Virus Deoxyribonucleic Acid Sequences in Subdiploid and Subtetraploid Revertants of Polyoma-Transformed Cells

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Polyoma-transformed cells can revert in the properties characteristic of transformation, although they maintain the polyoma-specific T antigen. Transformed cells contain the same number of copies of polyoma virus deoxyribonucleic acid (DNA) per cell (eight) as revertants with a subdiploid or a subtetraploid chromosome number. The results indicate that the duplication of chromosomes in the subtetraploid revertants did not include the chromosomes that carry the viral genome. The virus DNA in both transformed and revertant cells was associated with high-molecular-weight cell DNA. Reversion of the properties of transformed cells was, therefore, not associated either with a decrease in number of virus DNA copies per cell or with a lack of association of the virus DNA with cell DNA.

One of the major problems in understanding the mechanism of carcinogenesis is to determine the genetic basis of the difference between normal and malignant transformed cells. This difference results in the ability of transformed cells to form tumors *in vivo* and to multiply *in vitro* under conditions which inhibit the multiplication of normal cells. Once these transformed properties have been hereditarily expressed, they can again be hereditarily suppressed. This has been shown, for cells transformed by either viral or nonviral carcinogens can produce variants with a reversion of the properties of transformed cells (7, 8, 12-16). In cells transformed by polyoma virus or simian virus 40 (SV40), this reversion was not associated with the loss of the viral genome. The revertants from polyoma-transformed cells synthesize polyoma-specific T antigen, and virus-specific ribonucleic acid (RNA) (13). The chromosome composition of the revertants differs from that of the transformed cells. Reversion can be associated either with a decrease in chromosome number to subdiploidy or with an increase in chromosome number to subtetraploidy (7, 8, 14).

The present experiments were undertaken to determine whether viral deoxyribonucleic acid (DNA) is associated with high-molecular-weight DNA in the revertants, as in the transformed cells, and to determine the number of viral DNA copies in subdiploid and subtetraploid revertants. For the detection of virus DNA, *in vitro* synthesized

³H-polyoma complementary RNA (cRNA) was hybridized in solution with DNA isolated from transformed and revertant cells. The technique used ensured the detection of at least one viral DNA molecule per cell.

MATERIALS AND METHODS

Cells and cell cultures. The hamster embryo cells transformed by polyoma virus (H-polyoma) or after treatment with the chemical carcinogen dimethylnitrosamine (H-DMNA), and revertants of polyoma-transformed cells (variants 1 and 18-21) were cultured as described (7, 14, 16). The cells were used 4 days after subculture. There was no detectable mycoplasma contamination by testing the cultures on mycoplasma agar, according to the method of Chanock et al. (3).

Isolation of labeled and unlabeled polyoma DNA I. Labeled polyoma DNA was obtained by the following procedure. Cultures of confluent mouse kidney in 100-mm petri dishes were infected with a multiplicity of 5 to 10 plaque-forming units of polyoma per cell, and incubated in phosphate-free Eagle medium, with a fourfold concentration of amino acids and vitamins, and 2% dialyzed calf serum. Twenty-four hours after infection, each culture was labeled with 1 mCi of carrier-free ³²P (96 Ci/mg of P; The Radiochemical Centre, Amersham). At 48 to 72 hr after infection, the cells were harvested and polyoma DNA was selectively extracted (6). The extracts were treated with Pronase (0.5 mg/ml) for 3 hr at 37 C, followed by phenol-chloroform-isoamyl-alcohol extraction. The nucleic acids were precipitated overnight with 2 volumes of 95% ethanol at -20 C. The precipitate was collected by centrifugation, dissolved in 0.2 ml of 0.01 × SSC

(SSC is 0.15 M NaCl-0.015 M sodium citrate) and layered onto a 5 to 20% alkaline sucrose gradient containing 0.5 M NaCl and 1 mM ethylenediaminetetraacetic acid, pH 12.0. The gradients were centrifuged for 90 min at 45,000 rev/min at 20°C in a Spinco SW50.1 rotor. The quickly sedimenting peak of supercoiled DNA was pooled, neutralized with 2 M NaH₂PO₄, and dialyzed against 0.01 × SSC, pH 7.2, and stored at -20°C. This DNA had a specific activity of at least 7.5×10^6 counts per min per μ g.

Production and purification of unlabeled polyoma virus by equilibrium centrifugation in cesium chloride density gradient was carried out as described (18). Virus DNA I was extracted from purified virus by a procedure identical to that for labeled DNA.

Cellular DNA. DNA from whole cells propagated in tissue culture was extracted by the method of Marmur (10) with the following modification. Cell lysates were treated with 0.5 mg of Pronase per ml in the presence of 0.5% sodium dodecyl sulfate (the Pronase was preincubated for 4 hr at 37°C). The ratio of optical density at 260 to 280 nm was 1.90 or greater.

Synthesis of viral complementary RNA. *Escherichia coli* RNA polymerase was purified by the procedure of Chamberlin and Berg (2). Radioactive RNA complementary to polyoma DNA component I was synthesized and purified as described by Levine et al. (9). After precipitation in ethanol, the RNA was dissolved in 2 × SSC and passed through a Sephadex G-100 column, pre-equilibrated with 2 × SSC. The specific activity of the RNA (calculated from the specific activity of the precursor nucleotides) was 2×10^7 counts per min per μ g.

Alkaline sucrose gradient of whole cells. High-molecular-weight DNA was purified according to Sambrook et al. (17). The gradients were fractionated from the bottom of the tubes, as modified by Manor (*to be published*).

DNA-RNA hybridization. Hybridization was carried out according to Levine et al. (9) with the following modifications: ³²P-labeled control hamster cell DNA (from H-DMNA cells), with a specific activity of 5.2×10^5 counts per min per μ g, was added to each cellular DNA to be tested for the presence of polyoma virus genomes. This labeled DNA was used to calculate the exact amount of cellular DNA recovered on each filter after the hybridization. ³²P-polyoma DNA I was added in each reconstruction experiment to correct for the specific loss of complete hybrids (polyoma DNA I-³H-cRNA) from the filters (M. Haas, M. Vogt, and R. Dulbecco, *in press*).

DNA was denatured by boiling in 0.01 × SSC for 15 min followed by rapid cooling. For hybridization, the mixture containing ³H-polyoma cRNA was brought to a final concentration of 2 × SSC in a volume of 0.25 ml and was incubated at 65°C for 20 hr. After hybridization, the mixture was treated in a solution with 20 μ g of pancreatic ribonuclease per ml and 10 units of T₁ ribonuclease per ml for 1 hr at room temperature. Each sample was then passed through a Sephadex G-100 column pre-equilibrated with 2 × SSC. Fractions (2 ml) were collected and assayed for optical density at 260 nm. The fractions of the peak

corresponding to RNA-DNA hybrids were pooled, adjusted to 6 × SSC, and filtered slowly through nitrocellulose membranes (presoaked in 6 × SSC for 10 min). The filters were then washed with 150 ml of 3 × SSC at 60°C under suction filtration, dried, and counted. Under these conditions, the recovery of cellular DNA was more than 90%.

RESULTS

Reconstruction experiments. The number of viral copies per cell can be determined under nonsaturation conditions. In this case, as under conditions of saturation, ³H-polyoma cRNA counts bound to the filters after the hybridization depends only on the concentration of polyoma DNA sequences in each sample of cellular DNA. In order to use nonsaturation conditions, it was necessary to make appropriate reconstruction curves for each ³H-polyoma cRNA input.

Two reconstruction curves obtained by using mixtures of 100 μ g of nonpolyoma-transformed hamster cell DNA (H-DMNA) with known amounts of viral DNA are shown in Fig. 1. Specifically hybridized counts are those fixed by the DNA-containing filters after subtraction of counts fixed by H-DMNA-containing filters, and correcting for the loss of ³²P-polyoma DNA I from the filters.

Polyoma DNA equivalents in DNA extracted from polyoma-transformed cells and from subdiploid and subtetraploid revertants. The chromosome numbers of polyoma-transformed cells, and one subdiploid and two subtetraploid variants with a

FIG. 1. Hybridization between ³H-polyoma cRNA and increasing amounts of polyoma virus DNA I. Increasing amounts of polyoma DNA I were added to 100 μ g of H-DMNA DNA and hybridized with ³H-polyoma cRNA, as described in Materials and Methods. Broken line, ³H-polyoma cRNA at an input of 60,000 counts/min. Solid line, ³H-polyoma cRNA at an input of 30,000 counts/min. H-DMNA, hamster cells transformed after treatment with dimethylnitrosamine.

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 FIG. 3. Hybridization between polyoma-transformed cell DNA and increasing amounts of ^3H -polyoma cRNA. A 50- μg amount of polyoma-transformed cell DNA was hybridized with the indicated amounts of ^3H -polyoma cRNA. Each point represents the mean of two replicate filters.

reversion of transformed properties derived from these transformed cells, are shown in Fig. 2. The transformed cells had modal chromosome numbers of 44 and 45, the modal chromosome numbers of variant 1 was 39–42, and the modal chromosome numbers of variants 18 and 19 were 73–76.

100 μg of DNA from the variants and the transformed cells were hybridized with ^3H -polyoma cRNA. The results are shown in Table 1. The average number of polyoma DNA equivalents detected in the transformed line per 10^{-5} μg of cell DNA is 8, whereas in the revertant cells 8 equivalents per 10^{-5} μg of cell DNA were detected in variants 1, and 4 equivalents per 10^{-5} μg of cell DNA were detected in variants 18 and 19. It should be noted that the subtetraploid variants contain about twice as much DNA per cell compared to the transformed cells and subdiploid variants (Table 2). The viral DNA equivalents per cell (eight) was, therefore, the same in the subdiploid and subtetraploid variants as in the transformed cells (Table 1).

In order to obtain an estimate of the number of viral DNA equivalents in the polyoma-transformed line nearer saturation conditions, experiments were performed using 50 μg of polyoma-transformed cell DNA, instead of 100 μg used for the experiments in Table 1. About 0.1 μg of cRNA almost saturated all the available polyoma DNA

sites in the polyoma-transformed cell DNA (Fig. 3). About 10^{-4} μg of cRNA was bound to the DNA filters, corresponding to about 4 viral equivalents per cell. Since a plateau was not reached in these experiments and since the specific activity of the cRNA can only be estimated from the specific activity of the precursor nucleotides, the actual number of viral copies in these cells remains underestimated in the near saturation conditions. By plotting the reciprocal of the amount of hybrid counts against the reciprocal of the cRNA input counts, the saturation level can be calculated by extrapolation of the linear curve (11). This calculation gave about 4,000 counts/min as the saturation level. Taking 2×10^7 counts per min per μg as the specific activity of the cRNA, this level corresponds to about 8 viral equivalents per cell. This calculated result agrees with the results of the experiments performed at nonsaturation conditions.

Association of polyoma DNA sequences with high-molecular-weight cell DNA. DNA was extracted by the Hirt procedure (6) from polyoma-transformed cells and subdiploid variants 20 and 21, which have similar modal chromosome numbers as variant 1. The DNA was isolated and purified from the supernatant fluid and the pellet and was hybridized with ^3H -polyoma cRNA. Viral DNA sequences were detected in both fractions

TABLE 1. *Polyoma virus DNA equivalents in polyoma-transformed and variant cells^a*

Expt. no.	DNA	³ H-cRNA input	Counts per min bound per 100 µg of cell DNA	Specific counts per min bound per 100 µg of cell DNA ^b	Viral DNA equivalents per 10 ⁻⁵ µg of cell DNA	Viral DNA equivalents per cell ^c
1	H-DMNA	174,000	288	0		
	H-polyoma	174,000	1980	1,692	8	8
	Variant 18	174,000	1050	762	4	8
	Variant 19	174,000	1110	822	4	8
	H-DMNA + 4 × 10 ⁻⁴ µg of virus DNA	174,000	1998	1,710	8	
2	H-DMNA	170,000	300	0		
	H-polyoma	170,000	1720	1,420	7	7
	Variant 1	170,000	1860	1,560	8	8
	Variant 18	170,000	1170	870	4	8
	Variant 19	170,000	1080	780	4	8
	H-DMNA + 2 × 10 ⁻⁴ µg of virus DNA	170,000	1100	800	4	
3	H-DMNA	48,000	125	0		
	H-polyoma	48,000	639	514	8	8
	Variant 1	48,000	708	583	9	9
	Variant 19	48,000	410	285	4	8
	H-DMNA + 2 × 10 ⁻⁴ µg of virus DNA	48,000	385	260	4	
4	H-DMNA	30,000	108	0		
	H-polyoma	30,000	388	280	7	7
	Variant 1	30,000	380	272	7	7
	Variant 19	30,000	263	155	4	8
	H-DMNA + 2 × 10 ⁻⁴ µg of polyoma DNA	30,000	270	162	4	
	H-DMNA + 10 ⁻³ µg of virus DNA	30,000	884	776	20	

^a ³H-polyoma cRNA was hybridized with the DNA from DMNA and polyoma-transformed cells, and from variants 1, 18 and 19. The hybridized counts have been normalized to 100 µg of DNA.

^b Counts per minute after subtracting the background counts per minute bound to filters containing equal amounts of H-DMNA DNA.

^c Calculated from the DNA content per cell (Table 2).

TABLE 2. *DNA content per cell in variants and transformed cells^a*

Cell type	DNA (µg per cell × 10 ⁻⁵)
H-normal.....	1.22 ± 0.19
H-polyoma.....	1.18 ± 0.12
Variant 1.....	1.15 ± 0.10
Variant 18.....	2.14 ± 0.16
Variant 19.....	2.29 ± 0.11

^a Growing cells were washed with phosphate-buffered saline (PBS) and resuspended with the same buffer. The number of cells was determined in a hemocytometer. The cells were then lysed by adding sodium dodecyl sulfate (SDS) to a final concentration of 0.5%. The DNA content in the lysate was determined by the Burton reaction (1). A solution of 0.5% SDS in PBS was used as the control, and calf thymus DNA was used as the standard. All determinations were made at least in duplicate.

FIG. 2. Chromosome numbers in polyoma-transformed cells and variants 1, 18, and 19. For the chromosome analysis, 4 µg of colchicine per ml was added to exponentially growing cells for 2 hr. After trypsinization, the cells were centrifuged, suspended in a 1% sodium citrate solution for 20 min at 37 C, fixed in a solution containing 1 part glacial acetic acid and 3 parts absolute ethyl alcohol, air-dried, and stained with Giemsa. At least 50 cells were counted for the chromosome numbers.

TABLE 3. Hybridization between ^3H -polyoma cRNA and high-molecular-weight DNA or DNA extracted by the Hirt procedure^a

Cell type	Hirt pellet		Hirt supernatant		High-molecular-weight DNA ^b
	Counts per min hybridized ^c	Percent of total hybridizable counts	Counts per min hybridized ^c	Percent of total hybridizable counts	
Variant 20	424	3	254	97	246
Variant 21	455	3	246	97	296
H-polyoma	478	2	202	98	254
H-DMNA	76	4	76	96	78

^a ^3H -polyoma cRNA was hybridized with the indicated DNA. The cRNA input was 6.2×10^4 counts/min.

^b The hybridized counts have been normalized to 20 μg of DNA.

^c The hybridized counts have been normalized to 50 μg of DNA.

(Table 3). Since the DNA in the Hirt pellet represents 95 to 97% of the total cellular DNA, there were 40 to 100 times more viral-specific sequences in the pellet. The supernatant DNA had a sedimentation, in alkaline sucrose gradient, which corresponded to component II, and which presumably consisted of randomly broken DNA fragmented during the isolation procedure. This suggests that there was no detectable free virus DNA in either the transformed or the variant cells.

In order to provide direct evidence for the alkali-stable association of polyoma DNA to cell DNA, high-molecular-weight DNA was isolated from the transformed and variant cells in an alkaline sucrose gradient as described in Materials and Methods. Viral DNA was detected in the high-molecular-weight region of the gradient (Table 3). Reconstruction experiments using mixtures of cells and polyoma DNA under these conditions showed efficient separation between the virus and cell DNA (Fig. 4). Similar alkali-stable association has been shown previously in some SV40-transformed cell lines (5, 17).

DISCUSSION

The average number of viral DNA equivalents in polyoma-transformed cells and in subdiploid and subtetraploid revertants has been estimated by hybridization with synthetic polyoma ^3H -cRNA. We have utilized two approaches to quantitate the number of viral DNA equivalents. The first approach was based upon reconstruction experiments using nonsaturation conditions, where there are less complete hybrids and thus less loss from the filter (Haas et al., *in press*). To

confirm the results obtained under nonsaturation conditions, a second approach was used. This was based upon calculation of the number of viral DNA equivalents from the saturation level and the calculated specific activity of the ^3H -cRNA. Since both approaches produced similar results, we chose to work under nonsaturation conditions.

The comparison of the polyoma-transformed cells and subdiploid and subtetraploid revertants have shown that both transformed and revertant cells have an average of 8 viral equivalents per cell. The duplication of chromosomes in the subtetraploid variants was, therefore, not associated with the duplication of the chromosomes that carry the viral genome. The chromosomes that carry the viral genome may either not have been duplicated when the other chromosomes were duplicated, or may have been specifically lost after duplication of all the chromosomes. A more detailed study of the chromosomes of the subtetraploid and subdiploid variants may make it possible to identify the chromosomes that carry the viral genome.

The present results of 8 equivalents of polyoma virus in the cells studied can be compared to reports of 2 to 9 viral equivalents in some SV40-transformed cells detected by DNA-RNA hybridization (9), and 1 to 3 viral equivalents in some SV40-transformed cells detected by measuring nucleic acid reassociation kinetics (4).

FIG. 4. Velocity sedimentation of ^{32}P -polyoma DNA and ^3H -labeled cell DNA in alkaline sucrose gradient. A mixture of ^{32}P -polyoma DNA (1.2×10^6 counts per min per μg) and 3×10^6 cells were carefully mixed with 2 ml of 0.5 M NaOH containing 0.09 M ethylenediaminetetraacetic acid (EDTA) on top of a 15 to 30% alkaline sucrose gradient, containing 0.3 M NaOH, 0.01 M EDTA, and 0.5 M NaCl. The gradients were stored at 4°C for 16 hr and then centrifuged for 3 hr at 25,000 rev/min in a Spinco SW25 rotor at 4°C . Fractions were collected from the bottom of the tubes, precipitated with 5% trichloroacetic acid and collected on fiber-glass filters (Whatman GF/C) for counting.

Our data also show that the virus DNA in both polyoma-transformed and revertant cells was associated with high-molecular-weight cell DNA, and that there was no detectable free virus DNA in these cells. These results, therefore, indicate that the reversion of properties of transformed cells was not associated either with a decrease in the number of virus DNA equivalents per cell nor with a lack of association of the virus DNA with the cell DNA.

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LITERATURE CITED

1. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-323.
2. Chamberlin, M., and P. Berg. 1962. Deoxyribonucleic acid directed synthesis of ribonucleic acid by an enzyme from *Escherichia coli*. *Proc. Nat. Acad. Sci. U.S.A.* **48**:81-94.
3. Chanock, R. M., L. Hayflick, and M. R. Barile. 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as PPLO. *Proc. Nat. Acad. Sci. U.S.A.* **48**:41-49.
4. Gelb, L. D., D. E. Kohne, and M. A. Martin. 1971. Quantitation of simian virus 40 sequences in African green monkey, mouse and virus-transformed cell genomes. *J. Mol. Biol.* **57**:129-145.
5. Hirai, K., J. Lehman, and V. Defendi. 1971. Integration of simian virus 40 deoxyribonucleic acid into the deoxyribonucleic acid of primary infected Chinese hamster cells. *J. Virol.* **8**:708-715.
6. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
7. Hitotsumachi, S., Z. Rabinowitz, and L. Sachs. 1971. Chromosomal control of reversion in transformed cells. *Nature (London)* **231**:511-514.
8. Hitotsumachi, S., Z. Rabinowitz, and L. Sachs. 1972. Chromosomal control of chemical carcinogenesis. *Int. J. Cancer.* **9**:305-315.
9. Levine, A. S., M. N. Oxman, P. H. Henry, M. J. Levin, G. T. Diamandopoulos, and J. F. Enders. 1970. Virus-specific deoxyribonucleic acid in simian virus 40-exposed hamster cells: correlation with S and T antigens. *J. Virol.* **6**:199-207.
10. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208-218.
11. Perry, R. P., P. R. Srinivasan, and D. E. Kelley. 1964. Hybridization of rapidly labelled nuclear ribonucleic acids. *Science* **145**:504-507.
12. Pollack, R., H. Green, and G. Todaro. 1968. Growth control in cultured cells: Selection of sublines with increased sensitivity to contact inhibition and decrease tumor-producing ability. *Proc. Nat. Acad. Sci. U.S.A.* **60**:126-133.
13. Rabinowitz, Z., and L. Sachs. 1968. Reversion of properties in cells transformed by polyoma virus. *Nature (London)* **220**:1203-1206.
14. Rabinowitz, Z., and L. Sachs. 1970a. Control of the reversion of properties in transformed cells. *Nature (London)* **225**:136-139.
15. Rabinowitz, Z., and L. Sachs. 1970b. The formation of variants with a reversion of properties of transformed cells. IV. Loss of detectable polyoma transplantation antigen. *Virology* **40**:193-198.
16. Rabinowitz, Z., and L. Sachs. 1970c. The formation of variants with a reversion of properties of transformed cells. V. Reversion to a limited life-span. *Int. J. Cancer* **6**:388-398.
17. Sambrook, T., H. Westphal, P. R. Srinivasan, and R. Dulbecco. 1968. The integrated state of viral DNA in SV40 transformed cells. *Proc. Nat. Acad. Sci. U.S.A.* **60**:1288-1295.
18. Winocour, E. 1963. Purification of polyoma virus. *Virology* **19**:158-168.