THE INTEGRATED STATE OF VIRAL DNA IN SV40-TRANSFORMED CELLS*

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It has been shown by a modified DNA-RNA hybridization technique that the nuclei of cells transformed by either polyoma virus or SV40 contain viral DNA.[1] This method employs RNA synthesized *in vitro* with form I viral DNA[2-4] and *E. coli* RNA polymerase. The number of viral DNA equivalents per cell varies from 5 to 60, depending on the cell line. In this communication, we will report on the physical state of the viral DNA in SV3T3 cells, an SV40-transformed cell line that contains 20 SV40 DNA equivalents per cell.

Materials and Methods.—Viruses: The strains of polyoma virus and SV40 used and the methods of virus purification have already been published.[5-8]

Cells: The origins and the methods of cultivation of cell lines SV3T3-47 and Py3T3-6 have been described.[1]

DNA extraction: DNA was obtained from purified polyoma virus or SV40 by two extractions with phenol saturated with 0.5 *M* tris(hydroxymethyl)aminomethane (Tris), pH 7.8, and one with chloroform-isoamylalcohol (24:1). Forms I and II of the viral DNA were purified by sedimentation through 5–20% neutral sucrose gradients[9] containing 0.01 *M* Tris, pH 8.0, 0.001 *M* ethylenediaminetetraacetate (EDTA), and $^1/_{10}$ solution of 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7 (SSC/10). The gradients were centrifuged for 18 hr at 23,000 rpm in a Spinco SW 25.1 rotor at 20°.

DNA was prepared from isolated nuclei[10] by the method of Marmur,[11] except that the ribonuclease treatment was omitted.

High-molecular-weight DNA was isolated as follows: 2 ml of 0.5 *M* NaOH containing 0.1 *M* EDTA was layered on the top of a 54-ml alkaline sucrose gradient (10–30% sucrose in 0.3 *N* NaOH, 0.01 *M* EDTA, and 0.5 *M* NaCl) in polyallomer tubes. Low-optical-density sucrose (Harshaw Chemical Co., Cleveland, Ohio) was used in these experiments because sucrose from other sources caused high backgrounds in the hybridization experiments. From 2 to 4 $\times$ 10^6 cells in 0.3 ml of NaCl, 0.8%; KCl, 0.038%; Na_2HPO_4, 0.01%; Sigma 7–9, 0.3% (TD) were carefully layered into the alkaline layer on the top of the gradient. The gradients were stored at 4° for at least 12 hr to allow complete liberation of the DNA and then centrifuged in a Spinco SW 25.2 rotor at 25,000 rpm for 5 hr at 4°. The high viscosity of the DNA tends to create considerable turbulence in the tube during collection. To avoid this difficulty, fractions were collected from the top by pumping 40% sucrose into the bottom of the tube. The tube contents flowed through a nick in the side of the tube and were guided by a glass rod. This type of alkaline sucrose gradient has been used previously to isolate high-molecular-weight DNA from *E. coli*[12] and mammalian cells.[13]

DNA was also isolated from nuclei by the method of Hirt.[14] Freshly prepared nuclei from 7 $\times$ 10^7 cells were distributed in a thin film on the wall of a polyethylene tube. Twenty ml of 0.01 *M* Tris, pH 7.4, containing 0.01 *M* EDTA and 0.6% SDS were slowly added. After 15 min at room temperature, the cell lysate was adjusted to 1 *M* by the addition of 5 *M* NaCl. During the addition and subsequent mixing, care was taken to minimize the amount of shear of the DNA. After storage at 4° for 16 hr, the mixture was centrifuged at 35,000 $\times$ *g* in an angle head at 4°. The supernatant was carefully poured off; the pellet was washed with 5 ml of 0.01 *M* Tris, pH 7.4, and 0.01 *M* EDTA; and the washings were combined with the supernatant. DNA was extracted separately from the supernatant and the pellet by repeated extractions with chloroform-isoamyl-

alcohol mixture until no protein could be observed at the interphase, followed by dialysis against SSC/100 containing 0.001 *M* EDTA.

Chromosomes: Monolayers of cells were treated with colcemid (0.5 μg per ml) in calcium-free medium for 18 hr. The cells arrested in metaphase (about 40%) were selectively detached from the glass by shaking. Chromosomes were isolated by the method of Maio and Schildkraut.[15] DNA was extracted from the purified chromosomes by the method of Marmur.[11]

Equilibrium centrifugation was performed two ways: (1) with ethidium bromide[9, 16] in CsCl gradients in a Spinco 50 angle head rotor at 43,000 rpm for 48 hr at 20°; fractions were collected by puncturing the bottom of the tubes with a 13-gauge needle, and ethidium bromide was removed by passing the fractions through a column of Dowex-50 (Na form); (2) with alkaline CsCl gradients in a Spinco SW 50L rotor at 30,000 rpm for 60 hr at 20°; the DNA-CsCl solution was adjusted to pH 13 with 1 *N* NaOH, and the initial density of the solution was 1.74 gm/ml.

Labeling of virus and cells: P^{32}-labeled polyoma virus and SV40 were prepared by infecting cells in Eagle's medium containing low phosphate (10^{-5} *M*) and 10% dialyzed bovine fetal serum. Twenty-four hours after infection, carrier-free P^{32} as orthophosphate (100 μc/ml) was added. Five days later when the cytopathic effect was maximal, the virus was harvested, purified, and the DNA was immediately extracted. This DNA had a specific activity equal to or greater than 5×10^5 cpm per μg.

Cellular DNA was labeled by incubating exponentially growing cells for 8 hr in medium containing 0.2 μc of 2-C^{14}-thymidine per ml.

Cellular protein was labeled by incubating exponentially growing cells for 8 hr in Eagle's medium lacking leucine and lysine, supplemented with 10% dialyzed bovine fetal serum, and containing 1 μc each of H^3-lysine and H^3-leucine per ml.

DNA-RNA hybridizations: The method of preparation of H^3 complementary RNA (cRNA) has already been published, as have the details of the modified hybridization technique used in this laboratory.[1] The amount of DNA on each filter was determined after hybridization and counting by Burton's method.[17]

Radioactivity: Samples were precipitated with 5% trichloracetic acid onto Whatman GF/C paper and were counted in a Beckman scintillation counter using a toluene-based scintillation mixture.

Chemicals: Ethidium bromide was purchased from Boots Pure Drug Co., Nottingham England. Colcemid was a product of Ciba Pharmaceutical Co., New Jersey. Thymidine-2-C^{14} (52.8 mc/mmole) was obtained from Schwarz BioResearch, Inc., Orangeburg, New Jersey. L-leucine-4,5-H^3 (4.6 mc/μmole) and DL-lysine-4,5-H^3 (5 mc/μmole) were purchased from New England Nuclear Corp., Boston, Massachusetts.

Results.—(1) *Evidence that transformed cells do not contain supercoiled forms of viral DNA:* Centrifugation to equilibrium in neutral CsCl containing ethidium bromide has been shown to resolve unnicked circular DNA from the main mass of linear cell DNA.[9] The cyclic DNA binds less dye and bands at a higher density.[16] This method was used to find out whether transformed cells contain any form I viral DNA. The separation obtained when P^{32}-labeled SV40 DNA was mixed with H^3-thymidine-labeled cell DNA and centrifuged to equilibrium in CsCl-ethidium bromide is shown in Figure 1. All the unnicked circular viral form I is separated from the cellular DNA; viral form II behaves as linear DNA in these gradients and remains with the cell DNA. DNA's from 3T3 cells transformed by SV40 (i.e., SV3T3) and 3T3 cells transformed by polyoma virus (i.e., Py3T3) were centrifuged in a similar way. Then the gradients were divided into two fractions: one containing the cell DNA, and the other the rest of the heavy side of the gradient. Both fractions were first dialyzed against 0.001 *M* Tris, pH 8.0, 0.001 *M* EDTA, SSC/100 to remove CsCl and then

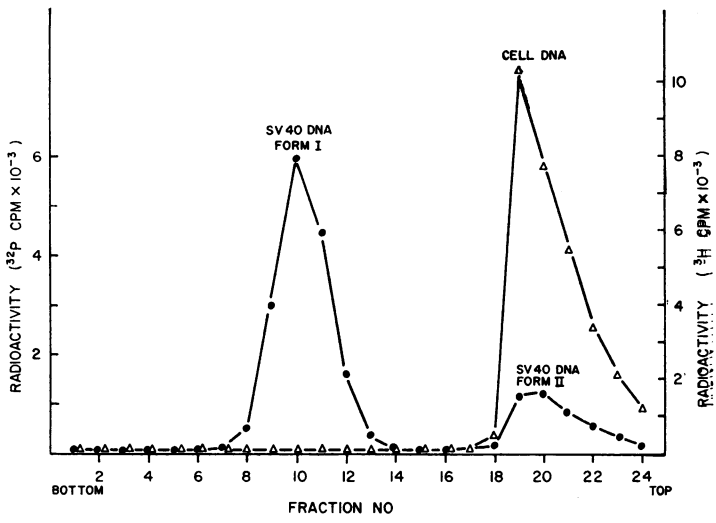

FIG. 1.—Equilibrium centrifugation of a mixture of 0.2 μg purified SV40 P^{32}-DNA (I and II) and 100 μg cell H^{3}-DNA in 5.00 ml CsCl, density 1.57 gm/ml, 100 μg/ml ethidium bromide for 36 hr at 43 krpm, 20°C in a Spinco 50 angle head. Fractions were collected and the DNA was precipitated and counted as described.

passed through Dowex-50 to remove ethidium bromide. The DNA was heat-denatured and then immobilized on Millipore filters and hybridized with H^{3}-cRNA prepared *in vitro* by using SV40 form I DNA as template. The results are shown in Table 1 where the counts hybridized with cellular DNA are in column *C* and those hybridized with the denser part of the gradient are in column *V*. There is no difference in counts in column *V* between SV3T3 and Py3T3 cells. If SV3T3 cells had contained free SV40 viral DNA form I, it would have been detected as shown by a reconstruction experiment whose results are given in line 3. In this experiment, SV40 DNA containing 80 per cent of form I and 20 per cent of form II was mixed with Py3T3 DNA and centrifuged. The results indicate that form I was entirely recovered in the *V* fraction and form II in the *C* fraction.

A highly significant difference between SV3T3 and Py3T3 cells is present in column *C*. All the virus-specific hybridizable counts are found in the band containing the cellular DNA. The question arises whether this result could be

TABLE 1. *Hybridization of DNA from transformed cells after centrifugation in CsCl/ethidium bromide gradients.*

Cell type	Fraction from Gradient (cpm) *V*	*C*
SV3T3	69 ± 5	358 ± 2
Py3T3	64 ± 7	129 ± 12
Py3T3 + 20 SV40 genomes per cell	385 ± 11	198 ± 5

H^{3}-RNA complementary to SV40 (form I) DNA was hybridized with the indicated fractions. The amount of DNA remaining on the filters after hybridization was determined and the figures in column *C* are the hybrid cpm normalized to 50 μg DNA. The figures in column *V* are uncorrected hybrid cpm. The H^{3}-cRNA input per filter was 1.2 × 10^{6} cpm in this experiment.

produced by selective loss of form I DNA or its conversion to form II during the extraction of DNA. This possibility was excluded by experiments in which labeled viral DNA was added to the nuclei before extraction of DNA. Under these conditions, there was no loss of viral form I DNA. We therefore conclude that there is no substantial amount of form I DNA in the transformed cells.

(2) *Evidence that transformed cells do not contain free viral DNA of a size similar to that present in virions:* The Hirt method of DNA extraction when applied to cells lytically infected with polyoma virus separates viral DNA irrespective of its form (i.e., I or II) from cell DNA.[14] The efficiency of the method was tested by adding P^{32}-labeled SV40 DNA to freshly isolated nuclei and extracting the mixture by the Hirt procedure. From 90 to 95 per cent of the P^{32} counts were found in the supernatant fraction. Less than 2 per cent of the cellular DNA is present in the supernatant if care is taken to minimize shear.

Nuclei from transformed cells were extracted by this method. DNA was isolated from the supernatant and pellet fractions, denatured, immobilized on Millipore filters, and hybridized. The results are shown in Table 2. There is

TABLE 2. *Hybridization of DNA extracted from transformed cells by Hirt's method.*

Cell type	Fraction	
	Pellet	Supernatant
SV3T3	694 ± 31	143 ± 20
Py3T3	287 ± 23	114 ± 20
Py3T3 + 140 SV40 genomes per cell	670 ± 18	3550 ± 360

H^3-RNA complementary to SV40 (form I) DNA was hybridized with the indicated DNA. The amount of DNA remaining on the filters after hybridization was determined, and the hybrid cpm given in the column under "Pellet" have been normalized to 50 μg DNA. The hybrid cpm for the supernatant fractions are uncorrected values. The H^3-cRNA input in this experiment was 1.7×10^5 cpm/filter.

no significant difference between the SV3T3 cells and the control, Py3T3, cells in the number of counts hybridized to DNA isolated from the supernatant fractions. However, marked differences are found in the hybridization to DNA obtained from the pellet fractions. Reconstruction experiments in which 140 SV40 DNA molecules per cell were added before extraction show that 90 per cent of the specifically hybridizable counts can be removed in the supernatant fraction. The viral DNA in transformed cells therefore is not in a free form of size comparable to that of DNA present in virions.

(3) *Evidence that the viral DNA is integrated in transformed cells:* (a) *Hybridizable material in isolated chromosomes:* Chromosomes were isolated from SV3T3 cells, the DNA was extracted, heat-denatured, immobilized on Millipore filters, and hybridized with SV40-cRNA. Control filters contained SV3T3 DNA isolated from cells that remained attached to the glass after the metaphase cells had been harvested, and also DNA's extracted from nuclei of Py3T3 and SV3T3 interphase cells. The results are presented in Table 3. The DNA isolated from chromosomes hybridized to the same extent as DNA from SV3T3 interphase cells and to a significantly greater extent than DNA from Py3T3 interphase cells. Nuclear contamination of the chromosome preparation could account for no more than 10 per cent of the DNA in the chromosome DNA preparation. The SV40 DNA in SV3T3 cells is therefore associated with chromosomes during the metaphase.

TABLE 3. *Hybridization of DNA extracted from chromosomes and nuclei.*

Source of DNA	Cpm hybridized
SV3T3 chromosomes	680 ± 63
SV3T3 nuclei*	690 ± 87
Py3T3 nuclei	290 ± 32
SV3T3 nuclei	740 ± 47

H^3-RNA complementary to SV40 (form I) DNA was hybridized with the indicated DNA. The amount of DNA remaining on the filters after hybridization was determined and the hybrid cpm have been normalized to 50 μg DNA. The H^3-cRNA input in this experiment was 2.1×10^5 cpm/filter.

* DNA extracted from nuclei of SV3T3 cells treated with colcemid.

(b) *Hybridizable material in high-molecular-weight DNA:* High-molecular-weight DNA was isolated by alkali treatment of both SV3T3 and Py3T3 cells at the top of an alkaline sucrose gradient followed by centrifugation. The denatured DNA had a sedimentation coefficient of about 110*S* (Fig. 2). The cellular DNA was well separated from viral forms I and II. Moreover, it was free of protein as evidenced by the absence of counts in the cellular DNA peak when the cells were labeled with a mixture of H^3-leucine and H^3-lysine (Fig. 3). Under these conditions, linear DNA of molecular weight of 6×10^7 equivalent to 20 viral genomes will have a sedimentation coefficient of about 50*S*.[18] The high-molecular-weight DNA obtained from the gradients was neutralized with 1.1 *N* HCl containing 0.2 *M* Tris, dialyzed against large volumes of SSC/100 containing 0.001 *M* EDTA, concentrated, redialyzed against the same buffer, adjusted to 6 × SSC, immobilized on Millipore filters, and hybridized against SV40 cRNA. The results are given in Table 4. The high-molecular-weight DNA isolated from SV3T3 cells hybridizes to the same extent as DNA isolated from the nuclei of SV3T3 cells. However, the counts hybridized are markedly higher than those

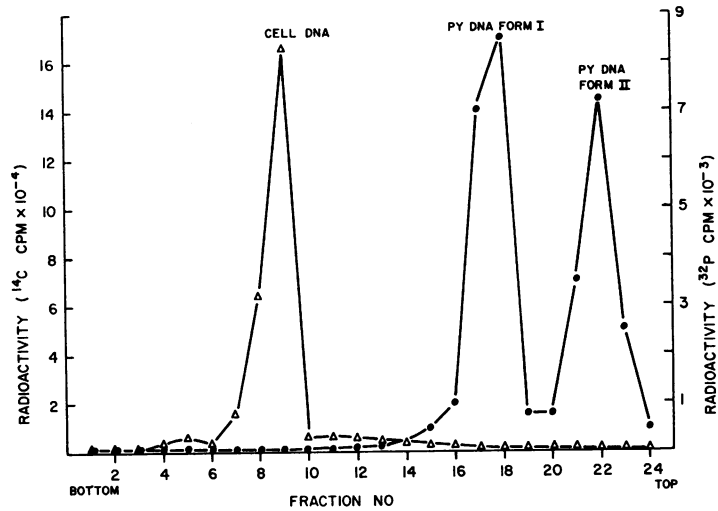

FIG. 2.—Velocity sedimentation of a mixture of polyoma P^{32}-DNA, I and II, and cell C^{14}-DNA in alkaline sucrose gradients. For details, see *Materials and Methods*.

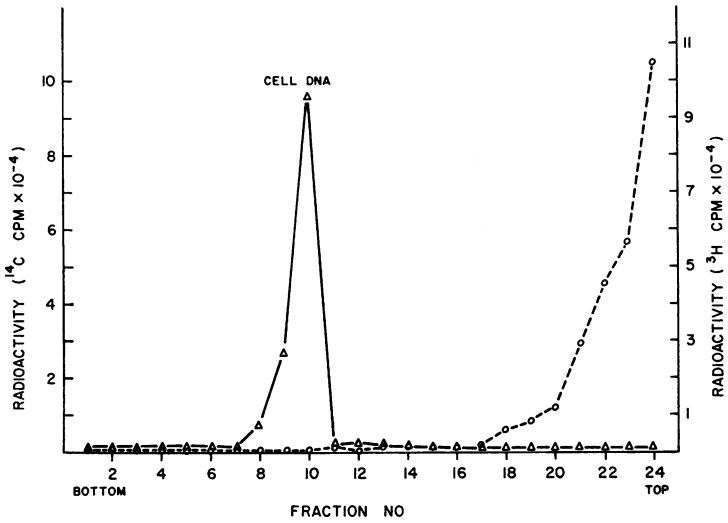

FIG. 3.—Velocity sedimentation of lysed cells labeled with C^{14}-thymidine and H^3-leucine and H^3-lysine. For details, see *Materials and Methods*.

obtained with either high-molecular-weight Py3T3 DNA or Py3T3 DNA isolated from nuclei. The hybridizable counts found with SV3T3 are comparable to the increase seen when 20 genomes of SV40 per cell are added either to high-molecular-weight Py3T3 DNA or to Py3T3 DNA isolated from nuclei.

In a further experiment, the high-molecular-weight DNA obtained from alkaline sucrose gradients was neutralized, dialyzed, concentrated, and centrifuged to equilibrium in alkaline CsCl gradients. At the end of the run, fractions were collected from the gradients, the refractive index of each fraction was measured, and the density calculated. The fractions of density 1.74–1.81 gm/ml were pooled, neutralized, and dialyzed. The DNA from these fractions was hybridized in the normal manner. The results are given in Table 4, experiment 2. SV3T3 DNA again hybridizes to a greater extent than Py3T3 DNA, and there is little difference in cpm hybridized between DNA's obtained directly from alkaline sucrose gradients and DNA's that were subsequently centrifuged to equilibrium in alkaline CsCl.

Discussion.—In these experiments, DNA's isolated by a variety of methods from SV3T3 and Py3T3 cells have been hybridized with RNA made *in vitro* with SV40 DNA as template. In every case, it was possible to isolate a DNA fraction from the SV3T3 cells that hybridized to a greater extent than the comparable DNA fraction from the control cell line, Py3T3. This increased hybridization is equivalent to 20 SV40 genomes per cell.

The results of the ethidium bromide experiments eliminate the possibility that viral DNA in SV3T3 cells is in the form of supercoiled circles of any size; and the fact that no hybridizable counts were present in the supernatant of the Hirt extraction eliminates the possibility that the viral DNA is in a free form of any configuration of molecular weight comparable to the DNA of the virion.

TABLE 4. *Comparison of hybridization of DNA obtained from transformed cells by alkaline sucrose gradients and by Marmur's method.*

	Source of DNA	Cpm hybridized
Expt. 1:	Alkaline sucrose gradients	
	SV3T3	354 ± 22
	Py3T3	144 ± 16
	Py3T3 + 20 genomes of SV40/cell	394 ± 36
	Nuclei extracted by Marmur's method	
	SV3T3	342 ± 15
	Py3T3	186 ± 13
	Py3T3 + 20 genomes of SV40/cell	386 ± 22
Expt. 2:	Alkaline sucrose gradients	
	SV3T3	593 ± 30
	Py3T3	210 ± 14
	Py3T3 + 25 genomes of SV40/cell	780 ± 30
	Alkaline sucrose gradients, followed by equilibrium centrifugation in alkaline CsCl	
	SV3T3	620 ± 12
	Py3T3	248 ± 2

H^3-RNA complementary to SV40 (form I) DNA was hybridized with the indicated DNA. The amount of DNA remaining on the filters after hybridization was determined, and the hybrid cpm normalized to 50 μg DNA. The H^3-cRNA input per filter was 1×10^5 cpm in expt. 1 and 1.5×10^5 cpm in expt. 2.

There are two further possible states of the viral DNA: (1) free form II molecules of a size larger than normal viral DNA, derived by tandem duplication; (2) covalently bound to cellular DNA or bound to other structural components of the nuclei.

The maximum size of a molecule described in the first possibility would depend on the average number of viral DNA equivalents per cell and on their distribution between individual cells. In the case of SV3T3 cells, there are about 20 viral genome equivalents per cell, and three lines of evidence suggest that they are uniformly distributed throughout the cell population. (*a*) The SV3T3 cells used in this study (SV3T3-47) are a clonal line; (*b*) all SV3T3-47 cells show a similar amount of T antigen in their nuclei as judged by immunofluorescence; and (*c*) the average number of SV40 DNA equivalents per cell does not differ in populations of cells grown under very different physiological conditions. So it is unlikely that any SV3T3-47 cell contains more than 60×10^6 daltons of viral DNA. If this DNA were present as a single molecule, it would have a sedimentation coefficient of about 50*S* in alkali,[18] and no hybridization would have been detected in the 110*S* DNA peak collected from the alkaline sucrose gradients.

Clearly the data support the alternative hypothesis that the SV40 DNA is attached to a larger molecule. It is unlikely that this is a structural component of the cell since the high-molecular-weight-DNA fraction that hybridizes with RNA complementary to SV40 has the same density as denatured DNA in alkaline CsCl (i.e., 1.78 gm/ml).

Thus the results presented here provide evidence that the viral DNA molecules in SV3T3 cells are integrated with cell DNA by alkali-stable covalent linkages. The viral DNA may be linked to the cellular DNA as one large piece at a single

site, or it may be connected at multiple sites after many individual insertions. This problem is currently under investigation.

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