Adenovirus Type 12 Gene 401 Function in Transforming Infection

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The temperature-sensitive DNA-minus mutant, H12ts401, transformed two to eight times more hamster embryo cells than wild-type 12 adenovirus at 38.5°C, but was unable to establish transformation of cultures of hamster embryo brain and rat 3Y1 cells at 41.5 and 40°C, respectively. Another H12ts406 DNA-minus mutant was not defective in cell transformation at these restrictive temperatures. Both mutants, however, induced T-antigen and cell DNA synthesis after infection of 3Y1 cells at 40°C.

The DNA-minus temperature-sensitive (*ts*) mutants of human adenoviruses exhibit a variety of capabilities in transformation induction at the nonpermissive temperature. The H12ts505 mutant (5) and the alleles H5ts36 and H5ts37 (15) transform cells at a significantly lower frequency than wild-type (WT) 12 and 5 viruses at the nonpermissive temperature; in contrast, H12ts401 (10), H5ts125 (2), and H5ts107 (3) transform at a two- to eightfold higher frequency than WT virus. On the other hand, H12ts406 (10) and H5ts149 (2), in different complementation groups from H12ts401 and H5ts125, respectively, transform at a frequency similar to WT at the nonpermissive condition. These findings imply that a specific adenovirus gene product affects transformation. The increased transformation potential of H5ts125 appears to be correlated with an enhanced integration of the viral genome (12). It has been suggested that a single-strand-specific DNA-binding protein encoded by H5ts125 (14) regulates transformation by normally modulating against transformation and, when mutated, permits increased viral DNA integration (3).

The H12ts401 mutant transforms hamster cells at frequencies two to eight times greater than WT virus at 38.5°C; at this restrictive temperature, *ts*401 cannot replicate its DNA in human cells (10). If the increased transformation potential of H12ts401 is correlated with a mutated viral gene product which naturally decreases transformation, it seems possible that incubation of cells, at a temperature higher than 38.5°C and tolerated by the cells, might result in a still higher transformation potential. Instead, the transforming ability of H12ts401, compared to H12WT or H12ts406, was suppressed at elevated temperatures.

The transforming ability of H12WT and the

*ts*401 and 406 mutants was compared using secondary cultures of hamster embryo brain (HEB) cells, purchased from BioLabs, Inc., Northbrook, Ill., and a cloned rat 3Y1 cell line (7) generously provided by G. Kimura. Infected cells were incubated at the permissive temperature (36°C) or at the nonpermissive temperatures tolerated by the cells: 40°C for 3Y1 cells and 41.5°C for HEB cells. The transformation procedure using hamster cells has been described (11). Cells from a primary HEB culture were removed with 0.2% trypsin, centrifuged, and suspended in Dulbecco-modified Eagle medium containing 10% fetal bovine serum. Then 6×10^5 HEB cells in 2.8 ml of medium were placed in a 60-mm petri dish, and, immediately after plating, 0.2 ml of virus was added at the indicated input multiplicities. The growth and titration of H12 in human embryo kidney cells have been described (8). After incubation at 36°C for 3 days, the cultures received 3 ml of Dulbecco-modified Eagle medium containing 10% fetal bovine serum and 0.1 mM CaCl_2 (1), and half of the cultures were transferred to 41.5°C. At 6 days postinfection, the medium was changed with the addition of 3 ml of an agar overlay medium which consisted of the above low Ca-containing medium in 0.3% agar (Difco). At 3- to 5-day intervals, 2 to 3 ml of additional agar medium was added. At 30 to 40 days, transformed foci were counted.

The transformation assay of H12 using 3Y1 cells has been described (5). We have used a modified procedure. The 3Y1 cells (10^5) were plated in medium and infected as described above. After incubation at 36°C for 2 days, the cells were trypsinized, and 10^4 cells were seeded in a 60-mm dish in 3 ml of the above low Ca-containing medium and incubated for 1 day at 36°C. Then half of the cultures were shifted to 40°C. At 5 to 6 days postinfection, the dishes

received 3 ml of the agar overlay medium described above. With this procedure, one transformation event is caused by 10^4 plaque-forming virions.

To determine the cell plating efficiency, after virus was adsorbed for 4 h, HEB cells were diluted further to contain the specified number of cells per 3 ml of the above low Ca-containing medium. After 7 to 9 days, the colonies were fixed with buffered Formalin and stained with Giemsa stain. The plating efficiency of 3Y1 cells was determined using cells trypsinized 2 days after infection, at the time when 10^4 cells were seeded in new cultures as described above.

The *ts401* mutant was defective in its ability to transform hamster and rat cells at 41.5 and 40°C, respectively (Table 1). *ts401* transformed these cells at an approximately 10-fold lower frequency than WT or *ts406* under the nonpermissive condition. The transformation efficiency of WT and *ts406* at the restrictive temperature was 70 to 90% of that at the permissive temperature. The much lower efficiency of transformation by *ts401* at the nonpermissive temperature was not due to cell death of *ts401*-infected cells, since the colony-forming efficiency of cells infected by all three viruses was similar under the nonpermissive condition (Table 1).

The opposite effects exhibited by the *ts401* mutant in its ability to transform HEB cells at 38.5 and 41.5°C are shown in Table 2. At 38.5°C, *ts401* transformed cells with approximately six times the WT efficiency; in contrast, at 41.5°C, the mutant was defective for transformation compared with WT virus. The *ts406* mutant, on the other hand, transformed cells with approximately the same efficiency as WT at both temperatures.

The *ts401* mutant also had a higher transformation frequency at 36°C than WT virus. From the data given in Table 1, the transformation efficiency (i.e., the ratio of total PFU added per dish per number of transformed foci counted per dish) of each virus can be calculated; *ts401* transformed approximately four times more hamster or 3Y1 cells than WT virus at 36°C.

Infection of 3Y1 cells with H12WT results in an abortive infection leading to the synthesis of T antigen but not infective virus (5). The expression of T antigen at the nonpermissive temperature was examined in 3Y1 cells infected with WT, *ts401*, or *ts406*. Cells growing on cover slips in Eagle medium containing 10% fetal bovine serum were infected at input multiplicities of 20 to 30. After virus was adsorbed for 1 h at 36°C, the cultures were incubated at 36 or 40°C for 3

TABLE 1. Transformation of HEB and 3Y1 cells by H12WT, H12*ts401*, or H12*ts406* virus

Cells	Virus	Virus multiplicity (PFU/cell)	Transformation efficiency (avg no. of foci per dish) ^a		Transformation frequency ^b	Plating efficiency (% of colonies plated per dish) ^c		Fraction of colonies plating ^d
			Permissive temp ^d	Restrictive temp ^d		Permissive temp ^d	Restrictive temp ^d	
HEB ^e	WT	60	26 ± 4	19 ± 3	0.73	5.1	2.8	0.55
	<i>ts401</i>	30	54 ± 9	3.7 ± 1	0.07	5.5	2.7	0.49
	<i>ts406</i>	50	38 ± 5	26 ± 7	0.68	7.2	3.1	0.43
3Y1 ^f	WT	60	27 ± 6	24 ± 4	0.89	19.1	16.8	0.88
	WT	30	12 ± 3	10 ± 2	0.83	20.4	17.2	0.84
	<i>ts401</i>	30	45 ± 8	4.2 ± 2	0.09	22.6	20.4	0.90
	<i>ts406</i>	50	29 ± 7	22 ± 4	0.76	21.9	18.1	0.83

^a Values represent the mean of foci counted on 5 to 8 dishes, ± the standard deviation.

^b At the restrictive relative to the permissive temperature.

^c From 10 to 12 dishes were each seeded with 1,000 HEB cells (diluted after virus had been absorbed for 4 h) or with 250 3Y1 cells, using cells trypsinized 2 days after infection, at the time when 10^4 cells were seeded in new cultures as described in the text. From five to six dishes of HEB and 3Y1 cells were incubated at the permissive and restrictive temperatures. After 7 to 9 days, colonies were fixed and stained with Giemsa stain (11).

^d Permissive temperature for HEB and 3Y1 cells was 36°C. The restrictive temperature for HEB cells was 41.5°C, and that for 3Y1 was 40°C.

^e From 10 to 16 dishes were each seeded with 6×10^5 HEB cells from a primary culture, and, immediately after plating, cells were infected with the indicated input virus multiplicities. After incubation at 36°C for 3 days, medium containing 0.1 mM CaCl_2 was added (see text), and half of the cultures were transferred to 41.5°C for the duration of the experiment. At 6 days postinfection, the medium was changed with the agar overlay medium as described in the text. Transformed foci were counted at 30 to 40 days.

^f Five dishes were each seeded with 10^5 3Y1 cells, and, immediately after plating, cells were infected with the indicated input virus multiplicities. After incubation at 36°C for 2 days, the cells were trypsinized, and 10^4 cells were seeded in each of 10 to 16 dishes in medium containing 0.1 mM CaCl_2 (see text). After incubation at 36°C for 1 day, half of the cultures were transferred to 40°C.

days and then stained for T antigen. The method for indirect immunofluorescent staining for T antigen, using antiserum from hamsters bearing WT-induced tumors, has been described (13). There was essentially no difference in nuclear fluorescent staining characteristics of WT- and the mutant-infected cells at the permissive or restrictive temperatures. At either temperature, approximately 50 to 70% of 3Y1 cells infected with WT or the *ts* mutants were T-antigen positive, while control uninfected cultures were T-antigen negative.

The effect of the 401 and 406 gene mutations was tested next on the stimulation of host cell DNA synthesis. 3Y1 cells were infected with WT, *ts*401, or *ts*406 viruses, and, at various times after infection at 36 or 40°C, the cultures were given [³H]thymidine for 1 h. The incorporation

of [³H]thymidine into cell DNA extracted by the method of Hirt (6) was determined. A parallel series of cultures was mock infected (Table 3). *ts*401 induced 3Y1 cell DNA synthesis with approximately the same efficiency as WT at 36 or 40°C (Table 3). At 2 days postinfection at 40°C, the rate of thymidine incorporation into the Hirt precipitate of cells infected by *ts*401 and WT viruses was 7.6 and 6 times greater, respectively, than that of control cells. After 3 days at the nonpermissive temperature, the incorporation rate in *ts*401- and WT-infected cells was five to six times higher than in controls. At these times, the levels of the rates of cell DNA synthesis after *ts*401 and WT infection at the permissive temperature were at least as high as at 40°C. The results also suggest that *ts*406 was somewhat more efficient in stimulating cell DNA

TABLE 2. Transformation of HEB cells by H12WT, H12*ts*401, or H12*ts*406 virus at 38.5 or 41.5°C^a

Virus	Total PFU added per dish (×10 ³)	Virus multiplicity (PFU/cell)	Avg no. of foci per dish ^b		Transformation efficiency ^c (×10 ³)		Relative transformation efficiency ^d	
			38.5°C	41.5°C	38.5°C	41.5°C	38.5°C	41.5°C
WT	5.1	85	22 ± 3	20 ± 4	23.2	25.5		
<i>ts</i> 401	2.4	40	58 ± 6	1.6 ± 1	4.1	150	5.7	0.17
<i>ts</i> 406	3.9	65	21 ± 4	18 ± 3	18.6	21.7	1.2	1.2

^a From 10 to 12 dishes were each seeded with 6×10^5 cells from a primary culture, and immediately after plating the indicated PFU were added to each dish. The transformation procedure was then carried out. Cells infected by all three viruses had cloning efficiencies of 6 to 7% at 38.5°C and 3 to 4% at 41.5°C.

^b Each figure represents the mean foci counted on five to six dishes, ± the standard deviation.

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

^d Transformation efficiency in mutant- relative to WT-infected cultures.

TABLE 3. Induction of cell DNA synthesis in 3Y1 cultures infected with H12WT, H12*ts*401, or H12*ts*406 virus^a

Days post-infection	CPM [³ H]thymidine incorporated/10 ⁴ mock-infected cells (×10 ⁻³)		Ratio of [³ H]thymidine incorporated in 10 ⁴ cells (infected/control)					
			WT		<i>ts</i> 401		<i>ts</i> 406	
	36°C	40°C	36°C	40°C	36°C	40°C	36°C	40°C
1	41.1	19.2	0.7	1.8	0.5	1.2	0.5	2.1
2	29.2	6.5	8.4	6.0	11.1	7.6	24.0	34.3
3	1.6	3.4	7.6	6.0	6.7	4.9	26.1	10.5
4	ND	4.2	ND	4.2	ND	4.4	ND	10.9

^a Subconfluent 3Y1 cultures growing in 75-cm² Falcon flasks were uniformly labeled by growth for 3 days in Eagle minimum essential medium containing 10% fetal bovine serum and [¹⁴C]thymidine (1 μCi per flask, 61 mCi/mmol). The [¹⁴C]-labeled cells were seeded in the same medium without the isotope into 16-mm wells in Falcon multiwell plates. When the cultures become confluent, the medium was replaced with 0.9 ml of Eagle medium containing 2% fetal bovine serum. After 3 days, 0.1 ml of virus was added at an input multiplicity of 20 to 30 to each well. Virus was adsorbed for 1 h at 36°C, and then the cultures were incubated at either 36 or 40°C. At 4 h postinfection, the medium in each well was replaced with 0.9 ml of Eagle medium containing 2% fetal bovine serum. At various times, 50 μl of Eagle medium containing [³H]thymidine (40 μCi/ml, 5 Ci/mmol) was added for 1 h. The cultures were washed twice with cold Tris-buffered saline, and DNA was extracted by the method of Hirt (6). The Hirt precipitate, consisting of cell DNA, was dissolved in 0.5 ml of 2 N NH₄OH, transferred to a glass fiber disk, dried, and counted by scintillation spectrometry (9). The ³H counts were normalized to a constant amount of [¹⁴C]DNA to correct for any variation in DNA recovery. ND, Not done.

synthesis at 36 or 40°C than was WT or *ts401* (Table 3). At 2 and 3 days postinfection, the *ts406*-infected cultures incorporated at least twice as much [³H]thymidine into cell DNA as did the other virus-infected cells at either temperature.

The *ts401* mutant is unable to replicate its DNA in human cells at 38.5°C (10); at this temperature, the mutant exhibits an enhanced transformation potential in hamster cells. At higher temperatures, however, the transformation capability of the mutant has been shown to be suppressed. These studies suggest that the host cell can influence differentially the temperature-dependent expression of the 401 mutation. Thus, the *ts401* gene product may exhibit "leakiness" in restrictive rodent cells, as reflected by an increased heat resistance of transformation. In another pertinent study, the host cell has been shown to influence strikingly the temperature at which the temperature-dependent cell transformation by an avian sarcoma *ts* mutant is expressed (4). The way the host cell affects the heat sensitivity of a mutated virus product remains to be ascertained.

The DNA-minus mutants H12*ts401*, H5*ts125* (2), and H5*ts107* (2) have been shown to transform cells at frequencies greater than WT virus at the nonpermissive as well as the permissive temperatures. The reason for the increased transformation property is unknown. The most direct interpretation of these findings, however, is that stocks of these mutants may contain a higher proportion of transforming defective genomes than WT virus.

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