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Persistence of type 5 adenovirus DNA in cells transformed by a temperature-sensitive mutant, H5ts125

(adenovirus transformation/adenovirus temperature-sensitive mutants/DNA-binding protein/DNA integration)

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ABSTRACT **The characteristic of H5ts125, a temperature-sensitive DNA-minus mutant, to transform 3 to 8 times more rat embryo cells than wild-type 5 adenovirus was correlated with the persistence of an increased proportion of the viral genome in cells independently transformed at the nonpermissive (39.5°) or semipermissive (36°) temperature. Reassociation kinetics of the hybridization of ^{32}P-labeled, *Hin*dIII restriction fragments of the viral genome and excess unlabeled, transformed cell DNA was used to measure the quantity of the viral genome in transformed cells. Three of four cell lines independently transformed and maintained at 36° contained all regions of the viral genome; one line transformed at 39.5° contained multiple copies representing all of the viral DNA; and one line contained a large proportion of the viral genome. The cell line transformed and maintained at 32° contained a quantity of viral genome consistent with that usually found in cells transformed by wild-type 5 adenovirus.**

Conditionally lethal, temperature-sensitive mutants of Rous sarcoma virus and simian virus 40 (SV40) have been critical for demonstrating that a viral gene product is required to transform susceptible cells (1, 2). Indeed, the SV40 ts A mutant identifies the viral gene responsible for transformation (3–6). Hence, the isolation and characterization of temperature-sensitive mutants of adenoviruses (7–10) suggested that they, too, may be useful to identify the critical gene(s) involved in, and to detect the gene product(s) essential for, transformation. In contrast to our expectation from data obtained with Rous sarcoma (1) and SV40 (2) viruses, however, an adenovirus mutant defective in DNA replication (5), H5ts125, was discovered that transformed rat embryo cells at frequencies 3 to 8 times greater than wild-type 5 adenovirus at the nonpermissive (39.5°) and semipermissive (36°) temperatures (11, 12). This finding implies that a specific viral gene product affects transformation, perhaps by modulating against it, so that, when the viral protein is nonfunctional or functions aberrantly, transformation is less restricted. A single-strand specific DNA-binding protein has been identified as the viral protein encoded in the ts125-mutated gene (13); this viral protein, therefore, may be a regulator of viral transformation.

Because only a portion of the left end of the type 5 adenovirus genome is required to establish transformation (14), and usually only a part of the type 2 or 5 genome persists in transformed cells (15–17), it seemed possible that functional adenovirus DNA-binding protein may decrease integration or promote excision of adenovirus DNA in the infected cells. It would then follow that, in rat embryo cells infected with H5ts125, effective integration might occur more frequently and more viral DNA might persist in the integrated state. The data from a test of this postulate form the basis of this communication. It will be shown that all or most of the viral genome was commonly present in cells transformed by H5ts125 at the nonpermissive or semipermissive temperature, in sharp contrast to the finding that only the left end of the genome persisted in cells transformed by wild-type 5 virus or by H5ts125 under permissive conditions (32°).

Abbreviation: SV40, simian virus 40.

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MATERIALS AND METHODS

Virus Infection and Cells. Wild-type 5 and H5ts125 adenoviruses were grown and titrated on KB cells, as described previously (8). Primary rat embryo cells were obtained from Flow Laboratories (Bethesda, Md.) and cultured in Eagle's basal medium supplemented with 10% fetal calf serum. For transformation assays, secondary rat embryo cells were infected at 40 plaque forming units per cell just before confluency was reached; low-calcium medium (18) was added to the cells 2 days after infection and changed twice weekly thereafter. The transformation frequency was calculated 4 weeks after infection. To obtain cell lines of transformed cells, transformed foci were picked with a pasteur pipette, trypsinized, transferred to an individual flask, and propagated in a low-calcium medium at the temperature at which each was originally transformed. Transformed cell lines were cultured for 6 to 11 passages before the viral DNA content was measured.

Extraction of DNA. DNA from transformed rat embryo cells was extracted by the procedure described by Gallimore *et al.* (16), using phenol extraction, chloroform/isoamyl alcohol extraction, and alkali digestion of RNA.

^{32}P-Labeled Adenovirus DNA. KB monolayer cells were infected with wild-type 5 adenovirus in phosphate-free Eagle's basal medium supplemented with 1% calf serum; ^{32}P-labeled phosphate (New England Nuclear), 200 μCi/ml, was added 3 hr after infection. Cells were harvested 40 hr after infection at 36°, and virus was purified by using two sequential CsCl gradients (19). DNA was extracted from purified virus as previously described (20). The viral DNAs used for hybridization had specific activities of 3 to 6 $\times$ 10^6 cpm/μg.

***Hin*dIII Endonuclease Restriction of ^{32}P-Labeled Adenovirus DNA.** *Hin*dIII restriction enzyme (21) with an activity of 2000 units/ml was purchased from Bio Labs (Beverly, Mass.). Enzymatic cleavage of viral DNA was carried out in 0.06 M NaCl/7 mM $MgCl_2$/7 mM Tris·HCl (pH 7.4) for 2 hr at 37°. The restricted DNA was incubated with Pronase at 37° for 60 min, extracted with phenol, precipitated with ethanol, and then dissolved in gel buffer (22). To separate the DNA restriction fragments, the restricted ^{32}P-labeled viral DNA was subjected to electrophoresis on 1.4% agarose gels, as described by Sugden *et al.* (22).

Hybridization Conditions and Hydroxylapatite Chromatography. Hybridization and hydroxylapatite chromatography were performed as described by Gallimore *et al.* (16).

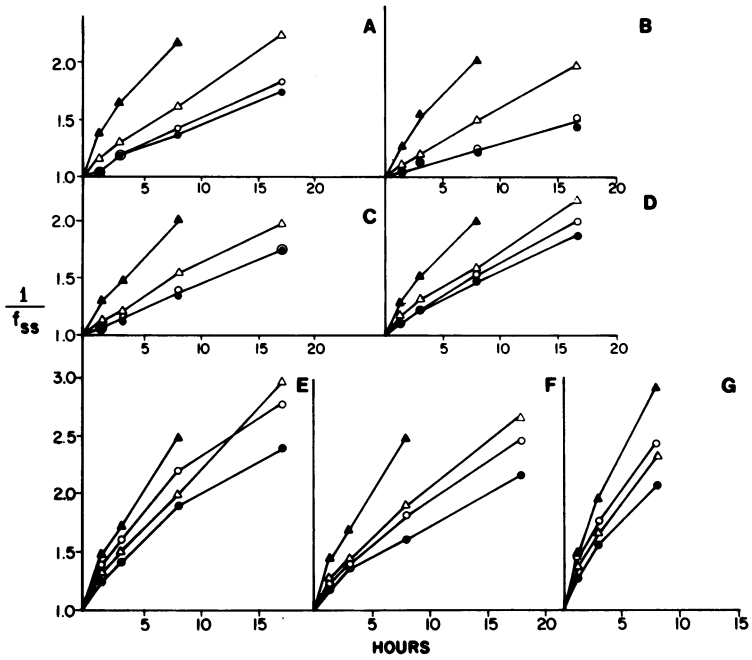

FIG. 1. Reassociation of seven (A-G) ^{32}P-labeled type 5 adenovirus DNA restriction fragments in the presence of: excess DNA from a rat embryo clone transformed by H5ts125 at 36° (Δ—Δ); excess DNA from a rat embryo clone transformed by H5 wild type at 36° (O—O); excess calf thymus DNA (●—●); excess calf thymus DNA plus 3.5 genome equivalents (1.27×10^{-2} μg) of unlabeled adenovirus type 5 DNA (▲—▲). ^{32}P-labeled adenovirus type 5 DNA (2×10^6 cpm/μg of DNA) was isolated and enzymatically cleaved with *Hin*dIII restriction endonuclease. Fragments were separated on 1.4% agarose gels. Each fragment (1250 cpm) was denatured and incubated with 600 μg of the appropriate unlabeled denatured DNA at 68° in 1 M sodium phosphate (pH 6.5)/0.2 M NaCl. At the indicated times, samples were removed and fractionated on hydroxylapatite columns to determine the fraction of the ^{32}P-labeled DNA that was single-stranded. The reciprocal of that fraction ($1/f_{ss}$) is plotted against time of incubation.

Renaturation was carried out in 1 M sodium phosphate/0.2 M NaCl (pH 6.5) at 68°.

RESULTS

To determine which portion of the adenovirus genome persisted in the various transformed cell lines, the DNA extracted from each of the cell lines was denatured and then reannealed in the presence of *Hin*dIII restriction fragments from denatured ^{32}P-labeled adenovirus DNA (16). The rates of renaturation of specific ^{32}P-labeled fragments of adenovirus DNA in the presence of excess transformed cell DNA were compared with the rates of renaturation of the ^{32}P-labeled fragments in the presence of calf thymus DNA. If the transformed cell line DNA contained adenovirus DNA sequences corresponding to a particular fragment, the rate of renaturation of this ^{32}P-labeled fragment was greater than its renaturation rate in the presence of calf thymus DNA. With each experiment, reconstruction analyses were done by using a known quantity of nonradioactive adenovirus DNA added to a mixture of calf thymus DNA and restriction fragments of ^{32}P-labeled adenovirus DNA. Seven cell lines independently transformed with H5ts125 were studied: two were transformed and propagated at 39.5° (nonpermissive temperature); four, at 36° (semipermissive temperature); and one, at 32° (permissive temperature). One cell line was studied that had been transformed by wild-type 5 adenovirus at 36°.

Fig. 1 summarizes the results of an experiment‡ comparing one H5ts125-transformed cell line with a wild-type 5 adenovirus-transformed rat embryo cell line. Both lines had been established and cultured at 36°. The data show the renaturation kinetics for each of seven *Hin*dIII restriction fragments of ^{32}P-labeled adenovirus DNA; the data for the H and I fragments are not presented because of the inaccuracy of the renaturation kinetics, which was perhaps a consequence of the small size of these fragments resulting in an unusually high ratio of ^{32}P-labeled probe to cell DNA. In the presence of the H5ts125-transformed cell DNA, the rate of renaturation of all ^{32}P-labeled adenovirus DNA fragments was increased; an increased renaturation was also noted in the presence of unlabeled adenovirus DNA. With the wild-type transformed cell DNA, however, only the restriction fragments from the two ends of the genome, G, E, and F, showed increased rates of hybridization (Table 1).

The concentration of ^{32}P-labeled adenovirus DNA in each incubation mixture was known and, from the increase in the rate of renaturation of the labeled probe, the concentration of unlabeled adenovirus DNA present (that contributed by the transformed cell DNA) was calculated. The data from Fig. 1 are expressed (experiment 1, Table 1) as the number of copies of each fragment per diploid quantity of cell DNA. The karyotypes of these cells have not been determined, however, and therefore these numbers may not be the exact numbers of copies per cell. The accuracy of these numbers must be judged in the light of the reconstruction experiment, in which the amount of cold adenovirus DNA added corresponded to 3.5 copies of adenovirus DNA per diploid quantity of cellular DNA. The data, although not quantitatively precise as to the number of viral genome equivalents present, are indicative of which portions of the adenovirus genome were present in the transformed cell lines studied. The evidence indicates that the increased potential of H5ts125 to transform cells is correlated with an enhanced integration potential of this mutant as compared with wild-type virus.

‡ The initial experiment was done through the generosity of Dr. J. Sambrook, Cold Spring Harbor Laboratory.

Table 1. Type 5 adenovirus DNA present in cells transformed with wild-type 5 or H5ts125 adenovirus at various temperatures

Exp. no.	Cell lines	Quantity of adenovirus present* *Hin*dIII restriction fragments of Ad5 DNA G	E	C	H†	D	A	B	F	I†
1	H5wt, 36°	1.9	1.8	0.14	—	0.03	0	0.02	0.73	—
	H5ts125, 36° (clone 7)	2.5	1.2	0.8	—	0.8	0.9	1.9	1.3	—
	Representative reconstruction 3.5 genome equivalents	5.1	4.6	3.5	—	3.8	3.5	5.5	4.9	—
2	H5ts125, 36°	1.1	1.6	0.9	—	0.7	0	0.23	0.23	—
	H5ts125, 36°	2.0	3.3	3.5	—	2.9	2.0	2.5	2.8	—
	H5ts125, 36°	2.6	2.5	1.9	—	1.3	1.4	1.6	2.2	—
3	H5ts125, 39.5°	2.7	1.1	0.9	—	0.7	1.2	0.5	0.8	—
	H5ts125, 39.5°	11.7	7.9	4.8	—	1.5	2.2	5.1	5.4	—
	H5ts125, 32°	2.0	1.0	0.5	—	0	0	0.4	1.1	—
*Hin*dIII map of Ad5 DNA (% of genome)		0	6	15	32	35.5	49.5	73.5	92.5	99.5 100

^{32}P-Labeled (specific activity of 3 to 6 × 10^6 cpm/μg), denatured *Hin*dIII restriction fragments of adenovirus type 5 were renatured in the presence of excess unlabeled, fragmented and denatured DNA isolated from various rat embryo cell lines which had been infected at the indicated temperature by adenovirus type 5 or H5ts125. Cells were maintained at the temperature of infection. Each experiment included a reconstruction mixture, which contained a known amount of unlabeled adenovirus DNA and unlabeled calf thymus DNA (Exp. 1: 1.27 × 10^{-2} μg of Ad5 DNA per 600 μg of calf thymus DNA). The table includes the order and approximate size of the *Hin*dIII restriction fragments of adenovirus 5 DNA (supplied by J. Sambrook and J. S. Sussenbach).

* Number of genome equivalents of each fragment per diploid quantity of cellular DNA.

† Data not presented (see *text*).

Additional H5ts125-transformed rat embryo cell lines infected and grown at 36° were studied (experiment 2, Table 1). At this semipermissive temperature, adenovirus DNA was synthesized in rat embryo cells (unpublished data), but the "125 gene" product, the DNA-binding protein (13), was present in markedly decreased quantities as measured by immunofluorescence (unpublished data). These results indicate that two of the three cell lines studied were similar to the one examined in the previous experiment in that they contained sequences representative of all the viral DNA *Hin*dIII restriction fragments tested. One of these cell lines, however, only contained approximately 50% of the viral genome corresponding to the left half of the molecule.

Experiments were next devised to investigate the temperature-dependence of the increased integration potential of the H5ts125 mutant. Of two cell lines transformed and maintained at 39.5° (experiment 3, Table 1), one clearly contained representative portions of the entire genome in larger amounts than previously observed; the second contained at least 75% of the genome, including the ts 125 gene present in restriction fragment A (J. Sambrook and T. Grodziker, personal communication), although it is unclear whether the right terminus was present. It is striking that the cell line transformed and maintained at 32° presented a pattern of viral DNA integration similar to that of wild-type virus. These data are consistent with the thesis that functioning DNA-binding protein not only decreases the frequency of viral transformation but also decreases the quantity of the viral genome that persists in the transformed cells.

DISCUSSION

In accord with the observation that H5ts125, a type 5 adenovirus temperature-sensitive mutant, transforms 3 to 8 times more rat embryo cells than does wild-type virus, H5ts125-transformed cells were found consistently to contain more of the viral genome than most lines of cells transformed with either type 2 (17) or type 5 wild-type viruses (17). Indeed, both cell lines transformed with H5ts125 and maintained at 39.5°, the nonpermissive temperature for this mutant, and three of four cell lines transformed and maintained at 36°, a semipermissive temperature, contained viral DNA representing all or most of the genome's *Hin*dIII restriction fragments. In contrast, the single H5ts125-transformed cell line tested which contained DNA consisting of no more than 18% of the left end of the genome was one that had been transformed and maintained at the permissive temperature (32°). Thus, the mutant under permissive conditions behaved like wild-type 5 virus in these experiments or similar to type 2 adenovirus as reported by Gallimore and colleagues (16, 17). It cannot be inferred from these data, however, that, in cells transformed by H5ts125 at 39.5° or 36°, the persistent DNA indicates the presence of an intact genome, although all portions of the genome are represented. Nor do the data imply whether the various portions of the genome present are integrated in tandem or are fragmented and integrated in scattered loci in the host's chromosomes. It should also be noted that infectious virus could not be detected in three of the transformed cell lines that carried the entire genome, and infectious virus was not rescued from the one cell line (clone 7) thus far tested by cell fusion.

It is striking that in wild-type 12-transformed cell lines, unlike type 2 (16) or type 5-transformed cells (17), multiple copies of the entire genome are present (23). Thus, H5ts125 appears to react like the so-called highly oncogenic adenoviruses in the transformation of rodent cells. H5ts125 cannot be compared with an oncogenic adenovirus, however, because it has not been possible to produce tumors in newborn hamsters with H5ts125—even this temperature-sensitive mutant is lethal to the animals (unpublished data).

Several possibilities, some even trivial, have been considered as explanations for the increased transforming property of the H5ts125 mutant.

1. Since rat embryo cells are semipermissive for type 5 ade-

novirus, and many cells undergo cytopathic changes after infection, the H5ts125 mutant may be less lethal than wild-type 5 adenovirus or the other mutants. Careful studies have shown this not to be the case (unpublished data).

2. The H5ts125 inoculum may contain many more defective particles than wild-type virus so that the actual amount of H5ts125 viral DNA used was much greater than the amount of wild-type viral DNA. Analyses have shown that the particle: plaque forming unit ratios of wild-type virus and H5ts125 are similar (measured by comparing protein and DNA concentrations of purified viral preparations with the same infectivities).

3. The H5ts125 virus may be an accidental double mutant in which the temperature-sensitive mutation of the DNA-binding protein gene (13), which is phenotypically expressed by inability of the mutant to replicate its DNA at the nonpermissive temperature, may be unrelated to the mutant's increased frequency of transformation. The major evidence opposing this hypothesis is the independent isolation of at least two other adenovirus temperature-sensitive mutants having the same biochemical and transformation phenotypes: H5ts107 (isolated in our laboratory); and a mutant of the $Ad2SV_{40}ND1^+$ (J. Williams, personal communication). In addition, the H12ts A DNA-minus mutant isolated by Shiroki *et al.* (10) has the same gene mutations as H5ts125 (Shimojo and Ginsberg, unpublished data); and the H12ts401 DNA mutant isolated by Ledinko (24) transforms 2 to 8 times more hamster cells than does wild-type 12 virus.

4. The high transformation frequency results from the mutant's inability to replicate its DNA at 39.5°. H5ts149 and H12ts406, however, are also DNA-minus mutants but both transform cells at a frequency similar to that of wild-type 5 and 12 viruses (11, 12, 24).

5. The adenovirus DNA-binding protein may be required for, and stringently controlled in, virus-host DNA recombination; if the mutated protein were less rigorously regulated, virus-host DNA recombination might be more frequent. This possibility is unlikely because recombination analyses done at 39.5° with H5ts125 and other type 5 temperature-sensitive mutants do not reveal unexpected recombination frequencies.

6. When mutated as in H5ts125 (13), the adenovirus-specific DNA-binding protein (25), which may naturally modulate against transformation, may permit increased viral DNA integration or antagonize excision of viral DNA, which recombines with host DNA during the early phase of viral infection (26).

These studies do not identify the presumed viral gene product required to effect and maintain transformation, nor do they suggest the mechanism by which adenoviruses transform cells. According to the last hypothesis, which cannot be discarded, the data presented imply that the virus-specific DNA-binding protein plays a governing role in transformation. Its precise action will probably not be understood until its structure and biochemical function in replication of adenovirus DNA are defined.

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