEK cells. The Gomen strain of Ad7 was kindly provided by C. Tibbetts. Mutant H5ts49 (11) was obtained from J. Williams, and H5ts125, H5ts149, H5ts116, H5ts128, H5ts147, and H5ts142 have been previously described (7).

Infectivity assays. Virus was assayed by means of an indirect immunofluorescence assay (30, 38) using antisera against purified Ad5 or Ad7 virions prepared in rabbits. Plates were incubated for 28 to 30 h at 39.5°C or for 60 to 70 h at 32°C.

Plaque assays were performed as previously described (21), with the following modification: at 39.5°C, 293 and KB cells were fed with 3.5 ml of medium on days 7 and 14 and stained on day 19 with overlay medium containing 0.01% neutral red, and plaques were counted on day 20. Assays using HF cells and gpt^+ KB cell line 18 followed the above feeding schedule, but they were stained on day 22 and counted on day 23. The nonpermissive temperature for the gpt^+ KB cell line 18 was 38.8°C, since these cells could not survive 39.5°C. HEK cells incubated at 38.8°C were fed on day 6, stained on day 7, and counted on day 8. At 32°C, KB

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and HF cells were fed on days 7, 14, and 21, stained on day 28, and counted on day 35. The overlay medium for HF and HEK cells were supplemented with 5% fetal bovine serum, and medium for the other three cell lines contained 2% chicken serum. Magnesium chloride at 12.5 mM was added to the medium for HF and 293 cells.

At the nonpermissive temperature, the plating efficiency of type 7 Wt on 293, KB, or gpt^+ KB 18 cell lines was 5- to 10-fold higher than on HF cells. Since KB cells often did not survive the 20-day incubation at 39.5°C, they were replaced in plaque assays by 293 cells, when these became available. The only exception was assays involving H7ts88, which replicated in 293 cells at 39.5°C (J. Praszkier and H. S. Ginsberg, manuscript in preparation).

Mutagenesis. Wt virus was mutagenized with hydroxylamine or nitrous acid as previously described (7). Mutants were selected from viral stocks treated with hydroxylamine for 27 h or with nitrous acid for 40 min; these mutagenesis treatments resulted in approximately a 3-log or 5-log reduction, respectively, in infectivity.

Isolation of *ts* mutants. Mutagenized virus was plated on KB or HF cells at 32°C, as described for the plaque assay. Randomly picked plaques were screened by cytopathic effect test on KB monolayers in 24-well plates, as previously described (7), except that cells were incubated for 12 days at 39.5°C or 19 days at 32°C. Viruses producing cytopathic effect at 32°C, but not at 39.5°C, were plaque assayed at the two temperatures on HF cells. Mutants were plaque purified two more times on HF cells, and to prepare viral stocks 0.3 ml of a plaque suspension was inoculated onto KB monolayers in 25-cm^2 flasks. Cells were harvested after the development of complete cytopathic effect, usually 14 to 20 days after infection at 32°C. Second-passage stocks were made by infecting KB cell monolayers in 175-cm^2 flasks with the above material at 0.1 focus-forming unit (FFU) per cell and harvesting the cells 9 to 10 days after incubation, when cytopathic effect was complete. All genetic studies were performed with second-passage virus.

Complementation analysis. Duplicate monolayer cultures of KB cells in 24-well plates were infected with 5 FFU of each of two mutants per cell, or with 5 FFU of each mutant alone per cell. After 2 h for adsorption at 32°C, cells were washed with Hanks balanced salt solution, incubated for 15 min with specific antiserum (diluted 1:100), washed twice more with Hanks balanced salt solution, and incubated in infecting fluid at 39.5°C for 2 days. Complementation analyses in KB cell spinner cultures were performed as described previously (7), except that cells were infected at a multiplicity of 5 FFU per cell. Viral yield was titrated by fluorescent focus assay at 32°C. Complementation indices were calculated using the following formula: (yield of double infection/sum of the greater of the two single infections).

Preparation of Ad7 Gomen DNA fragments. Adenovirus DNA was prepared from purified virus as previously described (3). Plasmid DNA was prepared according to the method of Figurski et al. (9). Restriction endonucleases were obtained from New England Biolabs, and the digestion conditions used were those suggested by the supplier.

Restriction endonuclease-generated fragments were fractionated in horizontal 0.8% agarose gels in E buffer (0.09 M Tris, 0.09 M borate, 0.0025 M EDTA; pH 8.0) by electrophoresis at 50 V for 20 h. The individual fragments were cut out of the gel, and the gel slices were placed in 5/8-in. (ca. 1.59-cm) dialysis bags containing 2 to 3 ml of E buffer. The bags were placed perpendicular to the direction of current in a horizontal gel apparatus and electrophoresed for 24 h at 100 V at 4°C. The agarose slices were then removed from the dialysis bags; liquid from each bag was added to a centrifuge tube, and each bag was rinsed with 2 ml of the E buffer. Pieces of agarose were sedimented by centrifugation; the supernatant fluid was extracted three times with buffer-saturated phenol (0.01 M Tris hydrochloride, pH 7.4, 1 mM EDTA, 0.15 M NaCl) and twice with chloroform-isoamyl alcohol (24:1) and then was ethanol precipitated. The DNA pellet was solubilized in 10 mM Tris (pH 7.4)–1 mM EDTA and stored at 4°C.

Restriction endonuclease *Bam*HI DNA fragments cloned in pBR322 (6) were kindly provided by T. Broker, and pBR322 clones of *Hin*dIII and *Pst*I fragments were prepared in this laboratory (J. Praszkier and H. S. Ginsberg, unpublished data).

Marker rescue. DNA-protein complex was prepared from purified virions disrupted by incubation for 5 min at 4°C in 4 M guanidine hydrochloride (ultra-pure grade; Bethesda Research Laboratories)–10 mM Tris-chloride–1 mM EDTA (pH 7.9). DNA-protein complex was used at 0.15 μg per plate, and the Ad7 Gomen fragments were added to the mixture at 0.8 μg molar equivalent per plate. Duplicate cultures of 293 cells in 3-cm plates were transfected (11, 16) and incubated at 39.5°C for 6 days. The viral yield from each plate was determined by plaque assay on 293 cells at 39.5°C, except for transfections involving H7ts88, which was assayed on KB cells or gpt^+ KB cell line 18 at 39.5 or 38.8°C, respectively.

Immunofluorescence. KB cells were cultured as monolayers on glass cover slips, infected at 1 FFU per cell, incubated for 28 h at 39.5°C or 60 h at 32°C, and stained as previously described (19).

Radioactive labeling and density gradient centrifugation. Infected cells were labeled with [^{35}S]methionine and [^{3}H]thymidine as previously described (3). For capsid assembly studies, cells were lysed by six cycles of freezing and thawing and analyzed on CsCl gradients as previously described (3), except that the cell extracts were centrifugated for 16 h instead of 3 h.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (20).

Immunoprecipitation. Immunoprecipitation reactions were performed as previously described (27).

RESULTS

Isolation of *ts* mutants. Thirty-six *ts* mutants were isolated from 398 plaques picked at random after treatment of Wt virus with hydroxylamine (mutation frequency, 9.0%), and 14 mutants were obtained from 367 plaques screened after mutagenesis with nitrous acid (mutation frequency, 3.8%). The reversion frequencies of the mutants ranged from less than 5.9×10^{-4} to less than 2.7×10^{-7}. Infectivity titrations of mutants at 32°C were performed by fluorescent focus assay, since Wt virus yielded values that were not significantly different from those obtained by plague assay. Comparison of the yields from single growth cycles at 32 and 39.5°C, in cells infected at a multiplicity of 5 FFU per cell, showed that most mutants exhibited relatively little leakiness. Only four mutants (H7ts1, -6, -39, and -70) had 39.5/32°C yield ratios greater than 10^{-3}. Table 1 shows the growth characteristics of those mutants that will be discussed in some detail in this paper.

TABLE 1. Multiplication of Ad7 *ts* mutants at 32 and 39.5°C

Mutant	Mutagenesis[a]	Yield (FFU/cell)[b] at:		Ratio of yields at 39.5°C/32°C	Reversion frequency[c]
		32°C	39.5°C		
H7ts93	HA	7.2×10^{1}	2.5×10^{-3}	3.5×10^{-5}	6.7×10^{-6}
H7ts17	HA	1.2×10^{2}	3.7×10^{-3}	3.1×10^{-5}	$<1.7 \times 10^{-5}$[d]
H7ts18	HA	1.4×10^{2}	3.3×10^{-3}	2.4×10^{-5}	$<2.1 \times 10^{-5}$
H7ts52	HA	2.7×10^{2}	3.3×10^{-4}	1.2×10^{-6}	$<8.3 \times 10^{-6}$
H7ts57	HA	8.9×10^{1}	9.3×10^{-5}	1.0×10^{-6}	$<2.9 \times 10^{-7}$
H7ts77	NA	6.7×10^{2}	1.3×10^{-2}	1.9×10^{-5}	$<2.7 \times 10^{-6}$
H7ts81	HA	5.3×10^{2}	2.7×10^{-3}	5.1×10^{-6}	$<2.7 \times 10^{-7}$
H7ts19	H7	3.4×10^{2}	1.3×10^{-3}	3.8×10^{-6}	$<1.3 \times 10^{-5}$
H7ts44	NA	1.3×10^{1}	1.7×10^{-4}	1.3×10^{-5}	$<7.1 \times 10^{-7}$
H7ts64	HA	3.2×10^{2}	1.0×10^{-3}	3.1×10^{-6}	6.1×10^{-7}
H7ts88	HA	9.7×10^{2}	8.7×10^{-2}	9.0×10^{-5}	$<1.4 \times 10^{-4}$
H7ts37	NA	1.0×10^{2}	3.0×10^{-3}	3.0×10^{-5}	$<2.2 \times 10^{-5}$
H7ts55	HA	8.6×10^{2}	2.3×10^{-4}	2.7×10^{-7}	$<1.4 \times 10^{-5}$
H7ts58	HA	7.2×10^{1}	2.3×10^{-3}	3.2×10^{-5}	$<4.1 \times 10^{-5}$
H7ts75	HA	9.5×10^{2}	6.0×10^{-3}	6.3×10^{-6}	$<8.7 \times 10^{-7}$
H7ts83	HA	7.8×10^{2}	1.8×10^{-1}	2.3×10^{-4}	3.3×10^{-5}
H7ts87	HA	2.8×10^{2}	2.7×10^{-3}	9.6×10^{-6}	$<1.1 \times 10^{-6}$
H7ts90	NA	1.1×10^{2}	1.5×10^{-3}	1.4×10^{-5}	5.3×10^{-6}
H7ts94	HA	3.7×10^{2}	6.7×10^{-3}	1.8×10^{-5}	1.5×10^{-5}
H7ts95	HA	2.8×10^{2}	1.0×10^{-3}	3.6×10^{-6}	2.0×10^{-6}
H7ts97	HA	4.4×10^{2}	1.7×10^{-2}	3.9×10^{-5}	$<9.1 \times 10^{-7}$
H7ts6	NA	8.4×10^{1}	1.3×10^{-1}	1.6×10^{-3}	$<2.0 \times 10^{-4}$
H7ts21	NA	3.8×10^{2}	3.3×10^{-3}	8.7×10^{-6}	$<2.4 \times 10^{-6}$
H7ts38	NA	4.9×10^{2}	1.6×10^{-2}	3.3×10^{-5}	$<1.3 \times 10^{-6}$
H7ts61	HA	5.1×10^{2}	2.7×10^{-3}	5.3×10^{-6}	$<2.0 \times 10^{-6}$
H7ts85	HA	1.2×10^{3}	9.0×10^{-3}	7.5×10^{-6}	$<8.3 \times 10^{-6}$

[a] Mutants were selected after hydroxylamine (HA) or nitrous acid (NA) mutagenesis.
[b] Final yield from monolayers of KB cells infected at a multiplicity of 5 FFU per cell and incubated at 32°C for 96 h or 39.5°C for 48 h. Virus was assayed on KB cells at 32°C, using the fluorescent-focus technique.
[c] Reversion frequency: viral infectivity at 39.5°C determined by plaque assay on KB or 293 cells, divided by infectivity at 32°C, determined by fluorescent-focus assay on KB cells.
[d] Maximum possible value.

Classification by complementation analyses. To group the isolated mutants according to functional defects, complementation analyses using pairs of the Ad7 *ts* mutants were carried out in KB cells to arrange the related mutants in groups. To obtain genetic evidence of the genes involved, intertypic complementation was done using well-characterized Ad5 *ts* mutants (7, 42).

Intratypic complementation. To facilitate the classification of the many mutants, the first 14 mutants isolated were tested in pairwise crosses and tentatively assigned to complementation groups. Representatives from each group were then used to screen the remaining mutants. Despite extensive evidence for intracistronic complementation (10), the results clearly classified the mutants into seven complementation groups (Table 2). The most ambiguous crosses were also analyzed by measuring the kinetics of viral replication in KB cell spinner cultures (7), and the results in every cross examined by this technique were in agreement with those by the standard technique. It is striking that the majority of mutants fell into two complementation groups, groups B and E, which contained 13 and 25 mutants, respectively. Each of these groups consisted of subsets of mutants that showed low degrees of overlapping complementation, a behavior that is characteristic of intracistronic or intragenic complementation (10). Marker rescue and phenotypic characterization studies supported this classification (see below).

Intertypic complementation analyses. To help identify the functional gene product affected by the mutation, complementation between the Ad7 *ts* mutants and well-characterized Ad5 *ts* mutants (7, 42) was employed. This approach seemed feasible since Ad12 Wt was able to complement some Ad5 *ts* mutants (4), and Ad5 and Ad7 are as related as the distantly related Ad5 and Ad12, which show only a low degree of DNA homology (12, 17).

Seven well-characterized Ad5 mutants, H5ts149 (DNA polymerase mutant; 7, 33), H5ts125 (DNA-binding protein mutant; 7, 14), H5ts49 (probably a mutant of protein IIIa; 11), H5ts128 and H5ts147 (hexon mutants; 7, 13; P. A. Luciw and H. S. Ginsberg, unpublished data), H5ts116 (a 100,000-molecular-weight [100K] protein mutant; 28), and H5ts142 (a fiber mutant; 3), were selected for use in these studies. Reproducible complementation was seen with all of the Ad5 mutants except for H5ts149, which failed to com-

TABLE 2. Classification of Ad7 *ts* mutants into complementation groups

Group	Mutants
A	H7ts93
B	H7ts5, H7ts9, H7ts17, H7ts18, H7ts27, H7ts42, H7ts52, H7ts57, H7ts59, H7ts66, H7ts77, H7ts80, H7ts81
C	H7ts19, H7ts39
D	H7ts88
E	H7ts2, H7ts8, H7ts13, H7ts32, H7ts37, H7ts40, H7ts48, H7ts51, H7ts55, H7ts58, H7ts62, H7ts68, H7ts70, H7ts74, H7ts75, H7ts82, H7ts83, H7ts87, H7ts89, H7ts90, H7ts94, H7ts95, H7ts97, H7ts44, H7ts64
F	H7ts1, H7ts6
G	H7ts21, H7ts38, H7ts61, H7ts85

TABLE 3. Intertypic complementation between *ts* mutants of Ad5 and Ad7[a]

Mutant	Yield[b] (FFU/cell)	Complementation index[c] (*n*)					
		H5ts125 (0.015)[b]	H5ts49 (0.11)[b]	H5ts128 (0.14)[b]	H5ts147 (0.10)[b]	H5ts116 (0.15)[b]	H5ts142 (0.07)[b]
H7ts93	0.007	0.78 (5)	70 (3)	18 (3)	21 (3)	11 (3)	45 (4)
H7ts81	0.087	302 (2)	28 (4)	0.94 (3)	3.4 (3)	1.3 (3)	46 (2)
H7ts52	0.018	73 (2)	122 (3)	1.2 (3)	0.5 (2)	0.65 (2)	144 (2)
H7ts19	0.002	17 (2)	291 (3)	0.12 (2)	0.32 (2)	0.17 (2)	83 (3)
H7ts88	0.092	26	154 (2)	43 (3)	30 (3)	52	32 (3)
H7ts55	0.085	26 (4)	99 (4)	52 (4)	84 (2)	27 (4)	187 (3)
H8ts37	0.010	136 (2)	193 (2)	30 (2)	46 (2)	48	30 (2)
H7ts75	0.007	433	214 (3)	534 (2)	216 (2)	579 (2)	104 (3)
H7ts87	0.093	48 (2)	97 (2)	43 (2)	25 (2)	19 (3)	60
H7ts94	0.111	40 (2)	120 (4)	86	91 (2)	21 (2)	169
H7ts85	0.002	91 (2)	176 (2)	215 (2)	89 (2)	54 (2)	61 (2)
H7ts6	0.19	78	202 (2)	13 (3)	24 (2)	10 (3)	21 (2)

[a] Complementation analyses were carried out in KB cell monolayers infected with 5 FFU of each mutant per cell or 100 FFU or with 5 FFU of each mutant alone. Cultures were incubated at 39.5°C for 47 h, and the yield was titrated by fluorescent-focus assay at 32°C.

[b] Geometric means of yields from single infection, expressed as FFU per cell.

[c] Complementation index, calculated as yield of double infection/sum of the two single infections, and presented as geometric mean of indices from several experiments. Values in parentheses represent the number of determinations upon which the mean is based.

plement many Ad7 mutants and complemented others poorly, although all of the Ad7 mutants, except for H7ts93, were defective in late viral genes (see below). Data for representative mutants from each of the seven complementation groups of Ad7 are summarized in Table 3. In summary, all 48 mutants examined complemented H5ts49, and all but H7ts93 (group A, Table 1) complemented H5ts125. The 15 mutants comprising groups B and C failed to complement either the hexon or the 100K protein mutants of Ad5. Of the remaining mutants, only H7ts44 and H7ts64 (group E) did not complement the hexon and 100K protein mutants of Ad5, and H7ts64 was also the only mutant that failed to make adequate amounts of functional fiber, suggesting that these two mutants carried multiple *ts* lesions.

Marker rescue. While genetic characterization of mutants permits their classification into functional groups, it fails to localize their defects on the genome. Marker rescue experiments were performed, therefore, to determine the physical sites of the mutations. Since no restriction endonuclease maps of the vaccine strain were available, the Gomen strain of Ad7, for which restriction cleavage sites of several enzymes are known (39), was used as the source of DNA fragments. The approximate locations of the mutations were established first, using the four *Xba*I restriction endonuclease fragments. Appropriate *Bam*HI, *Hin*dIII, and *Pst*I fragments, cloned in pBR322, were used subsequently to localize the position of each mutation more precisely.

Table 4 presents representative data obtained with the 14 mutants of the selected viruses mapped. None of the mutants was rescued by *Xba*I fragments C and D (68.5 to 80.5 and 80.5 to 100 map units, respectively) or by *Bam*HI fragment E (60.2 to 70.00 map units) (data not shown). Mutations of H7ts77 and H7ts81 (group B, see Table 2) and H7ts19 (group C) were found to lie between map coordinates 50.4 and 60.2 (the L3 region). The mutations of H7ts88 (group D) and H7ts37 and -58 (group E) were localized

TABLE 4. Marker rescue of Ad7 mutants with DNA fragments of Ad7 Gomen

Mutant	Virus yield (PFU/ml)[a] with fragment:											
	*Xba*I-A (0–44.0)[b]	*Xba*I-B (44.0–68.5)	*Bam*HI-A (36.7–60.2)	*Bam*HI-B (15.9–36.7)	*Hin*dIII-A (29.6–50.4)	*Hin*dIII-C (50.4–63.8)	*Hin*dIII-D (17.0–29.6)	*Pst*I-E (34.0–42.0)	*Pst*I-H (26.5–31.5)	*Pst*I-I (42.0–47.0)	*Pst*I-M (31.5–34.0)	Map limits[c]
H7ts77	<2	438	1,562	<2	<2	1,224	ND[d]	ND	ND	ND	ND	50.4–60.2
H7ts81	40	2,782	2,506	<2	<2	4,024	<2	ND	ND	ND	ND	50.4–60.2
H7ts19	<2	200	80	<2	<2	320	ND	ND	ND	ND	ND	50.4–60.2
H7ts88	800[e]	2[e]	42[f]	5,600[f]	13,000[f]	ND	52[f]	900[f]	16[f]	ND	26[f]	34.0–36.7
H7ts37	846	<2	<2	522	688	ND	<2	342	<2	ND	<2	34.0–36.7
H7ts55	1,160	<2	<2	598	756	ND	<2	<2	<2	ND	<2	29.6–36.7
H7ts58	ND	ND	<2	1,014	1,402	ND	<2	498	<2	ND	<2	34.0–36.7
H7ts83	92	<2	<2	288	134	ND	<2	<2	<2	<2	<2	29.6–36.7
H7ts90	ND	ND	<2	98	90	ND	<2	<2	<2	ND	<2	29.6–36.7
H7ts94	2,300	<2	<2	1,100	9,300	ND	<2	<2	<2	ND	<2	29.6–36.7
H7ts21	420	<2	570	<2	<2	ND	<2	38	ND	<2	ND	36.7–42.0
H7ts38	370	<2	312	<2	310	<2	ND	52	ND	<2	ND	36.7–42.0
H7ts61	788	<2	722	<2	670	<2	ND	186	ND	<2	ND	36.7–42.0
H7ts85	<2	102	380	<2	168	<2	ND	<2	ND	98	ND	44.0–47.0

[a] Yield of virus from transfected cells, harvested after 6 days of incubation at 39.5°C and plaque assayed on 293 cells at 39.5°C, unless otherwise indicated.

[b] Parentheses indicate map coordinates of DNA fragments used.

[c] Map coordinates within which the mutations are located, deduced from the overlap in the rescuing fragments.

[d] ND, Not done.

[e] Virus from transfected cells, harvested after 4 days of incubation at 39.5°C, was passaged once in KB cells at 39.5°C, and its titer was determined by plaque assay on KB cells at 39.5°C.

[f] As described in footnote *e*, except that plaque assay was performed on gpt$^+$ KB cell line 18 at 38.8°C.

between coordinates 34.0 and 36.7, and those of H7ts55, -83, -90, and -94 (group E) were localized between coordinates 29.6 and 36.7 (the L1 region). The map limits of the last four mutants could not be fixed more precisely, because they repeatedly failed to be rescued by any of the *Pst*I fragments within coordinates 26.5 to 42.0. Three of the mutants in group G (H7ts21, -38, and -61) were mapped between coordinates 36.7 and 42.0, and the fourth member of the group, H7ts85, was localized between coordinates 44.0 and 47.0 (the L2 region). H7ts93, the single member of group A, was rescued by *Sal*I-A (17.5-68.0) and *Xho*I-A (22.9-76.5), but not by any of the *Xba*I or *Bam*HI fragments subdividing this area of the genome. These data suggest that H7ts93 is a double mutant.

Immunofluorescence. Immunofluorescence, using antiserum prepared against purified Ad7 virions, was employed to test the ability of the *ts* mutants to synthesize the major structural proteins under permissive and nonpermissive conditions. Only the group A mutant, H7ts93, failed to make detectable amounts of immunologically reactive late antigens at 39.5°C and was therefore the only probable early mutant. This mutant was examined further for its ability to synthesize immunologically reactive DNA-binding protein, using antiserum made against Ad5 DNA-binding protein (14). No fluorescence was detected in cells infected with H7ts93 and incubated at 39.5°C whereas nuclear fluorescence was observed in H7ts93-infected cells at 32°C and in Wt-infected cells at 39.5 and 32°C.

To confirm the intratypic complementation data and to characterize more fully the phenotypic defects of group B and C mutants, the distribution of immunologically reactive hexons in cells infected at 39.5°C with representatives of the different complementation groups was examined, using antiserum prepared against purified Ad5 native (trimeric) hexons (34). It is important to note that the antibodies present in this antiserum only react with trimeric hexons and will not combine with the hexon polypeptide chains (34). In cells infected with group B and C mutants the fluorescence was predominantly cytoplasmic, whereas Wt virus and all other mutants examined accumulated trimeric hexons in the nucleus (Table 5).

TABLE 5. Immunofluorescence in KB cell monolayers infected with Ad7 *ts* mutants at 39.5°C and stained with antiserum prepared against Ad5 hexon[a]

Mutant	Group	Distribution of fluorescence	
		Nucleus	Cytoplasm
H7ts5	B	+/−	+++
H7ts9	B	+/−	+++
H7ts18	B	+/−	+++
H7ts27	B	+/−	+++
H7ts52	B	+/−	+++
H7ts57	B	+/−	+++
H7ts66	B	+	+++
H7ts77	B	+/−	+++
H7ts80	B	+/−	+++
H7ts81	B	+/−	+++
H7ts19	C	−	+++
H7ts39	C	+	+/−
H7ts88	D	+++	+/−
H7ts64	E	+/−	+++
H7ts37	E	+++	+/−
H7ts55	E	+++	+/−
H7ts75	E	+++	+/−
H7ts94	E	+++	+/−
H7ts6	F	+++	+/−
H7ts38	G	+++	+/−
H7ts61	G	+++	+/−
Wt		+++	+/−

[a] After 30 h of incubation, cover slips were fixed in acetone and stained with antiserum prepared in rabbits against purified trimeric Ad5 hexons. The indirect stain was fluorescein-conjugated goat anti-rabbit immunoglobulin G.

Assembly of capsids. Since mutants classified in complementation groups D, E, F, and G (see Table 2) produced functional hexons and fibers at the nonpermissive temperature, their ability to assemble capsids at 39.5°C was tested. Members of groups D, E, and F assembled empty capsids which banded in CsCl at a buoyant density of approximately 1.30 g/ml, as well as particles that banded at a buoyant density of approximately 1.27 g/ml. None of the mutants assembled DNA-containing capsids. Analysis by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels showed that the empty capsids of the mutants did not contain proteins V and VII and that their polypeptide pattern was indistinguishable from that of the empty capsids of the Wt virus. Proteins banding at a buoyant density of 1.27 g/ml consisted almost entirely of penton base and fiber and seemed to be identical to those proteins present in Wt virus-infected cell lysates (data not shown). All of the major viral structural proteins present in lysates of cells infected by members of groups D and E at 39.5°C were immunologically reactive in an immunoprecipitation test, and polypeptides made by these mutants at the nonpermissive temperature were stable during prolonged incubation at 39.5°C (data not shown).

Mutants assigned to complementation group G assembled DNA-containing particles as well as the empty capsids, but failed to produce material banding at a buoyant density of approximately 1.27 g/ml. Detailed characterization of these mutants will be presented elsewhere (Praszkier and Ginsberg, in preparation).

DISCUSSION

Fifty *ts* mutants of the vaccine strain of Ad7 were isolated after treatment of viral stocks with hydroxylamine or nitrous acid. The frequencies of mutant isolation, 9.0% for hydroxylamine and 3.8% for nitrous acid, were comparable to those reported by other investigators using similar techniques to select mutants of Ad2 (18, 25), Ad5 (7, 42), and Ad12 (31).

Forty-six mutants were assigned to seven nonoverlapping complementation groups (Table 1). Two mutants, H7ts7 and H7ts54, gave ambiguous results in complementation crosses and were not studied further. Characterization of H7ts44 and -64 revealed that, unlike other members of the complementation group to which they were assigned, they failed to assemble functional hexons, and H7ts64 also failed to assemble functional fibers at the nonpermissive temperature. The most plausible explanation for these observations is that H7ts44 and -64 are double mutants. Considerable intracistronic complementation (10) was observed with mutants classified in groups B and E. Crosses between pairs of mutants within these two groups gave complementation indices ranging from less than 1 to several hundred. However, the overall pattern was a series of overlapping groups strongly suggestive of intracistronic complementation. This view was supported by functional, phenotypic, and physical mapping studies. Intracistronic complementation has been suggested for Ad5 *ts* mutants (15, 43) and seems to explain the disparity between the large number of complementation

groups reported for Ad5 (43) and Ad2 (25) and the relatively few genes affected (15). Intracistronic complementation is a common feature of genes coding for multimeric proteins composed of identical subunits. Subunits altered in different sites may be able to compensate for each other's defects, resulting in partial restoration of function. Mutations which alter the subunits so that they are incapable of interacting with each other fail to complement.

Intertypic complementation experiments with Ad5 *ts* mutants showed that at the nonpermissive temperature, the group A mutant did not make functional DNA-binding protein and members of groups B and C failed to make functional hexons. None of the viruses containing single mutations was defective in production of fiber. Several additional points emerged in the course of these studies. First, the hexon-defective mutants of Ad7 failed to complement the 100K protein mutants of Ad5, supporting the findings that imply that both gene products are necessary for assembly of functional hexons (28) and suggesting that their interaction is highly specific. In contrast, interaction between the 100K protein and hexon of the closely related viruses Ad2 and Ad5 showed no type specificity (4). Second, gene functions of Ad5 were able to substitute for the defective hexon, 100K, IIIa, fiber, and DNA-binding protein gene products of the other serotype. Therefore, the functional relationship between Ad5 and Ad7 appears to be closer than that between Ad5 and Ad12, since Ad12 Wt was unable to complement Ad5 mutants defective in the IIIa, fiber, or DNA-binding protein genes (43).

The physical mapping studies of selected *ts* mutants of Ad7 showed that mutants H7ts77 and H7ts81 (group B) and H7ts19 (group C) mapped between coordinates 50.4 and 60.2, which is the physical location of the L3 region encoding the genes for protein VI and hexon (23, 26, 29). Since at the nonpermissive temperature these mutants were unable to assemble functional hexons and failed to accumulate trimeric hexons in the nucleus, their mutations are clearly in the hexon gene. The assignment of these mutants to two separate complementation groups was apparently due to intracistronic complementation.

Mutants H7ts88 (group D) and H7ts37 and H7ts58 (group E) mapped between coordinates 34.0 and 36.7, while H7ts55, -83, -90, and -94 (group E) were localized between coordinates 29.6 and 36.7. These data place members of groups D and E in the L1 family of genes, which codes for the 52/55K and IIIa proteins (26). It should be pointed out that the Ad5 mutant H5ts49, which complemented all members of groups D and E, mapped in the same area of the genome, between coordinates 31.5 and 37.3 (11). In addition, mutants of groups D and E assembled empty capsids at the nonpermissive temperature, as does H5ts58 (5), which is in the same complementation group as H5ts49 and maps between coordinates 31.5 and 37.3 (11). Since members of group E showed extensive intracistronic complementation, the possibility that they and H7ts88 (group D) are mutated in the same gene as H5ts49 and H5ts58 cannot be excluded.

Three of the mutants in group G (H7ts21, H7ts38, and H7ts61) mapped between coordinates 36.7 and 42.0, and the fourth member of the group, H7ts85, was located between coordinates 44.0 and 47.0. These data suggest that members of group G could be mutants in the penton base gene or other L1 or L2 region genes. However, the finding that they are phenotypically penton base defective (Praszkier and Ginsberg, in preparation) indicates that group G viruses are mutated in the penton base gene. Since the penton base mutants complemented the fiber mutant H5ts142, it appears that fiber proteins of Ad7 can interact with Ad5 penton bases to yield functional pentons.

Thus, of the 46 mutants studied, 26 were found to be in the L1 family of genes, 15 appeared to be hexon gene mutants, and 4 were penton base gene mutants. No mutants of the gene for 100K protein were found. The distribution of mutations along the Ad7 genome is clearly nonrandom. Indeed, it is striking that although similar procedures for mutagenesis and mutant isolation were employed for these studies with Ad7 and those for Ad5 (7, 42), Ad7 yielded a strikingly different set of mutations. Mutagenesis of Ad7 produced a predominance of mutations in late region 1 (L1) and many more in L2 (i.e., penton base mutants) than were obtained from Ad2 or Ad5 (2, 4, 7, 11, 15, 25, 42, 44). Moreover, no mutants defective in L4 were isolated, and a much smaller proportion of early mutants were obtained. Hence, this study did fulfill one of its original objectives, to obtain conditionally lethal mutations in genes not frequently affected by mutagenesis of Ad2 and Ad5.

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