Properties of the Polyoma Viruses Induced from BHK-21 Cells Transformed by A Gene Mutants

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Polyoma viruses induced to replicate at 31°C in BHK-21 cells transformed by polyoma A gene mutants retain the thermosensitive growth characteristics and genome structure of the original mutants used for transformation.

BHK-21-C13 cells transformed by polyoma A gene mutants do not contain detectable free viral DNAs when maintained at 39.5°C (1, 2). However, these cells harbor a small number of viral genomes in their nuclear DNA; many of these viral sequences are covalently joined with repetitive cell sequences (1, 2). Shifting the transformed cells to 31°C permits autonomous replication of viral DNA. By 72 h, approximately 30 to 100 genomes per cell are produced (1, 2). It is likely that the precursors of these molecules are derived from viral sequences covalently joined with cell DNA. In this report, we describe experiments indicating that the viral genomes induced from the BHK-21 cells at 31°C exhibit the thermosensitive gene function and characteristic structure of the polyoma genome of the viruses that were used initially for transformation. This suggests that the viral gene activity required for maintenance of the transformed state is functionally separable from the A gene activity required for the initiation of autonomous rounds of viral DNA replication.

BHK-21 cell lines Tsa-1b2, Tsa-2a2, Tsa-3b2, and Ts25-17E are independent transformants isolated in soft agar after infection of BHK-21-C13 cells by polyoma virus mutants Ts-a or Ts-25 (2). In each case, after infection and an incubation period of 5 days at 31°C, the cells have been grown continuously at 39.5°C. Tsa-1b2 clones 6, 7, and 8 are subclones of Tsa-1b2, cloned in soft agar at 39.5°C (2).

A variety of procedures were used to generate stocks of infectious virus from the inducible transformed BHK-21 cell lines to not select for a particular class of viral genome. However, all procedures contain as a common feature an incubation at 31°C. As this alone is sufficient to cause induction of virus (1), the procedures may not differ significantly from each other. A summary of the procedures is given in Table 1.

Previous experiments have demonstrated that viruses recovered from these inducible cell

lines are inactivated by antipolyoma virus antiserum, are resistant to DNase, and have a buoyant density in CsCl similar to that of poly-

TABLE 1. Virus stocks from induced cell lines

Virus stock	Cell line	Method of induction ^a	PFU/ml (31°C/37°C) ^b
1	Ts25-17E	a	ND ^c
2	Tsa-1b2 clone 7	c	>500
3	Ts25-17E	b	>500
4	Tsa-1b2 clone 8	a	>1,000
6	Tsa-1b2	d	>500
7	Tsa-3b2	a	>1,000
8	Tsa-2a2	a	ND
9	Ts25-17E	d	>500
10	Tsa-1b2 clone 6	a	>1,000
11	Tsa-1b2	a	>1,000
12	Ts25-17E	c	>500

^a Methods: (a) Subconfluent cells were shifted to 31°C for 6 days. Media and cells were removed from the plates and subjected to three cycles of freeze-thawing. Virus titers ranged from 3×10^4 to 9×10^4 PFU/culture. In each case, stocks were prepared by applying 1 ml of lysate to 10^7 whole mouse embryo (WME) cells and incubating the cells for 1 week at 31°C. Titters in these stocks ranged from 0.7×10^7 to 6×10^7 PFU/ml. (b) Covalently closed circular DNAs purified by banding in a CsCl gradient containing ethidium bromide from a culture of induced cells were used to infect WME cells at low multiplicity at 31°C (1). A single plaque was picked, and the virus was adsorbed to WME cells at 31°C to prepare a stock. (c) Single plaques were picked from infectious-center assays (1) in which BHK cells were placed in agar over monolayers of WME cells at 31°C. The viruses in these plaques were adsorbed to WME cells, and stocks were prepared at 31°C. (d) As in (c), except the BHK cells were first mixed with WME cells in the presence of inactivated Sendai virus and allowed to attach for 24 h to plates at 31°C. Then they were trypsinized and used in an infectious-center assay.

^b Assayed upon monolayers of WME cells. The ratio of the infectivity of Ts-a and Ts-25 viruses at 31 and 37°C is greater than or equal to 1,000.

^c ND, Not determined.

oma virus (1). Viruses present in the stocks described in Table 1 hemagglutinate guinea pig erythrocytes with the same efficiency as the parental virus. The ratio of PFU at 31°C to hemagglutinating activity of the virus stocks was in all cases between 0.5×10^5 and 2×10^5 . In addition, all the viruses examined in these experiments displayed thermosensitive plaque-forming ability on whole mouse embryo cells (Table 1). None of the stocks contained viruses capable of forming plaques on monolayers of cells from Syrian hamster embryos at 31 or 37°C.

As a further test of the identity of the viruses from the induced cells, their sequence complementarity with polyoma DNA was examined. Polyoma [^{32}P]DNA and covalently closed circular [^3H]DNAs were prepared from mouse 3T6 cells infected with plaque-purified large-plaque (LP) polyoma A-3 virus or with four separate, induced virus stocks. When a fivefold excess of the covalently closed circular DNAs generated by the stocks from the induced cells was fragmented, denatured, and incubated with polyoma [^{32}P]DNA, they accelerated the apparent reassociation of the polyoma DNA such that the two DNAs reassociated simultaneously. This indicates that they share considerable, if not complete, sequence complementarity.

To obtain a more complete picture of the types of DNAs produced by infection with the stocks of viruses induced from the transformed cells, they were examined after electrophoresis through agarose gels. Each virus stock was adsorbed to 3T6 cells (at a multiplicity of 1 to 6 PFU/cell), and the ^{32}P -labeled nucleic acids recovered in the Hirt supernatants were applied to agarose gels (Fig. 1). In this electrophoretic system, supercoiled form I polyoma DNA migrated fastest, followed by linear (form III) DNA and then open circular form II DNA.

Infection of 3T6 cells by each virus stock induced the synthesis of nucleic acids with mobilities approximately those of polyoma form I and form II DNA (first [a] slot in each set of three slots in Fig. 1). Since RNase treatment leaves the pattern of bands essentially unchanged, except for slightly shifting the migration of form I DNAs (second [b] slots, Fig. 1), they are not composed of RNA. Digestion of the nucleic acids in the Hirt supernatant with endonuclease *EcoRI* (which cleaves polyoma DNA once) converted most of the DNA in the Hirt supernatant that migrates at the form I and form II positions to the size of linear polyoma DNA (form III) (third [c] slots Fig. 1).

To characterize these DNAs more fully, the nucleic acids from each Hirt supernatant were

FIG. 1. Electrophoresis through agarose gels of [^{32}P]DNA from mouse cells infected with virus stocks. Mouse 3T6 cells were grown and infected at 1 PFU/cell with polyoma virus stocks as previously described (4). When cytopathic effect became evident $^{32}\text{P}_i$ was added, and after extensive cytopathic effect developed, viral DNA was extracted by a modification of the Hirt procedure. The nucleic acids in the Hirt supernatant were treated with Pronase, deproteinized with phenol, and precipitated with ethanol (2). The precipitated nucleic acids were dissolved in a small volume of 10 mM Tris-hydrochloride, 1 mM EDTA, pH 7.5. An aliquot of each sample was subjected to electrophoresis through an agarose slab gel (a) without further treatment, (b) after digestion with 2 μg of boiled pancreatic RNase for 30 min at 37°C, and (c) after digestion with excess *EcoRI* endonuclease. The slab gels contained 1% agarose (Seakem) and were subjected to electrophoresis and autoradiography as previously described (3). Extracts from mock-infected cells contain mitochondrial DNA (which migrates more slowly than polyoma form II), but no DNAs in the size range of polyoma form I and form II.

digested with *Hpa*II endonuclease, and the digests were fractionated by electrophoresis through 4% acrylamide slab gels. *Hpa*II endonuclease cleaves most strains of wild-type polyoma virus at eight sites (9). However, slight differences in the sizes of *Hpa*II fragment 5's make one strain of virus distinguishable from others (7).

Most of the DNAs generated by the rescued virus stocks produce *Hpa*II digestion patterns characteristic of the original transforming virus (Fig. 2). Virus produced by stocks 1, 3, 9, and 12 (which are derived from Ts-25-transformed cells) exhibit *Hpa*II fragments identical in size to the wild-type virus to which they are related, LP A-3. However, viruses produced by stocks 2, 4, 6, 7, 8, 10, and 11 are derived from

cells transformed by Ts-a and contain an *Hpa*II fragment 5 slightly larger than that of wild-type LP A-3. This difference is characteristic of the DNA of Ts-a virus, which is related to wild-type polyoma LP A-2 (7).

Infection of mouse 3T6 cells by some of the virus stocks caused the synthesis of discrete DNAs of lower molecular weight than polyoma DNA, most notably stocks 4, 6, and 7 (Fig. 1). Digestion of these mixtures of DNA with *Hpa*II produced some fragments that were not characteristic of mature polyoma DNA. (The unusual fragments in the digest of the stock 6 DNAs are apparent in Fig. 2. Unusual fragments in digests of stock 4 and stock 7 DNAs are less apparent, but are easily seen in the original autoradiogram.) Several of these DNAs were characterized more fully with additional restriction enzymes. They appear to contain deletions and rearrangements of sequences similar to those previously reported in DNAs of polyoma virus that have been passaged through mouse cells (3, 4, 7, 8, 10). The significance of these variant genomes is unclear, as they might have arisen during the passages through mouse cells that were required to produce virus stocks.

In summary, the viruses induced from the transformed BHK-21 cells were indistinguishable from the viruses used for transformation. The persistence of the transformed phenotype (growth in soft agar) in these polyoma A gene-transformed cells at a temperature in which initiation of rounds of viral DNA replication is blocked (in mouse cells) (5) and at which there is no detectable autonomous viral DNA replication (2, 5, 6) indicates that the activity required to maintain the transformed phenotype is functionally separable from the A gene product activity that is required for the initiation of autonomous viral DNA replication (5, 6). It is possible that this separation is real and due to distinct functions. However, as these cells were selected for their ability to grow in agar at 39.5°C, the possibility that their transformed phenotype may not be solely a function of viral gene expression cannot be excluded.

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FIG. 2. Fractionation of endonuclease *Hpa*II digestion products of [32 P]DNAs from induced virus stocks. Phenol-extracted Hirt DNAs (as in Fig. 1) were digested with excess endonuclease *Hpa*II at 37°C for 6 h and layered on 4% acrylamide gels. Electrophoresis and autoradiography were carried out as previously described (3). Polyoma viruses (such as Ts-25) related to wild-type LPA-3 have a slightly smaller fragment 5 than those such as Ts-a, which are related to LP A-2 (7).

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