Closed Circular DNAs with Tandem Repeats of a Sequence from Polyoma Virus

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The R_1 restriction endonuclease from bacteria containing the RTF-1 plasmid cleaves polyoma virus DNA at a single site. Virus stocks which have been passaged through cells several times at moderate or high multiplicities contain closed circular DNAs which are not cleaved by the R_1 endonuclease. One such population of DNAs, is not infectious, and when sheared and denatured, reassociates at a rate approximately four times faster than polyoma DNA. The predominant DNA in the population is 10% smaller than polyoma DNA and contains a fraction of the polyoma genome tandemly repeated three to four times. There is no significant contamination of the covalently closed circular DNAs by repetitive host sequences. A model explaining how such trandemly repeated molecules could arise by abortive rounds of polyoma DNA replication is discussed.

1. INTRODUCTION

The small size of the genomes of SV-40 and polyoma viruses provide some reason to hope that one or more viral gene products(s) responsible for neoplastic transformation might be identified. However, recent reports that the genomes of SV-40 and polyoma viruses are easily changed, acquiring host sequences or losing viral sequences, suggest that their size may misrepresent their complexity (Yoshiike 1968a,b; Thorne, 1968; Thorne et al., 1968; Aloni et al., 1969; Blackstein et al., 1969; Lavi and Winocur, 1972, 1974; Tai et al., 1972; Yoshiike et al., 1972.

Recently, several laboratories have made a great deal of progress in characterizing the structure of the SV-40 genome, using sequence-specific nucleases (Morrow and Berg, 1972; Mulder and Delius, 1972; Fareed et al., 1972; Danna et al., 1973; Khoury et al., 1973). In this report we demonstrate that multiple infection of cells by polyoma virus permits the accumulation of defective viral genomes. A frequent closed circular DNA molecule which is selected for, and which often predominates in the pool of replicating DNA, has lost or had modified the sequence (5') GAATTC

recognized and cleaved by the R_1 restriction endonuclease (Hedgpeth et al., 1972). We have characterized one such population of defective molecules, and show that they contain repetitions of a subset of the sequences of the polyoma genome, and are relatively homogeneous.

2. MATERIALS AND METHODS

(a) Cells and Media

Secondary whole mouse embryo (WME) cells, baby mouse kidney cells and 3T6 mouse cells were grown in AutoPOW MEM Medium (Flow) containing 0.03% glycine, 0.025% L-serine, 5% tryptose phosphate Broth (Difco) and 10% calf serum (Gibco). Viral DNA labeled with $^{32}P_1$ was purified from cells grown in a low phosphate medium containing the balanced salt solution of Dulbecco's Modified Eagle's Medium with the NaH_2PO_4 concentration reduced to 12.4 mg/liter. In addition it contained essential and nonessential amino acids, vitamins, and 3% calf serum (all from Gibco).

(b) Viruses

Viruses were concentrated from a mixture of cells and media by the procedure of

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Crawford (1969), or by pelleting at 80,000 g for 2 hr. The concentrated virus was purified by banding in CsCl (ρ_0 = 1.33 g/ml) containing 0.1 M Tris-Cl (pH 8.0) by centrifugation for 48 hr in a fixed angle 50 Ti rotor (Beckman) at 40,000 rpm, followed by dialysis against a buffer containing 0.15 M NaCl, 10 mM Tris-Cl, and 0.1 mM EDTA (pH 8.0).

Plaque assays were performed as previously described (Folk, 1973).

(c) Labeling and Purification of DNAs

Viral [^{3}H]DNA was prepared by adding [Me^{3}H]thymidine (1–100 μCi/ml, 15–20 Ci/mmole) to cells 24 hr after infection, and extracting the low molecular weight intracellular DNA 24–30 hr later. Generally, purified [^{3}H]DNAs had specific activities ranging from 10^4 to 10^6 cpm/μg.

Viral [^{32}P]DNA was prepared by adding ^{32}P$_i$ (100 μCi/ml) to cells at the time of infection in low-phosphate medium, and isolating the low molecular weight intracellular DNA at 72 hr postinfection. The specific activities of purified [^{32}P]DNA ranged from 2×10^5 to 5×10^5 cpm/μg.

The small, covalently closed circular DNAs from infected cells were extracted as previously described (Folk, 1973). After ethanol precipitation, the DNAs were dissolved in 10 mM Tris-Cl with 1 mM EDTA (pH 8.0) and banded in cesium chloride (CsCl; ρ_0 = 1.59 g/ml) containing ethidium bromide (EtBr; 200 μg/ml) by centrifugation for 48–72 hr at 40,000 rpm in a 50 Ti rotor at 20°. The fractions containing the covalently closed circular DNA were collected and extracted with isopropanol and diluted threefold with water. The DNA was precipitated by the addition of 2 vol of cold ethanol. The precipitate was collected by centrifugation at 20,000 rpm for 20 min in a SW27 Rotor, and dissolved in 1 ml of 1 mM Tris-Cl containing 0.1 mM EDTA (pH 8.0). The solution was made 10 mM in Na$_2$CO$_3$ (pH 11.3) and incubated at 40° for 24 hr, then neutralized with 1 N HCl, and layered on a 5–20% linear sucrose gradient containing 10 mM Tris-Cl with 1 mM EDTA (pH 8.0). The gradient was centrifuged in an SW27 rotor for 16 hr at 24,000 rpm at 4°, and the peak fractions

containing the form I DNA (20–22 S) were pooled, and the DNA precipitated by the addition of 2 vol of ethanol.

DNA from purified virus was extracted by making the dialysed virus 1% in sodium dodecyl sulfate (SDS) and shaking with phenol equilibrated with 0.1 M Tris-Cl (pH 8.0). The DNA was precipitated from the aqueous supernatant by adding NaCl to 0.1 M and 2 vol of cold ethanol. After several hours at $-20°$, the precipitate was collected by centrifugation, dissolved in 10 mM Tris-Cl containing 1 mM EDTA (pH 8.0) and the DNA was banded in CsCl-EtBr as described above.

Cellular DNA was purified from minced WME cells by first lysing the cells overnight with a solution containing 50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 mM NaCl, Pronase (Calbiochem, 100 μg/ml), and 0.4% SDS. The viscous solution was extracted three times with phenol equilibrated with 0.1 M Tris-Cl (pH 8.0). The aqueous supernatant was made 1 M in NaCl over which 2 vol of ethanol were overlaid. The DNA was isolated by winding on a rod, and was dissolved in a solution containing 1 mM Tris-Cl with 1 mM EDTA (pH 8.0), and digested with pancreatic RNase (Worthington, 10 μg/ml) for 4 hr at room temperature. The solution was extracted twice with phenol, and the DNA was precipitated with ethanol. The DNA was dissolved in a buffer containing 10 mM Tris-Cl with 1 mM EDTA (pH 8.0) and dialysed extensively against the same.

Salmon sperm DNA (Sigma) was further purified by phenol extraction and ethanol precipitation.

(d) Purification of R_I Endonuclease

R_1 endonuclease was purified from *Escherichia coli* R$_{204}$ RTF-1 using a modification of the procedure of Yoshimori (1971) made available to us by Dr. M. Yaniv. The purification required sonication of the cells, precipitation of nucleic acids from the sonicate with streptomycin sulfate, ammonium sulfate precipitation of the protein followed by chromatography on phosphocellulose and diethylaminoethyl cellulose. The enzymatic activity was monitored by measuring the extent of cleavage

of SV-40 [^{3}H] DNA either by selective binding of alkali (pH 12.2) denaturable DNA to nitrocellulose filters (Cuzin et al., 1973), or by visual inspection of the cleaved DNAs after agarose–EtBr gel electrophoresis (Sharp et al., 1973). The enzyme was concentrated by ultrafiltration using an Amicon PM-10 membrane, and stored in a solution containing 10 mM KPO$_4$, 1 mM β-mercaptoethanol, 0.4 M NaCl (pH 7.4) and 50% glycerol at $-20°$.

(e) Enzymatic Cleavage and Resealing of Viral DNAs

Polyoma DNA was incubated for 30 min at 37° with a three- to tenfold excess of R$_I$ endonuclease in a solution containing 50 mM Tris–Cl, 50 mM NaCl and 10 mM MgCl$_2$ (pH 7.5). The progress of the reaction was monitored by removing aliquots and measuring the denaturability of the DNA at pH 12.2. (Cuzin et al., 1973). After the reaction was complete, and further addition of enzyme did not produce more cleavage, EDTA was added to a final concentration of 10 mM and the DNA was purified either by banding in CsCl–EtBr, or by rate zonal sedimentation in linear neutral sucrose gradients. This procedure was generally repeated twice with each preparation of DNA to ensure complete cleavage.

Purified R$_I$ endonuclease cleaved DNA was resealed with E. coli DNA ligase by incubating 0.018 A_{260} (absorbancy at 260 nm) of DNA with 36 units of purified E. coli DNA ligase (Olivera and Lehman, 1967; a generous gift of Dr. P. Modrich) overnight at 15° in 1 ml of buffer containing 40 mM Tris–Cl (pH 8.0), 10 mM (NH$_4$)$_2$SO$_4$, 0.1 mM EDTA, 3 mM MgCl$_2$, 0.05 mM DPN, and 50 μg of bovine plasma albumin, and then stored at 0°.

(f) Fragmentation of DNAs by Sonication or by Hydrolysis

Solutions of DNA (10–100 μg/ml) in a buffer containing 10 mM Tris–Cl and 1 mM EDTA (pH 8.0) were sonicated for two 30-sec periods at 0°, using a Branson sonifier equipped with a microprobe at a setting of 2.4 A. Polyoma DNA sonicated in this manner sediments with a peak at 6 S

in linear alkaline sucrose gradients (compared to form III Polyoma DNA produced by R$_I$ endonuclease cleavage). This corresponds to a chain length of approximately 500 nucleotides (Abelson and Thomas, 1966).

Alternatively, DNA was nicked by boiling in 0.2 N NaOH for varying lengths of time. Approximately 60% of polyoma form I was converted to form II after 5 min at 100°, and after 25 min, the DNA fragments had a chain length of approximately 1000 nucleotides, as determined by sedimentation in linear alkaline sucrose gradients.

(g) DNA Reassociation

Labeled DNA was diluted into a solution containing 10 mM Tris–Cl, and 1 mM EDTA (pH 8.0) containing 0.8% SDS and sufficient salmon sperm DNA that the final DNA concentration was 10 μg/ml or higher. The DNA was denatured by heating to 100° for 2.5 min, then quickly chilled in ice water. A solution of 2 M sodium phosphate (pH 6.8) was added to bring the sodium concentration to between 0.1 M and 1.0 M, and the reannealing mix was quickly divided in 0.1 ml aliquots into 8 × 50 mm tubes. The tubes were capped with rubber stoppers and placed in a water bath at 68° and incubated for varying lengths of time. As each sample was removed, it was placed on ice. After all the samples were taken, 0.4% SDS was added to bring the sodium phosphate concentration below 0.14 M; the samples were fractionated on 0.5 ml columns of hydroxyapatite (Biorad HTP) in 2.5 ml syringes held in a Plexiglas box designed so that 60° water circulated around the syringes. Samples containing 0.25 mg of DNA or more were fractionated with additional hydroxyapatite so that the ratio of DNA to hydroxyapatite did not exceed 0.5 mg/ml. The DNA samples were adsorbed in 0.14 M sodium phosphate buffer (pH 6.8) containing 0.4% SDS, and washed with 5 ml of 0.14 M buffer. Then, the double stranded DNA was eluted with 5 ml of 0.4 M sodium phosphate buffer (pH 6.8) containing 0.4% SDS. Carrier salmon sperm DNA (to 10 μg/ml) was added to each fraction and the DNA was precipitated by adding trichloroacetic acid to

20%. The precipitated DNA was collected by filtration through nitrocellulose filters, dried and counted in a scintillation counter.

Rates of reannealing at different salt concentrations were normalized to 0.12 M sodium phosphate (pH 6.8) using the data of Britten (1970). A zero time (unincubated) sample was taken for every experiment and usually contained only 4–8% of the cpm eluting from hydroxyapatite as duplex DNA. C_0T values (Britten and Kohne, 1968) in moles $\times$ seconds $\times$ liters^{-1} were obtained by dividing the average of the sums of the single- and double-stranded cpm by the measured specific activity (cpm/A_{260}) and then multiplying that by the time/2 (in hours) (Kohne, 1969). No correction was made for zero time duplex DNA or for less than 100% duplex DNA at the end of the incubation. Values for the absolute rate of reannealing of R_I resistant and R_I sensitive DNAs were obtained from reannealing experiments using DNA of specific activity greater than 100,000 cpm/μg where complete annealing occurred in 20 hr or longer.

3. RESULTS

(a) Plaque-Purified Polyoma Virus DNA Contains a Sequence Cleaved by the R_I Endonuclease.

Eight preparations of radioactive Pasadena Large Plaque viral DNA were purified from whole mouse embryo (WME) cells which had been infected at an input multiplicity of 0.1 plaque-forming unit (PFU) per cell and incubated for 4 days. In each case, the infecting virus was prepared directly from independently isolated single plaques. After incubation with R_I endonuclease, greater than 95% of the molecules in each preparation were cleaved; the cleaved DNAs banded in CsCl gradients containing EtBr (200 μg/ml) at a density characteristic of polyoma DNA which is *not* covalently closed ($\rho = 1.55$ g/ml; Radloff *et al.*, 1967), and sedimented in a neutral linear sucrose gradient as a single peak with a sedimentation coefficient of approximately 14 S (Fig. 1). This is the sedimentation coefficient of unit length molecues (Vinograd *et al.*, 1965) indicating that polyoma DNA con-
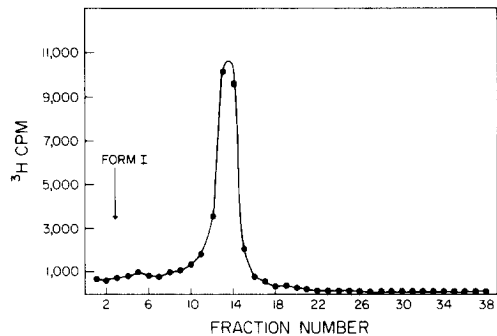

FIG. 1. Rate zonal sedimentation of R_I endonuclease cleaved polyoma DNA. [^{3}H]polyoma DNA purified from cells infected with virus derived from a single plaque was incubated with excess R_I endonuclease and layered on a linear 5–20% sucrose gradient containing 0.1 M Tris-Cl and 1 mM EDTA (pH 8.0) in a SW 50·1 tube. The gradient was centrifuged for 120 min at 49,000 rpm at 20°, and 6-drop fractions were collected on paper squares. The samples were dried and counted in scintillation fluid. In separate tubes in the same run, samples of uncleaved polyoma DNA and SV-40 DNA form I sedimented with peaks at fraction 3.

tains at least one nucleotide sequence recognized by the R_I endonuclease; the lack of any radioactivity sedimenting at a slower rate suggests that if there is more than one R_I site, there is little DNA between them.

(b) Heterogeneity of Closed Circular DNA from Passaged Virus

Seven different stocks of polyoma virus (including the Toronto Small Plaque virus, the Pasadena Large Plaque virus and the ts-A mutant derived from the Pasadena Large Plaque virus) which had been passaged several times through WME cells at moderate initial multiplicities (0.1–5 PFU/cell) for long times (7–10 days), were adsorbed to WME cells at a multiplicity of 1–10 PFU/cell. After 48–72 hr the intracellular covalently closed circular DNA was extracted, purified, incubated with excess R_I endonuclease, and rebanded in CsCl gradients containing EtBr. Each preparation contained both molecules which were cleaved by the R_I endonuclease, and molecules which were not. In different preparations the resistant fraction accounted for 20–90% of the total DNA.

To investigate further the R_I endonuclease resistant molecules, an attempt was

made to obtain a homogeneous population of DNA: a stock of Pasadena Large Plaque virus containing a small fraction of resistant molecules was prepared by passing virus from a single plaque three consecutive times through cells at low input multiplicity (0.01 PFU/cell) for relatively *short* periods of time (4–5 days at 37°). The virus used in the first passage contained no detectable amount of R_I resistant closed circular DNA. Virus from the third passage stock was used to infect WME cells at a moderate multiplicity (5–10 PFU/cell) and covalently closed circular [³H]DNA was extracted after 48 hr. This DNA contained 25–30% R_I endonuclease resistant molecules. It is not known at which passage these defective DNAs began to accumulate; however, one cycle of moderate multiplicity infection by single plaque virus does not generate any more R_I resistant molecules than low multiplicity infection.

(c) R_I Endonuclease-Resistant Molecules are Encapsidated.

The "third passage" stock of virus described above was concentrated and purified, and the "full" particles from CsCl banding were absorbed to WME cells at moderate multiplicity (5–10 PFU/cell). After addition of [³H]thymidine, the intracellular covalently closed circular DNA was extracted, purified, and digested with R_I endonuclease: it contained 25–30% R_I resistant molecules, the same amount found after infection by crude virus preparations.

In a separate experiment, [³H]thymidine labeled *virus* was purified from the supernatant of WME cells infected with banded "third passage" virus. The DNA was extracted from the purified virus, and purified further by banding in CsCl containing EtBr and by rate zonal sedimentation in neutral sucrose gradients. This DNA also contained 25–35% R_I resistant, covalently closed circular molecules. These experiments demonstrate that the R_I resistant molecules are encapsidated with the same amount of protein as infectious polyoma virus.

(d) Production of R_I Endonuclease-Resistant Molecules Requires Viral Infection and is Independent of Cell Origin

Mock infected cells contain less than 1% the amount of covalently closed circular DNAs that are detected in infected cells. A variety of cell types, including WME cells from T. O. mice, Swiss–Webster mice and Balb mice, baby mouse kidney cells from Balb mice, and the continuous cell line 3T6, all produced similar relative quantities of R_I-resistant and R_I-sensitive DNA upon infection with purified "third passage" virus. WME cells in a "resting" state imposed by low serum (Fried and Pitts, 1968) produced essentially the same fraction of R_I endonuclease resistant molecules as confluent cells in 10% calf serum. The overall production of polyoma DNA in confluent 3T6 cells was markedly reduced, which prevented a comparison between confluent and subconfluent cell populations.

No systematic study was made of the effect of multiplicity of infection upon production of R_I-resistant molecules. By comparing results from a number of different experiments, it was apparent however, that increasing the m.o.i. from 5–10 PFU/cell to 50 PFU/cell tends to raise the fraction of R_I endonuclease-resistant molecules from 30 to 50%. The ratio of intracellular R_I endonuclease-sensitive and -resistant molecules does not appear to change by more than 20% between 48 and 96 hr postinfection in WME cells infected with 5–10 PFU/cell of purified virus.

(e) Rate Zonal Sedimentation of the R_I Endonuclease-Resistant DNA

Prior to digestion with the R_I endonuclease, polyoma form I DNA has a sedimentation coefficient of 20 S in neutral sucrose gradients (Vinograd et al., 1965). The form I DNA extracted from cells infected with third passage virus is heterogeneous; its peak is broader than that of form I DNA extracted from cells infected with virus from a single plaque (Fig. 2a). After digestion with R_I endonuclease, the sedimentation coefficient of the R_I endonu-
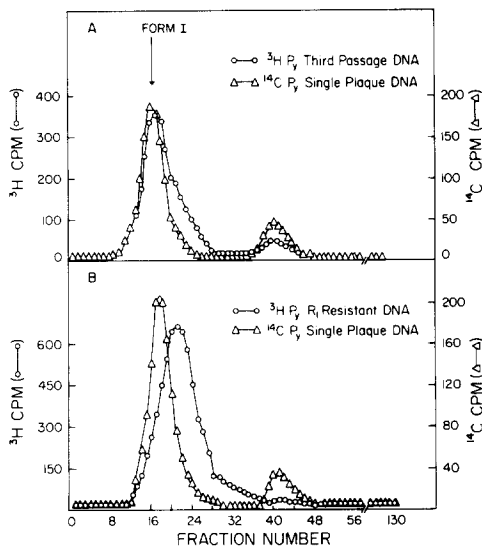
FIG. 2. Rate zonal sedimentation of polyoma DNAs. (A) [^{3}H]polyoma DNA from cells infected with the third passage virus at 5–10 PFU/cell was mixed with [^{14}C]polyoma DNA from cells infected with virus from a single plaque. The DNAs were layered on linear 5–20% neutral sucrose gradients containing 0.1 M Tris–Cl and 1 mM EDTA (pH 8.0) in a SW-41 tube, and centrifuged at 28,000 rpm for 16.5 hr at 19°. (B) The preparation of [^{3}H]DNA described above was incubated with R_I endonuclease, and resistant molecules were separated from cleaved molecules by CsCl-Etbr isopycnic centrifugation. The resistant (covalently closed) DNA was isolated, mixed with [^{14}C]polyoma DNA, and sedimented using the same conditions described in (A).

FIG. 3. Rate zonal sedimentation of polyoma DNAs in linear alkaline sucrose gradients. Covalently closed, R_I-resistant [^{32}P]DNA from cells infected with third passage polyoma virus at 5–10 PFU/cell was boiled for 5 min in 0.2 N NaOH and mixed with polyoma [^{3}H]DNA from cells infected with virus from a single plaque. The two DNAs were layered on linear 5–20% sucrose gradients containing 0.5 M NaOH and 1 mM EDTA in SW 50·1 tubes and centrifuged for 120 min at 49,000 rpm at 20°. Fractions containing 6 drops each were collected on paper squares, dried and counted. Fractions 25–29 from equivalent gradients containing only [^{32}P]DNA were pooled, neutralized with HCl and used as nicked, unit length R_I-resistant DNA for reannealing experiments (see Results). (□—□), [^{3}H]DNA; (●—●), [^{32}P]DNA.

(f) Isopycnic Banding of the R_I Endonuclease-Resistant DNA

To determine if the R_I endonuclease-resistant DNA differs from the R_I endonuclease-sensitive DNA in base composition, or superhelix density, the buoyant densities of the two types of DNA were compared.

R_I-resistant [^{32}P]DNA was mixed with R_I-sensitive [^{3}H]DNA and banded in a fixed angle 50 Ti rotor. In neutral CsCl, the peak of R_I-resistant DNA coincided with the peak of R_I-sensitive DNA, which has a density of 1.708 g/ml and a base composition of 48% GC (Sober, 1970). In a separate tube, the peak of polyoma R_I-resistant DNA was separated from a peak of SV-40 [^{3}H]DNA (ρ_{CsCl} = 1.701 g/ml; 40% GC (Sober, 1970) by three and one-half fractions, indicating that a 2% difference in base composition could have been detected

clease-resistant DNA is observed to be 4% less than that of the uncleaved low multiplicity viral DNA (Fig. 2b). This difference cannot be attributed to a change in superhelix density, as R_I endonuclease-resistant form II DNA produced after incubation with pancreatic DNase also sediments 4% slower than similarly treated low multiplicity viral DNA (not shown). In alkaline sucrose, the cyclic coil form of the DNA resistant to R_I endonuclease sediment approximately 6% more slowly than molecules which can be completely cleaved by the R_I endonuclease (Fig. 3). These reduced rates of sedimentation indicate that the R_I endonuclease-resistant form I DNAs are approximately 11–14% smaller than the R_I endonuclease-sensitive (low multiplicity) molecules (Abelson and Thomas, 1966).
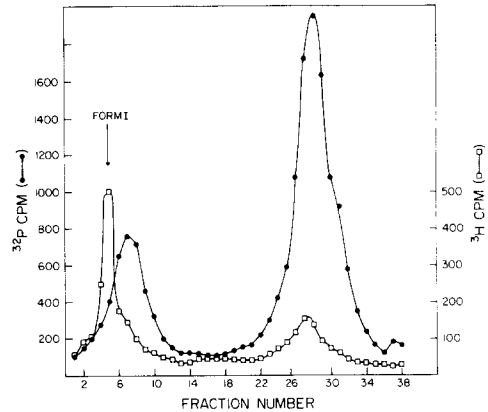
between R_I-sensitive and R_I-resistant DNAs.

To determine if the R_I-resistant DNA has more or fewer superhelical turns (superhelix density) R_I-sensitive form I and form II [^{32}P]DNA was mixed with R_I-resistant form I and form II [^{3}H]DNA and banded in CsCl containing EtBr (330 μg/ml; Bauer and Vinograd, 1971). The peaks of form I [^{32}P]DNA and [^{3}H]DNA coincided as did the peaks of form II DNA. These data indicate that the R_I-resistant DNAs have an average base composition of 48% GC and approximately -15 ± 1 superhelical turns in buoyant cesium chloride of neutral pH (Vinograd et al., 1968).

(g) Infectivity of the R_I Endonuclease-Resistant DNA

DNA molecules which have been cleaved by the R_I endonuclease have 16% the infectivity of undigested DNA. (Table 1). It is likely that the residual infectivity observed is due to resealing of the cohesive ends produced by the R_I endonuclease (Hedgpeth et al., 1972; Mertz and Davis, 1972) after the molecules have entered the cell; treatment of the cleaved molecules with E. coli DNA ligase restores their infectivity. This observation strongly sup-

TABLE 1

INFECTIVITY OF PURIFIED DNA[a]

DNA	Infectivity
Untreated	100%
R_I cleaved	16%
R_I cleaved + DNA ligase	200%
R_I resistant	<0.1%

[a] Covalently closed circular [^{3}H]DNA purified from WME cells infected with "third passage" virus was digested with R_I endonuclease, and the R_I-cleaved and R_I-resistant DNAs were separated by banding in CsCl. A sample of R_I-cleaved DNA was incubated with Escherichia coli DNA ligase (see Methods). The specific infectivity of each preparation of DNA was determined on WME cells using diethylaminoethyl dextran (Folk, 1973). Values given are the average PFU/cpm of input DNA from two separate assays. The untreated [^{3}H]polyoma DNAs used in these experiments had a specific infectivity of 4×10^5 PFU/μg.

ports the notion that each molecule contains only one sequence recognized by the R_I endonuclease: it is unlikely that infectious molecules could have been formed by treatment with DNA ligase had a segment of DNA been deleted by cleavage at two sites.

The R_I endonuclease-resistant molecules are not infectious when tested under the same conditions as the undigested, or R_I cleaved DNA (Table 1). Several experiments, however, suggest that the R_I-resistant molecules actively interfere with plaque production by the R_I-sensitive molecules (W. R. Folk and M. Fried, unpublished data). This may explain the increased infectivity of the purified, resealed R_I cleaved DNAs, and prevents a true measurement of any small amount of infectivity in the population of R_I-resistant DNAs.

(h) Sequence Complexity of the R_I Endonuclease-Resistant DNA

Polyoma DNA produced by low multiplicity infection of cells with virus from a single plaque (and completely sensitive to R_I endonuclease), when sonicated, denatured and incubated at 68°, reanneals at a rate such that the $C_0T_{1/2}$ (concentration × time for 50% reannealing) is 2.1 ($\pm$0.3) × 10^{-3} moles × sec/liter (Fig. 4). This is approximately the value expected for fragments of DNA derived from a genome with a sequence complexity (Britten, 1969) of 3 × 10^6 daltons (Wetmur and Davidson, 1968). In contrast, purified R_I endonuclease resistant DNA from cells infected with third passage virus, reanneals approximately fourfold faster, with a $C_0T_{1/2}$ of 0.6 ($\pm$0.1) × 10^{-3} moles × sec/liter. This increased rate of reassociation indicates the R_I resistant DNA is less complex than infectious polyoma DNA, and must contain repeated copies of a sequence substantially smaller than that of the polyoma genome. As the R_I resistant molecules are 85% the size of polyoma DNA, and the repeated sequence has a complexity equivalent to 25% of the polyoma genome, it must be repeated three to four times over in each molecule.
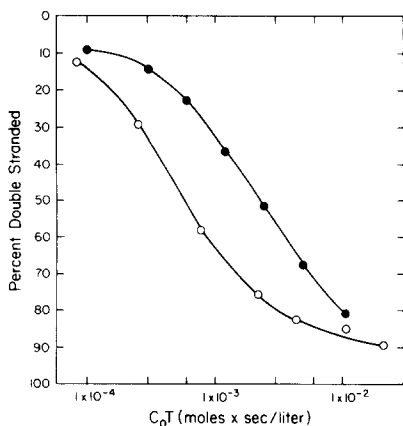
FIG. 4. Self-annealing of R_I-sensitive and R_I-resistant DNAs. R_I-resistant [^{32}P]DNA purified after R_I digestion of polyoma DNA from cells infected by 5–10 PFU/cell with third passage virus and R_I-cleaved [^3H]DNA purified from cells infected with virus derived from a single plaque were sonicated, boiled and allowed to reanneal *separately*. The two experiments are plotted together to permit comparison. These plots are representative of several independent preparations of [^3H]- and [^{32}P]DNAs, whose reannealing rates were measured in separate experiments. (O—O), R_I-resistant DNA; (●—●), R_I-sensitive DNA.

FIG. 5. Reannealing of excess R_I-sensitive with R_I-resistant DNAs. R_I-cleaved [^3H]DNA fragments ($1.1 \times 10^{-2} A_{260}$; 1.6×10^6 cpm/A_{260}) from cells infected with third passage virus were mixed with R_I-resistant [^{32}P]DNA fragments ($2.4 \times 10^{-4} A_{260}$; 7.8×10^7 cpm/A_{260}), boiled and allowed to reanneal together. The C_0 used for the abcissa is that of the R_I-cleaved DNA ($1.1 \times 10^{-2} A_{260}$). (O—O), R_I-resistant DNA; (●—●), R_I-sensitive DNA. The arrow indicates the point of 50% reannealing of the R_I-resistant DNA *alone*.

(i) Origin of the Sequences in R_I Endonuclease-Resistant DNA

If the R_I endonuclease resistant molecules are not infectious, what is their origin? Are they derived from the host genome, or from the polyoma genome? To answer this question, we determined whether the nucleotide sequences of the R_I endonuclease-resistant molecules were complementary to sequences in the polyoma genome, or to sequences in the cell DNA.

To determine if the sequences in the R_I-resistant molecules are complementary to those in polyoma DNA, R_I-resistant and R_I-sensitive DNAs labeled with different radioisotopes were mixed and allowed to reanneal together. As shown in Fig. 5, when R_I-resistant DNA fragments are incubated with an excess of R_I-sensitive DNA fragments, the rate of reannealing of the R_I-resistant DNA is increased so that the two DNAs become double stranded at essentially the same rate. The R_I-resistant DNA must be reannealing with the R_I-sensitive
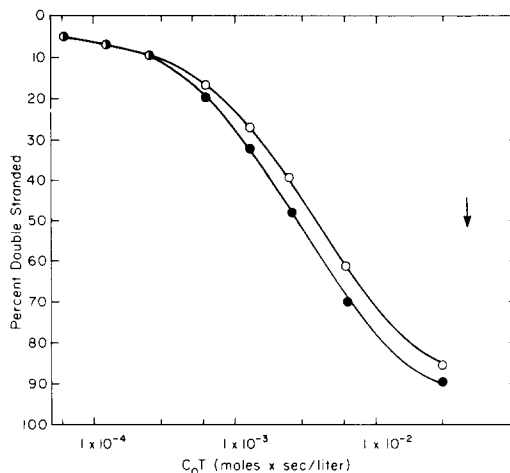
DNA, indicating that the two DNAs share common sequences.

When the ratio of the two DNAs is changed, and an excess of R_I-resistant DNA is incubated with R_I-sensitive DNA, the rate of reannealing of the bulk of the R_I-sensitive DNA is increased tenfold, but it is still 30 times less than the rate of the R_I-resistant DNA (Fig. 6). In fact, the reannealing of the R_I-sensitive DNA is heterogeneous in the presence of an excess of R_I-resistant DNA. Approximately 20% of the R_I-sensitive sequences reanneal at a faster rate than the rest of the R_I-sensitive DNA. This difference becomes clear when the reannealing data are replotted so that second order kinetics give a straight line (Fig. 7). In this plot, the self-annealing of both R_I-sensitive and R_I-resistant DNAs follow second order kinetics. When R_I-resistant DNA is used to "push" the reannealing of the R_I-sensitive DNA, the plot is biphasic. After subtracting the contribution of the slowly reannealing species, the rapidly reannealing minor fraction appears

to reanneal at approximately the same rate as the R_I-resistant DNA.

If, in the experiments described above, the minority (rapidly reannealing) fraction of R_I-sensitive DNA is part of the polyoma genome, then each molecule of polyoma
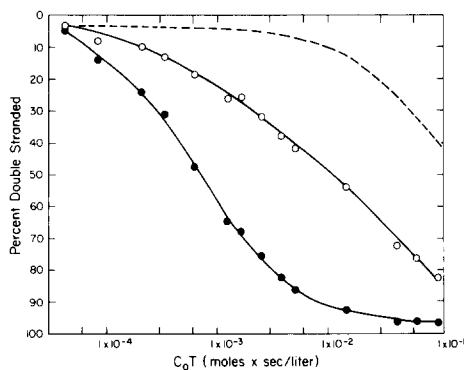

FIG. 6. Reannealing of R_I-sensitive with excess R_I-resistant DNAs. R_I-resistant [^{32}P]DNA fragments $(5.7 \times 10^{-3} A_{260}; 2.4 \times 10^7 \text{ cpm}/A_{260})$ were mixed with R_I-sensitive [^{3}H]DNA fragments $(8.2 \times 10^{-5} A_{260}; 1.4 \times 10^8 \text{ cpm}/A_{260})$ from cells infected with virus derived from a single plaque, boiled and allowed to reanneal together. The C_0 used for the abcissa is that of the R_I-resistant DNA $(5.7 \times 10^{-3} A_{260})$. The dashed line portrays the kinetics of self-annealing of the R_I-sensitive DNA in the absence of the R_I-resistant DNA. (●—●), R_I-resistant DNA (○—○) R_I-sensitive DNA.

DNA should contain one such sequence. When *unit length* polyoma DNAs are reannealed with an excess of sheared R_I-resistant DNA, the complementary sequences in the polyoma DNA should reanneal with the R_I-resistant DNA fragments. Because hydroxyapatite fractionates a DNA containing both single- and double-stranded regions as if it were completely double-stranded, the unit length polyoma DNAs containing a duplex region formed with the R_I-resistant DNA fragment should fractionate as double-stranded molecules. When this experiment is done, polyoma DNA is observed to become double stranded coincidentally with the R_I-resistant DNA (Fig. 8).

These data demonstrate that the population of R_I-resistant molecules is relatively homogeneous. The majority of the R_I-resistant DNA reanneals at the rate expected of a DNA with a sequence complexity approximately 25% that of polyoma DNA. Most R_I-resistant sequences are complementary to 18–20% of the polyoma genome, a value obtained by estimating the point in Fig. 7 where the rapidly reannealing fraction becomes dominated by the slowly reannealing fraction. However, 10–12% of R_I-resistant sequences
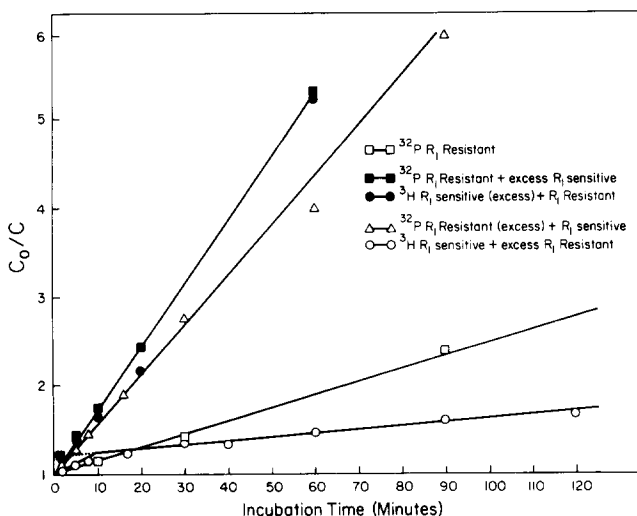

FIG. 7. Linear plots of reannealing kinetics. Data from Figs. 4, 5, and 6 is plotted so that second order kinetics will give a straight line intercepting the ordinate at 1. The second order rate equation which gives this plot is $1/(C_{0-x}/C_0) = kC_0'T + 1$ where C_0' is the initial concentration of nucleotides in single-stranded DNA and x is the concentration of nucleotides in double stranded DNA. (C_{0-x}/C_0) is the fraction of single stranded DNA (C) at time T.
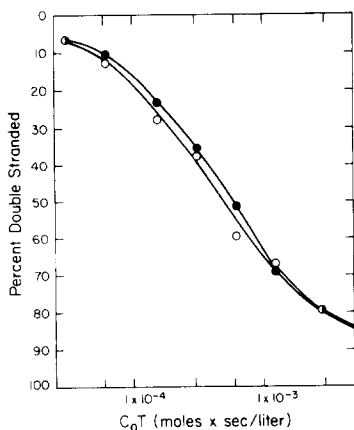
denatured, reannealed, and the duplex
DNA at 3 min (27% of the total) and the
single-stranded DNA at 17 min (30% of the
total) were compared with regard to their
complexity. It is clear that the two DNA
fractions differ (Fig. 9). Furthermore, when
the duplex DNA at 3 min was used to
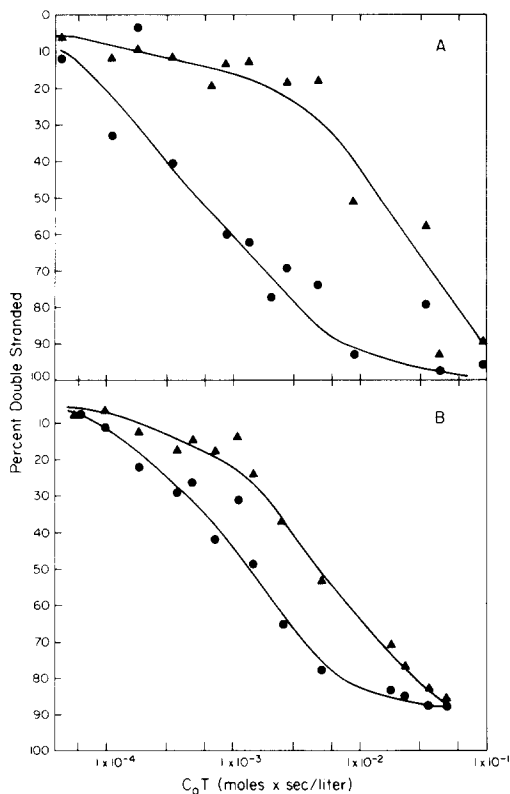"push" the reannealing of R_1-sensitive

FIG. 8. Reannealing of excess R_1-resistant DNA
fragments with R_1-sensitive unit length DNAs. R_1-
resistant [^{32}P]DNA fragments ($5.1 \times 10^{-3} A_{260}$; $2.4 \times$
10^7 cpm/A_{260}) were mixed with R_1-cleaved linear
[^{3}H]DNA ($9.1 \times 10^{-5} A_{260}$; 1.4×10^8 cpm/A_{260}) from
cells infected with virus derived from a single plaque,
boiled and allowed to reanneal together. The C_0 used
for the abcissa is that of the [^{32}P]DNA. (●—●), R_1
resistant; (O—O), R_1 sensitive.

(judged by the increased rate of reanneal-
ing of R_1-sensitive sequences in Fig. 6) are
complementary to the whole polyoma ge-
nome. These sequences may be present in
polyoma molecules which may simply lack
the R_1 site or they may be dispersed
throughout the molecules containing the
repeated sequences.

(j) Major and Minor Species of R_1 En-
donuclease-Resistant DNAs

If the sequences complementary to the
whole polyoma genome are dispersed
throughout all the molecules, then one-
third to one-quarter molecules of R_1-resist-
ant DNA should contain both repeated and
nonrepeated sequences. However, if the
population of R_1-resistant molecules is het-
erogeneous, and contains two types of cir-
cular DNAs, fragments from the molecules
with repeated sequences should not con-
tain nonrepeated sequences. These two
alternatives can be distinguished by deter-
mining if fragments of R_1-resistant DNA
which reanneal quickly, and presumably
contain repeated sequences, differ in com-
plexity from those which reanneal slowly,
and might contain nonrepeated sequences.

Fragments of R_1-resistant DNA were

FIG. 9. Reannealing of quarter length R_1-resistant
DNA with R_1-sensitive DNA fragments. R_1-resistant
[^{32}P]DNA was hydrolyzed to fragments of approxi-
mately 1000 nucleotides by boiling in 0.2 N NaOH.
After neutralization, the DNA was incubated at 68°,
and at 3 and 17 min aliquots were removed and
fractionated on hydroxyapatite. The duplex DNA at 3
min (27% of the initial DNA; $1.4 \times 10^{-3} A_{260}$) and the
single-stranded DNA at 17 min (30% of the initial
DNA; $1.6 \times 10^{-3} A_{260}$) were collected and mixed with
a small quantity of sonicated polyoma [^{3}H]DNA ($9.2
\times 10^{-5} A_{260}$) from cells infected with virus derived
from a single plaque. The mixed DNAs were boiled
and allowed to reanneal at 68°. (A). Duplex [^{32}P]DNA
at 3 min (●—●) mixed with [^{3}H]DNA (▲—▲). The
$C_0T_{\frac{1}{2}}$ of the [^{32}P]DNA was 0.6×10^{-3} moles × sec ×
liter^{-1}. (B). Single-stranded [^{32}P]DNA at 17 min
(●—●) mixed with [^{3}H]DNA (▲—▲). The $C_0T_{\frac{1}{2}}$ of
the [^{32}P]DNA was 1.4×10^{-3} moles × sec × liter^{-1}.

DNA, a fraction of R_I-sensitive sequences reassociated rapidly, but the majority of the sequences did not (Fig. 9A). This is in contrast to the effectiveness of the single-stranded R_I-resistant DNA at 17 min in "pushing" the reannealing of the R_I-sensitive DNA (Fig. 9B).

These results support the notion that the population of R_I-resistant DNA is composed of two types of molecules. A majority (88-90%) with repeats of a single sequence, and a minority with a variety of sequences complementary to the entire polyoma genome.

(k) Organization of Repeated Sequences in the R_I Endonuclease-Resistant DNA

The repeated sequences in the R_I-resistant molecules may be organized so that tandem repeats of the same sequence are present solely on one strand (and tandem repeats of the complementary sequence always on the other) or both sequences and complementary sequences might be interspersed on the same strand. Reannealing of unit length strands should give rise to extensive intrastrand duplexes by first order kinetics if the latter alternative is correct, but only interstrand duplexes should form if the former alternative is correct.

To distinguish between these two possibilities, R_I-resistant molecules were nicked by boiling in alkali and those molecules containing an average of one break per strand were isolated (Fig. 3). The reannealing of these strands was measured at two DNA concentrations differing by a factor of five. At both concentrations, reannealing followed second order kinetics at the same rate through 80% of the reaction, indicating that the repeated sequences are arranged in tandem, with no sequence and its complement on the same strand.

(l) Cellular Sequences in the R_I Endonuclease-Resistant DNA

The kinetics of reannealing of the R_I endonuclease-resistant DNA in the presence of an excess of polyoma DNA (R_I sensitive) are unimodal, suggesting that large quantities of cellular sequences are

not present. To reinforce this notion, sheared R_I-resistant DNA was reannealed in the presence of a 10^6-fold excess of salmon sperm DNA, or WME DNA. The reannealing kinetics of the [^{32}P]DNA were exactly the same in both cases (Fig. 10); reannealing of half the [^{32}P]DNA occurred in 15 hr at 65°, and a 50% difference in rates would have been detected. Thus, significant contamination of these R_I-resistant DNAs by any repetitive sequences in the cell genome can be excluded. Other defective polyoma DNAs, however, have been reported to contain repetitive sequences derived from the host (Lavi and Winocour, 1974).

(m) Sequence Heterogeneity of the R_I Endonuclease-Sensitive DNA.

DNA from cells infected with the third passage virus which is cleaved by the R_I endonuclease reanneals at a marginally faster rate (10%) than DNA from cells infected at low multiplicity with virus from a single plaque. This difference in rates is only significant when an excess of the
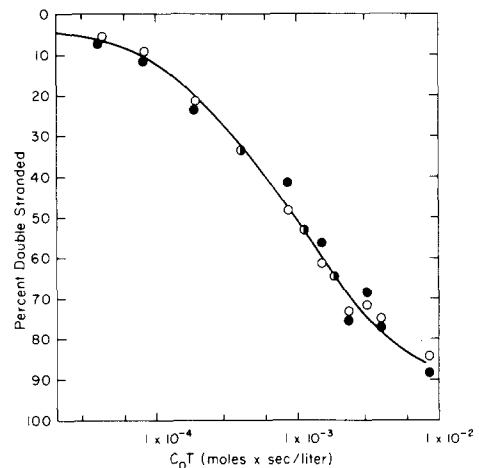

FIG. 10. Reannealing of R_I-resistant DNAs in the presence of salmon sperm or WME DNA. R_I-resistant [^{32}P]DNA fragments (1.4 × 10^{-4} A_{260}; 5.3 × 10^7 cpm/A_{260}) purified after R_I digestion of polyoma DNA from cells infected by 5-10 PFU/cell with third passage virus were fragmented and mixed with sonicated (unlabeled) WME DNA (149 A_{260}) or salmon sperm DNA (110 A_{260}), boiled and allowed to reanneal. The C_0 used for the abcissa is that of the R_I-resistant DNA, (O—O) with salmon sperm DNA; (●—●) with WME DNA.

former DNA is reannealed with the latter DNA. As it is likely that this DNA is somewhat contaminated by R_I-resistant DNA converted to Form II by strand scission (through radiation damage or hydrolysis) it is difficult to determine if this increased rate of reannealing is significant.

4. DISCUSSION

These experiments demonstrate that, in cells multiply infected with polyoma virus, large quantities of covalently closed circular DNA lacking portions of the polyoma genome are produced. All such molecules are likely to be defective because they have lost the R_I site, a sequence present in infectious molecules. However, they must derive some selective advantage from the loss of part of the genome, or else they would not recur so frequently, and in such high amounts. Although we did not detect any significant quantity of R_I endonuclease-resistant molecules in DNA preparations one passage removed from a single plaque, it is likely that some were present, for the formation of a single plaque must involve multiple infection of cells with the progeny virus from the first infected cell.

One preparation of defective DNA which we have extensively characterized contains circular DNAs of which the majority had repeated copies of a sequence comprising approximately 20–25% of the polyoma genome. This estimate of the size of the repeat comes from two independent experiments: the kinetics of reannealing of the R_I-resistant DNA indicate a complexity approximately one fourth that of the infectious polyoma genome (Fig. 4); and when the R_I-resistant DNA is used to push the reannealing of fragments of polyoma DNA, approximately 18–20% of the polyoma genome reanneals with a rate similar to the R_I-resistant DNA (Fig. 7). Further characterization will be required to determine which polyoma virus function(s) are directed by this sequence. However, it is of interest to speculate how such a molecule might evolve, and why it possesses a selective advantage in a pool of replicating DNAs.

One process which might give rise to such tandemly repeated molecules is rep-

lication of the covalently closed circular viral genome.[1] Polyoma replication proceeds bidirectionally from a point which is separated from the R_I site by approximately 29% of the genome (Crawford et al., 1973). Newly replicated DNA is not covalently joined to the parental strands (Bourgaux et al., 1969; Bourgaux et al., 1971; Pigiet et al., 1973) which appear to be covalently closed throughout most of replication. However, the parental strands must unwind as replication proceeds, so that intermittent opening and closing undoubtedly occurs at a fast rate. An enzymatic activity which might perform such a "swivel" function has been described in WME cells (Champoux and Dulbecco, 1972).

Molecules of SV-40 DNA undergoing replication have been visualised in the electron microscope (Sebring et al., 1971); they have two "relaxed" loops which have been replicated, and a segment of DNA which is supercoiled and not yet replicated. This suggests that the "swivel" activity does not unwind the parental molecule at a rate much faster than the rate of chain elongation. If the same is true for polyoma replication, and if the forces which induce supercoiling when DNA is extracted from cells act in the same way upon DNA within the cell, then the two growing forks in a molecule that is being replicated bidirectionally will be forced close to each other.

During replication, a tandemly repeated sequence could be formed by the joining of a nascent strand in one replicating fork to a parental strand in a nearby opposite fork which is being unwound. (Fig. 11A). Joining could occur by a DNA ligase (part of the swivel activity) simply mistaking the 3′ terminus of a nascent strand for the 3′ terminus of the parental strand. One growing fork in the replicating molecule would presumably abort, and DNA ligase could seal the interruption (Fig. 11A, part II. One of the tandem repeats would form a bubble with a partially replicated strand (Fig. 11A, part III). A daughter molecule with a tandem repeat could be formed by elonga-

[1] The suggestion that abortive rounds of replication might generate defective DNAs was originally made by Dr. Don Robberson.

tion of the partially replicated strand, and initiation of a new strand at the base of the bubble (Fig. 11A, part IV_A). Alternatively, initiation of replication could recur at the original sites, and the bubble would be copied by asymmetric strand elongation (Fig. 11A, part IV_B or IV_C). Following the formation of a circular molecule with one tandem repeat, molecules with additional repeats could be formed by a second round of abortive replication, or by two unequal crossovers between tandemly repeated sequences (Fig. 11B).

This model for the production of tandemly repeated sequences requires bidirectional replication, and proximity of the two growing forks, constraints which are not likely to be met by all biological systems. The model predicts that the repeated sequences should contain a site for the initia-

tion of replication. In polyoma, the R_I site is approximately 0.29 genomes from the point of initiation of DNA synthesis. The R_I endonuclease-resistant molecules, with a sequence corresponding to 25% of the polyoma genome repeated severalfold, would be expected to lack the R_I site if the repeat centers about the initiation site.

Multiple repeats of the site for initiation of DNA synthesis may provide a strong selective advantage to molecules competing in a pool of replicating DNA where initiation is limiting. As long as cells are multiply infected, presumably only a few viral molecules need to maintain the genes coding for structural proteins.

Other, less obvious, advantages may accrue from gene duplication. In *E. coli*, frequent duplication of the genes coding for glycyl tRNA synthetase provides a means
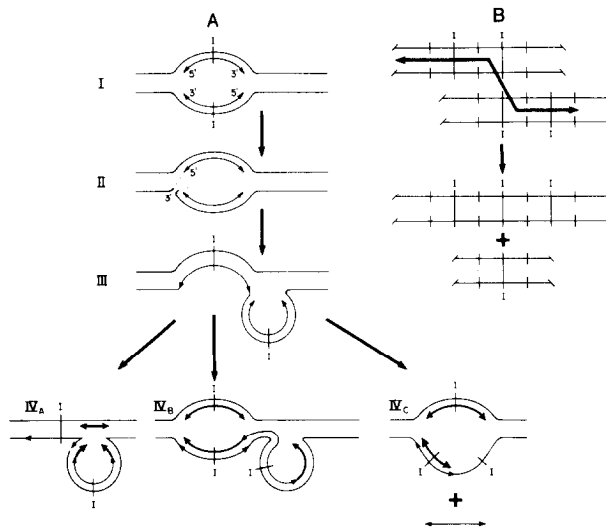

FIG. 11. Model for the formation of multiple tandem repeats during abortive rounds of replication.

Part A. I. A replication loop with forks proceeding bidirectionally from the initiation site (I). II. The parental strand is opened by the "swivel" activity, but is mistakenly rejoined to the ends of a progeny strand. Superhelical turns in the unreplicated portion of the molecule might force the two growing forks closer together than is illustrated. III. Heterozygote DNA containing hybrid parental and progeny strand (forming a tandem repeat) and a base paired fragment of DNA. IV. Replication of heterozygote DNA could continue by: A. Extension of ends of the fragment of DNA and reinitiation of the complementary strand at a new site (heavy lines indicate new synthesis). B. Reinitiation at the original site followed by joining of the fragment of DNA to the newly synthesized DNA. C. Reinitiation at the original site followed by displacement of the fragment of DNA.

Such an asymmetric replication loop has many features in common with the proposed mechanism for the replication of circular mitochondrial DNA in mouse L cells (Robberson *et al.*, 1972).

Multiple tandem repeats could be formed by several such events occurring independently in the same molecule. Alternatively, unequal crossing over among duplicated sequences between different molecules would also generate multiple tandem repeats (part B).

to increase the quantity of enzyme if it is mutationally altered (Folk and Berg, 1971). The structural genes for the glycyl tRNA synthetase are located close to the site for the initiation of DNA replication in *E. coli* (Bird *et al.*, 1972), and recently, replication has been shown to proceed bidirectionally around the chromosome (Bird *et al.*, 1972; McKenna Masters, 1972; Prescott and Kuempel, 1972). This may be an instance in which duplication of the site for DNA synthesis initiation includes other genes whose increased quantity provide a selective advantage. Also, there is substantial evidence that in Chinese hamster cells, DNA replication is bidirectional and is initiated at numerous sites throughout the chromosomes (Huberman and Riggs, 1969; Huberman and Tsai, 1973). Sequence duplication by a process such as that described above might generate the families of repeated sequences which have been observed in all species above the fungi (Britten, 1968).

Other instances of "illegitimate" recombination (formation of tandem repeats in DNA) have been described, and have on the whole, gone unexplained (Franklin, 1971). Slippage of the DNA polymerase along the template strand, or crossing over between nonhomologous sequences (Hershey, 1970) are two other ways by which tandem duplications might be generated.

The homogeneity in size of the preparation of polyoma defective DNAs which we have characterized may reflect a limitation imposed by encapsidation. It is known that polyoma oligomers that are 1.6 times the size of polyoma are not encapsidated (Mulder and Vogt, 1973). Consequently, duplication and reduplication of a variety of sizes of DNA containing the same sequence may occur any number of times, but if the defectives are passaged from cell to cell by encapsidation with coat protein, the maximum limit may be approximately 3×10^6 daltons.

Other types of defective molecules occur in polyoma stocks which are passaged through cells at high multiplicities. In an independent study, Fried has found several different types of defective polyoma DNAs, some of which have a majority of the genome deleted (personal communication). Most such defective DNAs lack the sequence cleaved by the R_I endonuclease (Robberson and Fried, personal communication). The class of defective DNAs which are reported here may be similar to one of the classes characterized by Fried and Robberson. However, as many such stocks of defective viruses may arise from events having a low probability, but a high selective value, independent isolates may vary markedly from each other. After most of our experiments were completed, a circular defective SV-40 DNA with tandemly repeated sequences was described in stocks of virus which were serially passaged without dilution through monkey cells (Martin *et al.*, 1973).

An important conclusion from this work is that unless great care is taken with the production of polyoma virus, its genome becomes heterogeneous, with loss and redistribution of its DNA sequences. The selectivity of the R_I endonuclease provides a simple and sensitive method to evaluate whether a DNA preparation is likely to be infectious and homogeneous. The homogeneity of the DNA is correlated with the quality of the virus. It has been our experience that virus with a high ratio of plaque forming units to physical particles (1/10–1/40) produce DNA which is completely sensitive to the R_I endonuclease, whereas defective stocks (1/100 or lower) produce DNA which is resistant to the R_I endonuclease.

One interesting and potentially important question posed by such defective viruses is whether or not they are capable of transforming cells. The target size for inactivating the transforming activity of the polyoma genome is about 20% that for inactivating the plaque-forming activity (Latarjet *et al.*, 1967). Thus, defective molecules with only a fraction of the genome may be capable of transformation, even though they cannot code for all the products required to produce mature virions. It is conceivable that the defective viruses are more efficient at transforming cells, if they have lost genes whose expression prevents maintenance of stable transformation (Folk, 1973) or reduces the

probability of the cell surviving. We are presently testing such a possibility.

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