State and Organization of Polyoma Virus DNA Sequences in Transformed Rat Cell Lines

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Polyoma virus-transformed rat cell lines were isolated as colonies growing in agar after infection of F2408 cells with low multiplicities of wild-type virus. Viral DNA present in the transformed cells was analyzed by fractionating the cellular DNA on agarose gels before and after digestion with various restriction endonucleases, followed by detection of the DNA fragments containing viral sequences using the procedure described by Southern (E. Southern, *J. Mol. Biol.*, **98**: 503-515, 1975). Five lines, independently derived, were studied in detail. All five lines, when examined after a minimum number of passages in culture, contained both free and apparently integrated viral DNA. The free polyoma DNA in three of the lines was indistinguishable, by restriction enzyme analysis, from wild-type viral DNA, whereas the two other lines also contained smaller free DNA molecules which lacked parts of the wild-type genome. The integrated DNA in the five lines studied existed as head-to-tail tandem repeats of unit-length polyoma DNA covalently attached to nonviral DNA. The same five polyoma-transformed rat lines were examined after further passages in culture. Free viral DNA was then either undetectable or greatly reduced in amounts, whereas the high-molecular-weight, integrated units persisted after passage of the cells. Ten subclones, derived from one of the five lines selected for detailed analysis, showed some variations in the quantity and size of the free viral DNA as well as minor alterations in the pattern of the apparently integrated sequences.

Infection of certain cells, typically of rat or hamster origin, with either simian virus 40 (SV40) or polyoma virus (Py) can result in the oncogenic transformation of a fraction of the cell population (see 61 for a review). Genetic evidence has suggested that at least one viral gene product is necessary for the establishment of transformation (12, 13, 16, 35, 44, 60) and that in some cases it is also required for the maintenance of the transformed phenotype (6, 35, 44, 47, 56, 60).

The interaction of mouse Py with rat cells is rather complex. Rat cells are semipermissive for Py, as indicated by the synthesis of capsid antigen in a very small proportion of the cells after infection at high multiplicity (50). Transformed rat cell lines which spontaneously produce small amounts of virus have been described (14, 64), although these are exceptional. In nearly every line, fusion with permissive mouse cells induces virus production (36, 50).

Populations of Py-transformed rat cells com-

monly contain many (10 to 50) copies of viral DNA per diploid equivalent of cellular DNA. The majority of this viral DNA exists in a non-integrated state, although integrated viral DNA is also present (42, 64). In situ hybridization experiments have demonstrated that most, if not all, of the nonintegrated Py DNA occurs in a minor fraction of the cells, presumably as the result of spontaneous induction of viral DNA replication (46, 64).

On the other hand, rodent cells transformed by the monkey virus SV40 have shown a simpler situation. Rodent cells, except when physically injected with massive quantities of viral DNA (19, 20), are nonpermissive for SV40 replication. SV40-transformed rat cells do not produce virus unless they are fused with permissive monkey cells. All detectable viral DNA (usually between 1 and 10 genome equivalents per diploid quantity of cell DNA) is covalently integrated into high-molecular-weight host DNA (5, 34, 54). SV40-transformed semipermissive human cells, however, may have properties more similar to Py-transformed rat cells (M. Botchan, J. MacDougall, and J. Sambrook, personal communication).

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It has recently become feasible to study the molecular organization of the viral DNA sequences in transformed cells. Ketner and Kelly (34) and Botchan et al. (5) have used the Southern (59) blotting procedure to examine the integration sites and sequence arrangements of SV40 DNA in transformed mouse and rat cells. The technique used involves digestion of small quantities of transformed cell DNA (ca. 10 μ g) with various restriction endonucleases, fractionation of the resulting fragments by agarose gel electrophoresis, transfer of the DNA onto a sheet of nitrocellulose, and the subsequent autoradiographic detection of the bands containing viral sequences by annealing to highly radioactive viral nucleic acid. Less than one copy of viral DNA per diploid equivalent of cellular DNA can be detected, and restriction enzyme mapped in this way. The conclusion common to these studies was that integration of SV40 DNA was not specific with respect to either the cellular or the viral genome. The general impression obtained was of considerable complexity. Several of the rat lines studied had multiple independent insertions of SV40 DNA into cellular DNA, making detailed characterization difficult. Nevertheless, it was possible to determine restriction maps of the integrated genomes in the simpler lines (5, 33).

We report herein the results of experiments using the Southern (59) procedure to examine the state of viral DNA in a series of Py-transformed rat cell lines selected by growth in soft agar. The F2408 line of Fisher rat fibroblasts (15), used in some of the studies discussed above (5, 50, 64), was chosen for transformation. An important motivation for this project was to determine whether the known differences between SV40- and Py-transformed rat cells reflected a different organization of the integrated viral DNA which might result from the relative permissivity of the recipient cell for the transforming virus.

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MATERIALS AND METHODS

Cells and virus. F2408 rat cells (15) were maintained in Dulbecco-modified Eagle medium supplemented with 5% fetal calf serum, as were the transformed derivatives of this line. Py large plaque A2 (24) was grown in whole mouse embryo (WME) cells, using plaque-purified virus, and was assayed to demonstrate the absence of defective viral DNA as described (30).

Transformation assay and isolation of the transformed cell lines. A monolayer of F2408 cells was infected with Py at a multiplicity of 1 PFU/cell. Adsorption was for 2 h at 37°C; unadsorbed virus was

then removed and replaced by Dulbecco-modified Eagle medium containing 5% fetal calf serum. After a further 2 h at 37°C, the cells were trypsinized, and 5×10^4 cells per 50-mm dish were plated in soft agar (40). Three weeks later, macroscopically visible colonies were picked, one from each of ten dishes. Transformation frequency was 0.14%, in agreement with reported values (50); mock-infected cells plated in agar in the same conditions did not form colonies. The colonies were transferred to Limbro dishes in Dulbecco-modified Eagle medium containing 5% fetal calf serum plus 2×10^5 hemagglutinating units of anti-Py antiserum (a gift from P. Nicklin) per ml. The growing colonies were trypsinized (this was counted as passage no. 1) and passaged in the same medium containing antiserum. The transformed cell lines were maintained in culture by replating at a dilution of 1/20 or 1/40 each Monday and Friday with an intermediate medium change each Wednesday. We refer to each replating as a "passage."

Plaque assay and virus rescue. All virus titrations were done on secondary WME cells in the presence of dexamethasone (45). Spontaneous virus production by the transformed cell lines was checked by assaying either sample of the culture medium or freeze-thawed cells by plaque formation onto WME cells.

Transformed lines were also checked for their ability to produce infectious virus upon Sendai virus-mediated fusion with WME cells. A total of 5×10^5 cells were fused with 2×10^6 WME cells. After 5 days in culture, the cells with the medium were harvested, freeze-thawed three times, and assayed by plaque formation onto WME cells.

Characterization of the transformed lines. Five of the 10 lines thus isolated were chosen for a detailed study. They showed strong nuclear immunofluorescence when stained with anti-Py T serum; this serum was a gift of Y. Ito (28). None of the lines contained any V-antigen-positive cells detectable by immunofluorescence.

Spontaneous virus induction and rescue after fusion with permissive WME cells were studied at both an early time in culture (passage 7 for line B5, passage 5 for the other four lines) and a later time (passage 18 for line B4, passage 31 for lines B6, C6, and D5, and passage 34 for B5). At the early passage numbers, all the lines were tested for spontaneous virus release by assaying samples of the culture medium for plaque formation onto WME cells; three of them (B5, B6, and D5) released low but measurable amounts of virus (2 to 40 PFU/ 10^6 cells) in one particular experiment. When freeze-thawed cells were used instead of medium (lines B5, B6, C6, and D5), the titers obtained were somewhat higher (4 to 130 PFU/ 10^6 cells).

Virus from lines B5, B6, C6, and D5 was rescuable after fusion with WME cells at an early passage and from the late passages of lines B4, B5, B6, and D5. Titers of rescued virus ranged from 7.5×10^6 to 2.5×10^7 PFU/ 10^6 cells when the early passages were tested and from 50 to 10^4 PFU/ 10^6 cells in the case of the late passages. All the lines grew in agar, though the efficiencies of cloning were different, ranging from 8.5% (line B4) to 30% (line B6) when 10^3 transformed cells were plated per 60-mm dish.

Preparation of viral DNA. Form I Py DNA was purified from infected 3T6 cells (24) by selective extraction (27) followed by equilibrium centrifugation in CsCl containing ethidium bromide (9) and velocity sedimentation through neutral sucrose gradients. DNA to be used for in vitro labeling by nick translation was further purified by agarose gel electrophoresis (39). Adenovirus 2 (Ad2) DNA was the gift of L. Crawford, and bacteriophage λ DNA was the gift of I. Molineux.

Isolation of high-molecular-weight cellular DNA. Cellular DNA was isolated by a slight modification of the procedure described by Gross-Bellard et al. (25). DNA concentrations were measured by mithramycin fluorescence (26). Yields of DNA were in the ranges of 15 to 20 and 50 to 100 μg /confluent 90-mm tissue culture dish of normal and transformed cells, respectively. The size of the DNA in each preparation was monitored by electrophoresis in 0.8% agarose gels; the DNA did not enter such gels, demonstrating that its molecular weight was several times larger than that of the 35-kilobase Ad2 DNA (21) included as a size marker.

In vitro labeling of Py DNA. Highly purified form I Py DNA (see above) was labeled in vitro by the nick translation reaction (31) used for the preparation of hybridization probes (41, 51), with some modifications (5, 41). In our further modification, a typical reaction (400 μl) contained 20 μmol of Tris-hydrochloride (pH 7.5), 2 μmol of MgCl_2 , 4 μmol of 2-mercaptoethanol, 20 μg of bovine serum albumin (Armour, crystallized), 100 μCi each of the four α - ^{32}P -labeled deoxyribonucleoside triphosphates (150 to 300 Ci/mmol; Radiochemical Centre), 4.2 μg of Py DNA, and 14.7 U of *Escherichia coli* DNA polymerase I (Boehringer grade I, 2,500 to 5,000 U/mg). Incubation at 12 to 14°C was continued for 120 to 150 min, until 35 to 40% of the radioactivity was incorporated into DNA. The labeled DNA (specific activity, 6×10^7 to 7×10^7 cpm/ μg) was stored at -20°C in 50% ethanol containing 0.25 M NaCl and 80 μg of yeast RNA carrier per ml. Results presented elsewhere (4) have shown that the probe DNA prepared in this way was representative of the entire genome.

Agarose gel electrophoresis and transfer of the DNA to nitrocellulose. A horizontal slab gel apparatus (4), containing gels 15 by 20 by 0.5 cm, was used throughout. Trial experiments showed that the buffer described by Shinnick et al. (58), A buffer (0.16 M Tris-acetate, 80 mM NaCl, 80 mM EDTA, pH 8.0), afforded the best resolution of large fragments at high local DNA concentration. Unless otherwise specified, 1% agarose (Seakem) gels were used, and electrophoresis was for 18 h at 0.5 V/cm at room temperature (standard conditions).

The DNA in the gel was transferred onto sheets of nitrocellulose (Schleicher & Schuell, BA 85) with minor modifications of the procedure described by Southern (59).

DNA annealing and detection of the hybrids by autoradiography or fluorography. Before annealing the blots were incubated for 4 h at 68°C in hybridization buffer (6 $\times$ SSC containing 0.02% bovine serum albumin [Schwarz/Mann], 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) (11). Each blot was in-

serted into a plastic bag (4), the heat-denatured ^{32}P -labeled Py DNA (5×10^6 cpm/blot) dissolved in 3 ml of hybridization buffer containing 0.5% sodium dodecyl sulfate and 50 to 100 μg of heat-denatured calf thymus DNA per ml was added, and the bag was sealed. Annealing was for 60 to 65 hours at 68°C in a shaking water bath. Blots were washed extensively at 68°C with 2 $\times$ SSC containing 0.5% sodium dodecyl sulfate and rinsed in 2 $\times$ SSC. They were dried and exposed for autoradiography or fluorography. Kodak Kodirex film was used for direct autoradiography, and pre-flashed Fuji RX or Kodak RP-Royal films combined with Ilford Fast Tunstate or Fuji Mach II intensifying screens were used for fluorography at -70°C (37).

RESULTS

The Southern blotting procedure (59) is the basis of the method we have used to examine the Py DNA present in transformed rat cells. Reconstruction experiments were done initially to estimate the sensitivity and background level of the procedure described in Materials and Methods. A fixed quantity (10 μg) of high-molecular-weight untransformed F2408 rat cell DNA was mixed with various quantities (from 10^{-5} to 10^{-6} μg) of form I Py DNA and digested with different restriction endonucleases. *EcoRI* and *BamI* make one cut in Py DNA (23, 24), *HhaI* makes three cuts (22), and *HpaII* makes eight cuts (24), whereas the restriction endonuclease *HpaI* does not cut Py DNA (24) (Fig. 1). The products of the digestions were fractionated by electrophoresis in an agarose slab gel, the DNA in the gel was transferred to a sheet of

FIG. 1. Physical map of Py DNA. In the inner circle are indicated the positions of cleavage by the restriction endonucleases *EcoRI* (24) and *BamI* (23). The middle and outer circles show the positions of the *HhaI* (22) and *HpaII* (24) sites, respectively.

nitrocellulose, and the resulting blot was annealed with *in vitro* labeled ^{32}P -Py DNA.

This reconstruction experiment (data not shown) demonstrated that (i) there was no detectable annealing between the labeled DNA probe and the untransformed rat cell DNA, (ii) the autoradiographic intensities of the bands increased with increasing inputs of viral DNA, and (iii) DNA fragments as small as 6 to 7% of the viral genome were detected at a viral-to-cellular DNA ratio of 10^{-6} , equivalent to about one polyoma genome per diploid equivalent of rat DNA.

Detection of viral sequences integrated into cellular DNA. The transformation of F2408 rat cells with wild-type Py (A2 strain) and the characterization of the cell lines thus obtained are described in Materials and Methods. Five lines, designated B4, B5, B6, C6, and D5, were analyzed; DNA was prepared from these lines at both "early" (passages 5–7) and "late" (passages 18–34) times during the maintenance of the lines in culture.

The strategy used to characterize the state of the viral DNA sequences in these lines involved initial comparison of the sizes of the DNA molecules containing viral sequences before and after digestion with restriction endonucleases which do not cut Py DNA. The molecular weight of nonintegrated viral DNA would be unaltered by digestion with such enzymes, whereas the sizes of cellular DNA fragments containing integrated viral sequences would be drastically reduced, generating one specific fragment for each insertion of Py DNA between two appropriate restriction sites in the cellular DNA. The results of such an experiment, using restriction endonuclease *HpaI*, are shown in Fig. 2. Ethidium bromide staining of the gel before blotting (data not shown) revealed that most of the DNA in the unrestricted samples (slots 1 and 3 for each cell line) was of very high molecular weight and stayed at the top of the gel, whereas after *HpaI* digestion, a continuum of DNA fragments was found. A complex pattern of bands containing viral sequences was obtained for the four lines analyzed here (Fig. 2). We provisionally define as "free" Py DNA molecules those bands which are visible both before and after *HpaI* digestion and which have mobilities similar to or greater than that of the Py-*EcoRI* linear DNA marker. We will show later that these bands correspond to the superhelical and nicked circular forms of wild-type and defective viral DNA molecules; it should be noted, however, that such bands were far more intense in the early-passage DNA preparations for all the four lines analyzed in this experiment (slots 3 and 4 for

each line). We will first discuss the high-molecular-weight bands additionally found and return to consideration of the putative free DNA in a subsequent section.

Each of the four lines analyzed in this particular experiment contained one or more high-molecular-weight fragments specifically generated by *HpaI* digestion. Some hybridization in the region near the top of the gel slots was obtained with the unrestricted DNAs, but since it is known that very high-molecular-weight DNA transfers poorly from agarose gels to nitrocellulose (5), it was not surprising that the intensities of these bands were low. Because Py DNA lacks an *HpaI* site, the high-molecular-weight DNA fragments found only after enzyme digestion meet the criteria stated above for viral insertions into the host DNA. The protocol used in this experiment (see legend to Fig. 2), moreover, excluded the possibility that the high-molecular-weight bands specific to the restricted samples were generated by shearing of oligomeric viral DNA molecules, because the bands were absent from the unrestricted samples which had been mock-digested and processed in precisely the same manner as the digested samples.

Table 1 lists the approximate sizes of the fragments containing Py DNA sequences generated by *HpaI* digestion. Molecular weights were estimated by comparison with the mobilities of *EcoRI* fragments of Ad2 DNA run in the same gels but must be considered only as rough approximations, since nearly all of the fragments were of too high molecular weights for an accurate determination under the electrophoresis conditions used. In general, the fragment sizes differed among the five lines, but strikingly all of them were two to more than five times the size of the 5.2-kilobase viral genome. It is interesting to note that the fragments obtained from early- and late-passage DNA preparations of each line were similar but showed quantitative differences in band intensities. Line B5, in particular, showed a complex pattern of multiple high-molecular-weight fragments which could result from either multiple insertions or a heterogeneity among the cells in the population used; we shall return to this problem in a subsequent section which describes the analysis of subclones of this line.

The large size of the viral DNA-containing fragments generated by *HpaI* digestion could either reflect the distance between *HpaI* sites in the cellular DNA or result from the integration of multiple copies of viral DNA between two *HpaI* sites. The latter explanation would be consistent with the observed intensities of the

FIG. 2. Comparison of the sizes of the fragments containing Py DNA sequences in the unrestricted and *HpaI*-cleaved DNA preparations from various transformed cell lines. DNA samples were processed as follows. Duplicate samples from each preparation were either restricted with *HpaI* or mock incubated in the digestion buffer (10 mM Tris-hydrochloride, pH 7.5, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 M NaCl) for 4 h at 37°C. *HpaI*, purified as described (57), was further purified by affinity chromatography on a DNA-agarose column (55). Amounts of enzyme identical to those used for the digestion were then added to the mock-digested samples, and all the reactions were stopped by addition of sodium dodecyl sulfate (SDS) and EDTA to final concentrations of 0.5% and 1 mM, respectively. Samples were then dialyzed overnight against 100 volumes of 0.1 mM Tris-hydrochloride buffer, pH 7.9, containing 0.01 mM EDTA and 0.05% SDS before lyophilization nearly to dryness in the presence of the solution used for loading in the gel (60% [wt/vol] sucrose containing 0.5% SDS, 1 mM EDTA, and 0.5% bromophenol blue). Amounts of the 60% sucrose solution added were chosen so as to have a final concentration of 20% sucrose. Samples were loaded in an 0.6% agarose gel in A buffer; electrophoresis was for 36 h at 0.5 V/cm. *EcoRI* fragments of Ad2 DNA (48) were included in a marker track, and their positions were measured on the ethidium bromide-stained gel. The sizes (in kilobases [KB]) and the migrations of the two largest fragments are indicated. The gel was processed as described in the text; this film was exposed for 3 weeks, using fluorography. Lines B5, B6, C6, and D5 were analyzed. For each cell line, slots 1 to 4 in each set contained: slot 1, unrestricted DNA, and slot 2, *HpaI*-cleaved DNA from the late-passage preparations; slot 3, unrestricted DNA, and slot 4, *HpaI*-cleaved DNA from the early-passage preparations. Slot Py contained marker *EcoRI*-Py DNA. (The figure is a combination of two separate fluorograms obtained in the same experiment.)

HpaI fragments, which were far darker than would be expected for high-molecular-weight DNA containing a single viral genome when compared with the *EcoRI*-Py DNA marker (Fig. 2). It was thus of interest to digest the DNA preparations with *BglII* (49), a second enzyme that does not cut Py DNA (J. Arrand, personal communication). Figure 3 shows the results of this experiment.

High-molecular-weight fragments (13 to >35 kilobases) were generated by *BglII* digestion of early- and late-passage DNA preparations from all five lines examined (Fig. 3, Table 1). As noted for *HpaI* digestion, more than one fragment was obtained for most of the lines; line B5, for example, contained at least two fragments in the early preparation and at least three fragments in the late one. Lines B6 and D5 shared apparently common *BglII* fragments but differed in

the molecular weights of the fragments generated by *HpaI*. In general, the sizes of the *BglII* and *HpaI* fragments found in each line were different. It is therefore probable that the fragments obtained with the five different cell lines represent distinct sites of integration of the viral DNA into the rat genome.

As previously mentioned in the case of the *HpaI* digestion (see Fig. 2), *BglII* digests of each of the early-passage DNA preparations (Fig. 3, E slots) contained one or more bands migrating in the region of the linear *EcoRI*-Py DNA marker or farther, which we have defined as free DNA and will discuss more fully below.

Integrated viral sequences exist as tandem repeats of unit-length Py DNA. The digestion of the DNA from five different Py-transformed rat cell lines with restriction enzymes which do not cut Py DNA generated

TABLE 1. Sizes of the fragments containing viral sequences^a generated by *Hpa*I and *Bgl*II digestion of early- (E) and late- (L) passage DNA preparations of lines B4, B5, B6, C6, and D5

Enzyme	Fragment size (kilobases) ^b									
	B4-E	B4-L	B5-E	B5-L	B6-E	B6-L	C6-E	C6-L	D5-E	D5-L
<i>Hpa</i> I		22	22	22	13.5	13	>30	>30	27	27
		24	25	25	20	22.5			29	
			28	28	23.5					
				>30						
<i>Bgl</i> II	22	28	17	17	13	14	35	35	13	13
	29	>35	30	24.5	23				23	21
	>35			>30	>35				>35	>35

^a The putative free viral DNA species (see text) have not been listed here.

^b The sizes of the fragments were estimated from the migrations of marker DNA fragments (see Fig. 2 and 3). Data for line B4 were obtained in an experiment not shown here.

FIG. 3. Detection of fragments containing Py DNA sequences after *Bgl*II cleavage of the early- and late-passage DNA preparations of the five Py-transformed rat cell lines B4, B5, B6, C6, and D5. The DNA samples were digested with *Bgl*II in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 5 mM MgCl₂ and 1 mM dithiothreitol for 4 h at 37°C. The digested DNAs were concentrated by ethanol precipitation and redissolved in 30 µl of 10 mM Tris-hydrochloride buffer, pH 7.9; 20 µl of loading solution (see Fig. 2) was added, and the samples were loaded in a 0.6% agarose gel in A buffer. Electrophoresis was for 30 h at 0.5 V/cm. *Eco*RI fragments of λ DNA (1) were included in a marker channel (slot λ); their migrations, measured on the ethidium bromide-stained gel, are indicated on the fluorogram, together with their sizes in kilobases (KB). The gel was processed as described in the text; the above film was exposed for 3 weeks, using fluorography. The arrows indicate the origin; marker *Eco*RI-Py DNA was included in slot Py. Slots E and L for each cell line contained DNA from the early- and late-passage preparations, respectively. (The figure is a combination of two fluorograms obtained in the same experiment.)

DNA fragments between two and five times the length of Py DNA. Although it is possible that the integration sites in each line are remote from both *Hpa*I and *Bgl*II sites, the alternative explanation of multiple adjacent integrations seems

more likely. Experiments were therefore done to further characterize the integrated high-molecular-weight DNA by digesting with enzymes which cut Py DNA once, *Eco*RI and *Bam*I (see Fig. 1). If the viral DNA were integrated in the

various lines as an exact, head-to-tail tandem repeat of unit-length molecules, then digestion with *EcoRI* or *BamI* should generate unit-length linear molecules plus two additional "linker" fragments containing both viral and host sequences. The presence of free circular Py DNA molecules would complicate the interpretation of the results obtained with such enzymes because they too could give rise to unit-length linear DNA. Consideration of quantitative differences between early-passage DNA preparations, which contained considerable quantities of free Py DNA, and late-passage preparations, in which the amount of free DNA was drastically reduced (see below), will be important in distinguishing the contributions from free versus integrated DNA.

Figure 4 shows the hybridization patterns obtained after *EcoRI* digestion of early- and late-passage DNA preparations. In each of the five lines, the late-passage DNA preparations (Fig. 4, L slots) contained a major *EcoRI* fragment which comigrated with the unit-length Py DNA marker. The coelectrophoresis of the major B5 *EcoRI* fragment and unit-length viral DNA was confirmed in reconstruction experiments (data

not shown). Since little or no free Py DNA was detected in the late-passage DNA preparations (see Fig. 2 and 3 and below), the unit-length DNA most probably originated from the integrated DNA. In this regard, it should be noted that the major high-molecular-weight viral DNA bands seen after *HpaI* or *BglII* digestion were absent from the *EcoRI* patterns. Visual comparison of the intensities obtained after digestion of 10 μg of transformed cell DNA with those of the marker tracks in Fig. 4, which contain either one (10^{-5} μg) or five (5×10^{-5} μg) genome equivalents of viral DNA, readily demonstrated that multiple copies of Py DNA were present in late-passage preparations from lines B4, B5, and C6, whereas digestion of DNA from line B6 generated about one copy of unit-length Py DNA. These results were consistent with the sizes of the *HpaI* and *BglII* fragments obtained with the different lines; late line B6 DNA contained (Table 1) a single *BglII* fragment, approximately 2.6 times the length of Py DNA, whereas the other four lines contained fragments at least five times longer than the Py genome.

The *EcoRI* digestions of the late-passage preparations shown in Fig. 4 also contained mi-

FIG. 4. Comparison of the DNA fragments containing Py DNA sequences after *EcoRI* cleavage of the early- and late-passage DNA preparations from cell lines B4, B5, B6, C6, and D5. The DNA samples were digested with *EcoRI* (purified as described in reference 5) in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 5 mM MgCl_2 , 1 mM dithiothreitol, and 0.125 M NaCl. The samples were then processed as described in the legend to Fig. 3 and electrophoresed under standard conditions. The gel was processed as described in the text; the film was exposed for 3 days, using fluorography. Slots E and L for each cell line contained DNA from the early- and late-passage preparations, respectively. Marker *EcoRI*-Py DNA (about 5×10^{-5} and 10^{-5} μg , respectively) was included in four channels of the gel (Py slots).

nor bands which might represent viral/host linker fragments from the ends of the integrated units. Line B5 late DNA, for example (Fig. 4, slot L of B5), had two such fragments, one of higher molecular weight than the Py genome and a second one slightly smaller than 5.2 kilobases, which was obscured on the particular exposure shown here because it was very close to the dark *Eco*RI linear band; it was apparent in other experiments in which a better resolution was obtained. Line B6 late DNA had a single major putative linker fragment somewhat larger than Py DNA. In line C6, which appeared to have the largest integrated unit of the five lines examined, it was difficult to identify any putative linker fragment in this experiment. *Hpa*I and *Bgl*II, however, generated different-sized fragments containing viral sequences, which suggested that line C6 late DNA did not contain free oligomers. Furthermore, no oligomeric forms could be found in the supernatant fraction of a selective extraction (27) performed on this line (data not shown).

We have not attempted extensive studies to characterize the viral/host joins in these lines, since in most cases it would be difficult and probably not of general significance because each line has at least one continuous viral genome. Furthermore, it cannot be directly demonstrated that the minor bands are linkers and not separate insertions of viral DNA fragments.

Comparison of the intensities of the *Eco*RI unit-length linear DNA bands obtained with early and late preparations of line B6, D5, and C6 DNA (Fig. 4) revealed darker bands in the early preparations than in the late ones. We attribute this difference to the presence of free DNA species containing a single *Eco*RI site in the early-passage preparations which were absent from the late-passage preparations (see below). Line B5 early contained, as we shall describe below, a free defective genome which lacked an *Eco*RI site. The smallest fragment visible in B5 early, but absent from B5 late (Fig. 4, slots E and L for this line), is the nicked circular form of this free defective (see below).

To demonstrate directly that the unit-length DNA in the early-passage preparations originated in part from the high-molecular-weight integrated units, partial digestion experiments were attempted. Figure 5 shows the results of such an experiment, using line C6 DNA, in which it is very clear that *Eco*RI progressively cleaved the high-molecular-weight DNA to yield the unit-length linears. Similar data were obtained for B5, B6, and D5 early DNA preparations.

Digestion with *Bam*I, an enzyme which makes a single cut in Py DNA at a site distant from

FIG. 5. Analysis of the fragments containing Py DNA sequences obtained by partial *Eco*RI digestion of DNA from the early-passage of cell line C6. A 10- μ g amount of DNA from line C6 was digested with 1, 2, and 5 μ l of the *Eco*RI preparation for 2 h (slots 2, 3, and 4) and with 10 and 20 μ l of enzyme preparation for 4 h at 37°C (slots 5 and 6), respectively. Standard *Eco*RI incubation was 10 to 15 μ l of enzyme per 10 μ g of cellular DNA per 4-h incubation in the buffer described in the legend to Fig. 4. The samples were electrophoresed under standard conditions, and the gel was processed as described in the text. The film was exposed for 8 days, using fluorography. Slot 1 contained about 5×10^{-5} μ g of *Eco*RI-Py DNA.

*Eco*RI (see Fig. 1), was next used to further analyze the structure of the integrated units of Py DNA among the different lines. Figure 6 shows the results of *Bam*I digestion of late-passage DNA preparations from each of the five cell lines, as well as the patterns obtained by double digestion with *Eco*RI and *Bam*I. In every case, the fragments obtained were those expected from the digestion of either circular Py DNA or a tandemly repeated linear array of viral DNA molecules. *Eco*RI and *Bam*I digestion of the early DNA preparations of lines B5, B6, C6, and D5 in each case generated a major fragment of the same size as unit-length linear Py DNA (data not shown). Minor bands were also visible, which might be either virus/cellular DNA linker fragments or separate insertions of parts of the viral genome. The free defective species, which we shall describe more fully be-

FIG. 6. Analysis of the fragments containing Py DNA sequences produced by cleavage with *Bam*I and double digestion with *Eco*RI and *Bam*I of the late-passage DNA preparations from cell lines B4, B5, B6, C6, and D5. A 5- μ g amount of DNA from each preparation was digested with either *Bam*I (slot 1 for each cell line) or *Bam*I and *Eco*RI (slot 2 for each cell line). *Bam*I was purified according to a published procedure (63); digestions with this enzyme were performed in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM dithiothreitol. In the case of the double digestions, samples were first restricted with *Bam*I; NaCl concentration was then adjusted to 0.125 M and digestions were continued with *Eco*RI. Samples were electrophoresed under standard conditions. Film was exposed for 1 month, using fluorography.

low, were also present.

We conclude from the results obtained by digestion with *Eco*RI and *Bam*I that the high-molecular-weight integrated units of Py DNA present in each of the five cell lines must contain exact head-to-tail tandem repeats of the viral genome.

Nonintegrated viral DNA in the transformed cell lines. We previously identified as free viral DNA those bands (see Fig. 2 and 3) with mobilities similar to or greater than that of the marker linear Py DNA. In considering the band pattern expected for free Py DNA, it is important to recall the relative mobilities of superhelical, linear, and nicked circular DNAs of Py length on loose agarose gels: linear unit-length DNA (on 1% gels) migrates about 0.75 times as fast as superhelical DNA, and nicked circular DNA migrates very slightly slower than

the linears. A further complication is that all preparations of superhelical DNA usually contain variable amounts of the nicked circular form, the proportion of which can increase because of nonspecific nicking during digestion with enzymes that do not restrict the closed circular DNA. Moreover, since defective Py DNA molecules shorter than unit length are readily generated during lytic infection (17), there is no a priori reason to expect free DNA in transformed cells to exist only as wild-type-length molecules.

In our discussion of the results of the *Hpa*I digestion (see Fig. 2), we noted the appearance of viral DNA species in all of the early-passage preparations, which were unaltered by *Hpa*I digestion. The same bands were noted after *Bgl*II digestion (see Fig. 3). Since these DNA molecules resolved from the host DNA with or

without restriction and did not contain *HpaI* or *BglII* sites, they are candidates for nonintegrated forms of viral DNA. Figure 7 shows the results of fractionating early (Fig. 7a)- and late (Fig. 7b)-passage DNA preparations restricted with *HpaI* under conditions which ensure that free DNA does not run off the gels, but which do not resolve the high-molecular-weight integrated forms. Early preparations of B4, B5, B6, C6, and D5 DNA all contained variable amounts of DNA with the mobilities expected for unit-length superhelical or nicked circular Py DNA (Fig. 7a). Line B5 (slot 2) additionally contained large quantities of DNA species which had the mobilities expected for the superhelical and nicked circular forms of defective molecules shorter than unit length. A different defective species was apparent in line B6 (slot 3). The late-passage DNA preparations (Fig. 7b), by contrast, lacked free DNA and showed only the high-molecular-weight integrated DNA discussed above.

Confirmation that the DNA species tentatively identified as free molecules were not integrated into high-molecular-weight host DNA was obtained by analyzing the DNA prepared from the lines by selective extraction (27) (data not shown). Early DNA preparations had bands with the same mobilities as the free DNA observed after *HpaI* digestion but lacked the high-molecular-weight component previously identified as integrated DNA. Analysis of Hirt supernatant DNA prepared at later times in the history of the lines showed that the free DNA was either greatly reduced in amount (lines B4, C6, and D5) or absent (lines B5 and B6).

The defective DNA in line B5 has been characterized in some detail. From *EcoRI* and *BamI* digestions, and other experiments not shown here, we deduced that it had no *EcoRI* site and one *BamI* site. The molecular weight of the *BamI* linear was about 2×10^6 ; digestion of DNA from early B5 preparations with *HhaI* (Fig. 8, slot 2) yielded, in addition to the three fragments expected from the integrated DNA (see Fig. 1), a very dark band with a molecular weight of 2×10^6 . Double digestion of the DNA preparation with *EcoRI* and *HhaI* did not alter this band (Fig. 8, slot 1). These data suggested that the defective DNA had a single *HhaI* site. If the viral origin of DNA replication is maintained in this defective species, the *HhaI* site retained should be the one at 73 map units (see Fig. 1). Restriction of B5 DNA with *HpaII* (Fig. 8, slot 3) yielded, in addition to the wild-type DNA fragments, a new band of molecular weight 1.1×10^6 . Moreover, fragments *HpaII*-3 and *HpaII*-5 were present in excess. We tentatively con-

clude that the B5 defective molecule, 55% of the length of Py DNA, comprises *HpaII* fragments 5 and 3 (which include the origin of viral DNA replication) (8, 24) and further sequences probably originating from *HpaII* fragments 1 and 4. It was not possible to determine whether host sequences are included in the molecule.

Viral DNA in 10 subclones derived from line B5. Ten clones (B5-1 to B5-10) were derived from early line B5 by plating cells at high dilution in the presence of Py antiserum and selecting individual colonies growing on the plastic. DNA prepared from each clone was analyzed by restriction with *BglII* (Fig. 9), *EcoRI* (Fig. 10), and *HpaI* (data not shown). The complex patterns of high-molecular-weight integrated DNA found in both the early- and late-passage preparations of uncloned line B5 were present in each of the 10 clones, but the molecular weight of the fragments containing integrated Py DNA varied among the clones. This is most apparent in Fig. 9, which shows the pattern obtained by *BglII* digestion. It is clear that the clones are related to B5, but the relative abundance of the different high-molecular-weight fragments varied. This result implies that the multiplicity of integrated high-molecular-weight tandem repeats of viral DNA units was not caused by heterogeneity in the uncloned B5 population in a simple way. It may result from either several independent integrated units existing in each cell, which tend to segregate upon cloning, or an inherent instability in the length of the integrated unit causing continual cell-to-cell variation.

Eight of the 10 B5 clones contained large amounts of free as well as integrated viral DNA, as shown by digestion with *BglII* (Fig. 9) and *HpaI* (data not shown). Clone B5-5 had no detectable free DNA, as shown by the absence of low-molecular-weight bands and by the relatively high proportion of the 9-kilobase linker fragment apparent after *EcoRI* digestion (Fig. 10). Clone B5-3 contained very little free DNA, but a band corresponding to the nicked circular form of the free defective genome of the early B5 was just visible in Fig. 10. Among the eight clones which contained large amounts of free Py DNA, a band with the mobility of unit-length nicked circular Py DNA was present in each case, but additional bands corresponding either to the defective species present in early B5 or to new defective species (as in clone B5-2) were found. It is interesting to point out that the late-passage B5 cells apparently lacked free DNA (as did late passages of B4, B6, C6, and D5), whereas 80% of the cloned derivatives of B5 had maintained nonintegrated DNA.

FIG. 7. (a) Analysis of the free viral DNA molecules after *Hpa*I cleavage of the early-passage DNA preparations from cell lines B4, B5, B6, C6, and D5. The DNA samples were digested with *Hpa*I under the incubation conditions described in the legend to Fig. 2; they were electrophoresed under standard conditions, and the gel was processed as described in the text. The film was exposed for 15 days, using fluorography. Marker *Eco*RI-Py DNA was included in a marker channel (slot Py). Slots 1 through 5 contained DNA from cell lines B4, B5, B6, C6, and D5, respectively. (b) Analysis of the DNA fragments which contain viral sequences after *Hpa*I cleavage of the late-passage DNA preparations from cell lines B4, B5, B6, C6, and D5. A 10- μ g amount of DNA from each preparation was digested with two different *Hpa*I preparations and electrophoresed under standard conditions. The gel was processed as described in the text; the film was exposed for 1 week, using fluorography. Slots 1 and 14 contained 5×10^{-5} μ g of form I and II Py DNA, and slots 2 and 13 contained 5×10^{-5} μ g of *Eco*RI-Py DNA. Slots 3 through 12 contained DNA from cell lines D5 (slots 3 and 8), C6 (slots 4 and 9), B6 (slots 5 and 10), B5 (slots 6 and 11), and B4 (slots 7 and 12).

FIG. 8. Analysis of the defective DNA species present in the early-passage preparation of cell line B5. DNA samples, digested with *HhaI* (in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM dithiothreitol), sequentially digested with *HhaI* and *EcoRI*, or digested with *HpaII* (in the same buffer as used for *HhaI*), were loaded in a 1.4% agarose gel in A buffer. Electrophoresis was for 18 h at 0.5 V/cm. The gel was processed as described in the text. The film was exposed for 3 weeks, using fluorography. Slot 1, 10 μ g of DNA, digested with *HhaI* and *EcoRI*; slot 2, 5 μ g of DNA cleaved with *HhaI*; slot 3, 5 μ g of DNA digested with *HpaII*. The arrows indicate the positions of the extra fragments originating from the defective DNA.

DISCUSSION

We have analyzed by restriction endonuclease digestion the Py DNA sequences present in T-antigen-positive transformed rat cell lines isolated by growth in soft agar. An important motivation for this study was to determine whether the state of the papovavirus genome(s) present in these semipermissive cells differed from that found previously for SV40-transformed nonpermissive rodent cells (5, 33, 34). The SV40 genome in transformed rat cells has only been induced to replicate by fusion with permissive cells (36, 52), whereas a minority of the cells in populations of Py-transformed rat cells are normally "induced" for viral DNA replication (46, 64). It was thus not surprising that in our analysis of

five different transformed lines and 10 subclones of one of these lines, free as well as integrated Py DNA was detected. A significant new observation, however, emerged from our examination of the integrated Py DNA. In each of the five cell lines examined in detail (and probably in three out of four other less well-characterized lines isolated in the same conditions; unpublished data) the integrated DNA contains tandem repeats of full-length viral DNA comprising between two and more than five copies of the Py genome.

The correlation between the presence of tandemly repeated integrated genomes and the relatively high frequency of spontaneous induction of viral DNA replication in Py-transformed semipermissive rat cells might imply a causal relationship. Tandemly integrated sequences would be expected to be unstable, because they could readily produce free circles through homologous recombination, leading to partial excision. Alternatively, induction might readily occur without excision by multiple rounds of *in situ* replication of the integrated DNA as proposed for bacteriophage μ (7), leading to the segregation of tandemly repeated free linear DNA molecules which might then circularize by homologous recombination (53; J. Sambrook and M. Botchan, personal communication). Although our detection of tandemly repeated integrated sequences thus provides a probable molecular basis for the induction process, it is by no means proven that nonintegrated DNA persists in the transformed cell population solely by occasional spontaneous induction. It is not known whether induction is a lethal event to the cell. Free DNA could therefore originate by induction but then persist in the population as autonomously replicating "plasmids." Depending on the synchrony between viral and cellular DNA replication, such plasmids might persist for many generations or be rapidly diluted out. Free DNA was detected in our cell lines when they were analyzed at early passage numbers. After 100 to 300 generations of further growth in culture, the cell lines lost virtually all of the free DNA molecules. We cannot distinguish between the alternative hypotheses of selection against clones with high spontaneous induction frequency and that of autonomously replicating plasmids originally present eventually being lost. When subclones of line B5 were examined 25 to 30 divisions after cloning, the free DNA persisted in most but not all of the subclones. The difference between this result and that obtained simply by passaging the parental line in mass culture suggests that selective pressures exerted by the culture conditions are important in the maintenance of free DNA

FIG. 9. Analysis of some subclones of the early cell line B5 by *Bgl*II cleavage. DNA samples digested with *Bgl*II as described in the legend to Fig. 3 were electrophoresed in a 0.6% agarose gel in A buffer for 30 h at 0.5 V/cm. The migrations of marker *Eco*RI- λ DNA fragments were measured on the ethidium bromide-stained gel and are indicated on the figure, together with their sizes in kilobases (KB). The gel was processed as described in the text; the film was exposed for 3 weeks, using fluorography. *Eco*RI-Py DNA was included in a marker channel (slot Py). Slots E and L contained DNA from the early- and late-passage preparations of line B5; slots 2, 3, 4, 6, 7, 8, 9, and 10 contained DNA from clones B5-2, B5-3, B5-4, B5-6, B5-7, B5-8, B5-9, and B5-10, respectively.

molecules or in the retention of high spontaneous induction potential. The situation in line B5 is further complicated by the fact that in this particular cell line, the free DNA is a mixture of wild-type-length DNA and a defective species with a restriction map distinct from that of the bulk of the tandemly repeated wild-type-length integrated DNA found in the cells. Further experiments with line B5, designed to determine whether the original cells additionally contained an integrated form of the defective genome, which was lost upon passaging (and in the subclone lacking free defective DNA), are required to resolve the alternative hypotheses of continual induction or plasmid persistence.

The correlation between tandemly repeated, integrated Py genomes and induction of free DNA has striking parallels in other systems. Nonpermissive SV40-transformed rodent cells have not been found to contain repeats of two or more genomes, but partial tandem repeats

comprising approximately 1.5 genomes have been found (5). By contrast, the adenovirus-SV40 defective hybrid, Ad2⁺⁺HEY, which resulted from recombination between the two viruses in cells permissive to SV40, exists as an unstable mixed population of Ad2 DNA containing 0.43, 1.43, or 2.39 SV40 genomes arranged as repeats (32). Infection of monkey cells permissive to SV40 with Ad2⁺⁺HEY gives rise to infectious SV40 virions with high efficiency (38). A second defective hybrid, Ad2⁺⁺LEY, contains only either 0.03 or 1.05 SV40 genomes; this hybrid produces infectious SV40 in monkey cells with far lower efficiency than Ad2⁺⁺HEY. The difference in efficiency of SV40 DNA production has been attributed to the longer tandem repeat in Ad2⁺⁺HEY, which would have a higher probability of an intramolecular recombination event (32). A rather similar result has recently been reported for the integration of the transposon Tn9 into bacteriophage P1 in *E. coli* (10). The

FIG. 10. Analysis of the 10 subclones of the early cell line B5 by *EcoRI* digestion. DNA samples, digested with *EcoRI* as described in the legend to Fig. 4, were electrophoresed under standard conditions, and the gel was processed as described in the text. The film was exposed for 3 days, using fluorography. The position of marker *EcoRI*-Py DNA, included in the two outer channels, is indicated by the arrow. Slots E and L contained DNA from the early- and late-passage preparations of B5, respectively, and slots 10-1 contained DNA from the respective clones B5-10 to B5-1.

mouse 3T3 cells transformed by the Py *tsa* mutant characterized by M. Vogt and colleagues (3, 9, 62) may contain at least partial tandem repeats of the viral genome in an integrated form, since induction of viral DNA replication by shifting these cell lines to the permissive temperature leads to the production of oligomeric circular viral DNA molecules (9).

We can only speculate on the molecular mechanism(s) involved in the generation of integrated tandem repeats. It is possible that the viral DNA which initially integrated was an oligomer. Several groups (18, 29, 43) have indeed reported that oligomeric DNA accumulates late during the SV40 lytic cycle. Alternatively, the tandem repeats could be the result of amplification after the initial integration event. Such amplification might occur by secondary recombination between a single integrated genome and free copies of viral DNA. The demonstration that superinfection of SV40-transformed rat cells with a physically distinguishable SV40 results in independent integration of the second viral genome (M. Botchan, personal communication) argues somewhat against this hypothesis. Amplification could also occur, however, by recombination between integrated genomes. Tandem duplications commonly occur under selective pressure in bacteria and in bacteriophages (see 2 for review); the models developed to explain these

phenomena can readily be applied to the present situation. Any initial event resulting in the integration of slightly more than one copy of the viral genome in tandem could be readily amplified by subsequent legitimate but unequal recombination between the repeated portions of the viral DNA. If the initial integration event involved only unit-length DNA, amplification by legitimate recombination is more difficult to imagine. Illegitimate unequal recombination could generate tandem repeats, but in general these would not comprise exclusively viral DNA. It is possible that the tandem arrays studied here in fact contain very small insertions of host DNA between the viral genomes; such a proposition entails testable predictions about the structure of circular DNA molecules recoverable from the cell lines by induction or rescue. However, under special assumptions it would be possible to amplify a single integrated viral genome to an exact, exclusively viral tandem repeat. If one proposes that the initial recombinational event leading to integration is a legitimate exchange between homologous sequences in the viral and host genomes, then a subsequent unequal legitimate crossover of the same type between the virus/host juncture preceding and following the integrated Py genome would produce one daughter chromosome containing an exact viral duplication. This duplication could

then be amplified by further legitimate unequal recombination.

The frequency with which Py-transformed rat cell lines containing tandem repeats are isolated by growth in soft agar suggests that some selective pressure is involved. An obvious explanation is gene dosage. Seif and Cuzin (56) have demonstrated that rat cells transformed with Py temperature-sensitive *a* gene mutants are not temperature sensitive for the maintenance of the transformed state if soft-agar selection is used to isolate the lines. By contrast, similar transformed rat cell lines isolated by focus formation on plastic surfaces exhibit a temperature-sensitive transformed phenotype. It will be interesting to determine whether the apparent difference in the role of the *a* gene product in the maintenance of the transformed state is the result of major variations in the level of *a* gene expression caused by alternative arrangements of the integrated viral genomes.

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LITERATURE CITED

- Allet, B., P. G. N. Jeppesen, K. J. Katagini, and H. Delius. 1973. Mapping the DNA fragments produced by cleavage of λ DNA with endonuclease RI. *Nature (London)* **241**:120-122.
- Anderson, R. P., and J. R. Roth. 1977. Tandem genetic duplications in phages and bacteria. *Annu. Rev. Microbiol.* **31**:473-505.
- Bacheler, L. T. 1977. Virus-specific transcription in 3T3 cells transformed by the ts-a mutant of polyoma virus. *J. Virol.* **22**:54-64.
- Birg, F., J. Favaloro, and R. Kamen. 1977. Analysis of polyoma virus nuclear RNA by miniblot hybridization. *Proc. Natl. Acad. Sci. U.S.A.* **74**:3138-3142.
- Botchan, M., W. Topp, and J. Sambrook. 1976. The arrangement of SV40 sequences in the DNA of transformed cells. *Cell* **9**:269-287.
- Brugge, J. S., and J. S. Butel. 1975. Role of simian virus 40 gene A function in maintenance of transformation. *J. Virol.* **15**:619-635.
- Bukhari, A. I., E. Ljungquist, F. de Bruijn, and H. Khatoun. 1977. The mechanism of bacteriophage mu integration, p. 249-261. In A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), *DNA insertion elements, plasmids and episomes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Crawford, L. V., A. Robbins, and P. M. Nicklin. 1974. Location of the origin and terminus of replication in polyoma DNA. *J. Gen. Virol.* **25**:133-142.
- Cuzin, F., M. Vogt, M. Dieckmann, and P. Berg. 1970. Induction of virus multiplication in 3T3 cells transformed by a thermosensitive mutant of polyoma virus. II. Formation of oligomeric polyoma DNA molecules. *J. Mol. Biol.* **47**:317-333.
- De Bruijn, F., and A. I. Bukhari. 1978. Analysis of transposable elements inserted in the genomes of bacteriophages mu and P1. *Gene* **3**:315-331.
- Denhardt, D. 1966. A membrane filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**:641-646.
- Di Mayorca, G., J. Callender, G. Marin, and R. Giordano. 1969. Temperature sensitive mutants of polyoma virus. *Virology* **38**:126-133.
- Eckhart, W. R. 1969. Complementation and transformation by temperature sensitive mutants of polyoma virus. *Virology* **38**:120-125.
- Fogel, M., and L. Sachs. 1969. The activation of virus synthesis in polyoma transformed cells. *Virology* **37**:327-334.
- Freeman, A. E., R. V. Gilden, M. L. Vernon, R. G. Wolford, P. E. Hugunin, and R. J. Huebner. 1973. 5-Bromo-2 deoxyuridine potentiation of transformation of rat embryo cells induced *in vitro* by 3-methyl-cholanthrene: induction of rat leukemia virus gs antigen in transformed cells. *Proc. Natl. Acad. Sci. U.S.A.* **70**:2415-2419.
- Fried, M. 1965. Cell-transforming ability of a temperature-sensitive mutant of polyoma virus. *Proc. Natl. Acad. Sci. U.S.A.* **53**:486-491.
- Fried, M. 1974. Isolation and partial characterization of different defective DNA molecules derived from polyoma virus. *J. Virol.* **13**:939-946.
- Goff, S. P., and P. Berg. 1977. Structure and formation of circular dimers of simian virus 40 DNA. *J. Virol.* **24**:295-302.
- Graessmann, A., M. Graessmann, and C. Mueller. 1976. Regulatory mechanism of simian virus 40 gene expression in permissive and nonpermissive cells. *J. Virol.* **17**:854-858.
- Graessmann, A., M. Graessmann, and C. Mueller. 1977. Regulatory function of simian virus 40 DNA replication for the late viral gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **74**:4831-4834.
- Green, M., M. Pina, R. Kimes, P. C. Wensick, L. A. McHattie, and C. A. Thomas. 1967. Adenovirus DNA. I. Molecular weight and conformation. *Proc. Natl. Acad. Sci. U.S.A.* **57**:1302-1309.
- Griffin, B. E., and M. Fried. 1975. Amplification of a specific region of the polyoma virus genome. *Nature (London)* **256**:175-179.
- Griffin, B. E., and M. Fried. 1976. Structural mapping of the DNA of an oncogenic virus (polyoma viral DNA). *Methods Cancer Res.* **12**:49-86.
- Griffin, B. E., M. Fried, and A. Cowie. 1974. Polyoma DNA: a physical map. *Proc. Natl. Acad. Sci. U.S.A.* **71**:2077-2081.
- Gross-Bellard, M., P. Oudet, and P. Chambon. 1973. Isolation of high molecular weight DNA from mammalian cells. *Eur. J. Biochem.* **36**:32-38.
- Hill, B. T., and S. Whatley. 1975. A simple, rapid microassay for DNA. *FEBS Lett.* **56**:20-23.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
- Ito, Y., N. Spurr, and R. Dulbecco. 1977. Characterization of polyoma virus T antigen. *Proc. Natl. Acad. Sci. U.S.A.* **74**:1259-1263.
- Jaenisch, R., and A. Levine. 1973. DNA replication in SV40-infected cells. VII. Formation of SV40 catenated and circular dimers. *J. Mol. Biol.* **73**:199-212.
- Kamen, R. I., D. M. Lindstrom, H. Shure, and R. W. Old. 1974. Virus-specific RNA in cells productively infected or transformed by polyoma virus. *Cold Spring Harbor Symp. Quant. Biol.* **39**:187-198.
- Kelly, R. B., N. R. Cozzarelli, M. P. Deutscher, I. R. Lehman, and A. Kornberg. 1970. Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single strand break. *J. Biol. Chem.* **245**:39-45.
- Kelly, T. J., A. M. Lewis, A. S. Levine, and S. Siegel. 1974. Structure of two adenovirus simian virus 40 hy-

- brids which contain the entire SV40 genome. *J. Mol. Biol.* **89**:113-126.
33. Kelly, T. J., and D. Nathans. 1977. The genome of simian virus 40. *Adv. Virus Res.* **21**:85-174.
 34. Ketner, G., and T. J. Kelly. 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. *Proc. Natl. Acad. Sci. U.S.A.* **73**:1102-1106.
 35. Kimura, G., and A. Itagaki. 1975. Initiation and maintenance of cell transformation by simian virus 40: a viral genetic property. *Proc. Natl. Acad. Sci. U.S.A.* **72**:673-677.
 36. Kimura, G., A. Itagaki, and J. Summers. 1975. Rat cell line 3Y1 and its virogenic polyoma and SV40-transformed derivatives. *Int. J. Cancer* **15**:694-706.
 37. Laskey, R., and A. D. Mills. 1977. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized films. *FEBS Lett.* **82**:314-316.
 38. Lewis, A. M., and W. P. Rowe. 1970. Isolation of two plaque variants from the adenovirus type 2-simian virus 40 hybrid population which differ in their efficiency in yielding simian virus 40. *J. Virol.* **5**:413-420.
 39. Lewis, J. B., J. F. Atkins, C. W. Anderson, P. R. Baum, and R. F. Gesteland. 1975. Mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. *Proc. Natl. Acad. Sci. U.S.A.* **72**:1344-1348.
 40. Macpherson, I., and L. Montagnier. 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. *Virology* **23**:291-294.
 41. Maniatis, T., S. G. Kee, A. Efstratiadis, and F. Kafatos. 1976. Amplification of a β -globin gene synthesized *in vitro*. *Cell* **8**:163-182.
 42. Manor, H., M. Fogel, and L. Sachs. 1973. Integration of viral into chromosomal deoxyribonucleic acid in an inducible line of polyoma transformed cells. *Virology* **53**:174-185.
 43. Martin, M. A., P. M. Howley, J. C. Byrne, and C. R. Garon. 1976. Characterization of supercoiled oligomeric SV40 DNA molecules in productively infected cells. *Virology* **71**:28-40.
 44. Martin, R., and J. Y. Chou. 1975. Simian virus 40 function required for the establishment and maintenance of malignant transformation. *J. Virol.* **15**:599-612.
 45. Morhenn, V., Z. Rabinowitz, and G. M. Tomkins. 1973. Effect of adrenal glucocorticoids on polyoma virus replication. *Proc. Natl. Acad. Sci. U.S.A.* **70**:1088-1089.
 46. Neer, A., N. Baran, and H. Manor. 1977. *In situ* hybridization analysis of polyoma DNA in an inducible line of polyoma-transformed cells. *Cell* **11**:65-71.
 47. Osborn, M., and K. Weber. 1975. Simian virus 40 gene A function and maintenance of transformation. *J. Virol.* **15**:636-644.
 48. Pettersson, U., C. Mulder, H. Delius, and P. Sharp. 1973. Cleavage of adenovirus type 2 DNA into six unique fragments by endonuclease R. RI. *Proc. Natl. Acad. Sci. U.S.A.* **70**:200-204.
 49. Pirrotta, V. 1976. Two restriction endonucleases from *Bacillus globiggi*. *Nucleic Acids Res.* **3**:1747-1760.
 50. Prasad, I., D. Zouzias, and C. Basilico. 1976. State of the viral DNA in rat cells transformed by polyoma virus. I. Virus rescue and the presence of nonintegrated viral DNA molecules. *J. Virol.* **18**:436-444.
 51. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**:237-252.
 52. Risser, R., D. Rifkin, and R. Pollack. 1974. The stable classes of transformed cells induced by SV40 infection of established 3T3 cells and primary rat embryonic cells. *Cold Spring Harbor Symp. Quant. Biol.* **39**:317-324.
 53. Sambrook, J., M. Botchan, P. Gallimore, B. Oزانne, U. Petterson, J. Williams, and P. A. Sharp. 1974. Viral DNA sequences in cells transformed by simian virus 40, adenovirus type 2 and adenovirus type 5. *Cold Spring Harbor Symp. Quant. Biol.* **39**:615-632.
 54. Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco. 1968. The integrated state of viral DNA in SV40-transformed cells. *Proc. Natl. Acad. Sci. U.S.A.* **60**:1288-1295.
 55. Schaller, H., C. Nusslein, F. J. Bonhoeffer, C. Kurz, and I. Nietzsche. 1972. Affinity chromatography of DNA-binding enzymes on single stranded DNA-agarose columns. *Eur. J. Biochem.* **26**:474-481.
 56. Seif, R., and F. Cuzin. 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *tsa* mutant of polyoma virus. *J. Virol.* **24**:721-728.
 57. Sharp, P., B. Sugden, and J. Sambrook. 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry* **12**:3055-3063.
 58. Shinnick, T. M., E. Lund, O. Smithies, and F. R. Blattner. 1975. Hybridization of labeled RNA to DNA in agarose gels. *Nucleic Acids Res.* **2**:1911-1929.
 59. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-515.
 60. Tegtmeyer, P. 1975. Function of simian virus 40 gene A in transforming infection. *J. Virol.* **15**:613-618.
 61. Tooze, J. 1973. Transformation by polyoma and SV40, p. 350-402. In J. Tooze (ed.), *The molecular biology of tumor viruses*. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
 62. Vogt, M. 1970. Induction of virus multiplication in 3T3 cells transformed by a thermosensitive mutant of polyoma virus. I. Isolation and characterization of *Tsa*-3T3 cells. *J. Mol. Biol.* **47**:307-316.
 63. Wilson, G. A., and F. E. Young. 1975. Isolation of a sequence-specific endonuclease (Bam I) from *Bacillus amyloliquefaciens* H. *J. Mol. Biol.* **97**:123-125.
 64. Zouzias, D., I. Prasad, and C. Basilico. 1977. State of the viral DNA in rat cells transformed by polyoma virus. II. Identification of the cells containing nonintegrated viral DNA and the effect of viral mutations. *J. Virol.* **24**:142-150.