Polyoma Virus Minichromosomes: Associated DNA Molecules

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Electron microscopy was used to identify and quantitate DNA molecules associated with ^3H -labeled polyoma minichromosomes which had been fractionated on a sucrose gradient. The percentage of replicating DNA molecules observed in the fractions of the gradient normally designated the replicative intermediate region was up to ninefold higher than in fractions from the mature region. Nevertheless, because of the higher overall concentration of polyoma DNA molecules in the mature region, nearly as many replicating DNA molecules were computed to be in the mature region as in the replicative intermediate region. The replicating molecules in the mature region were predominantly early replicative intermediates. Almost all late replicative intermediates were found in the replicative intermediate region. Under aqueous spreading conditions, a substantial fraction of the replicating DNA structures appeared to be asymmetrical or otherwise unusual, suggesting that extensive single-stranded regions may exist in replicating polyoma minichromosomes.

The enzymes associated with replicating and nonreplicating simian virus 40 or polyoma minichromosomes have recently been the subject of several studies (10, 20, 24, 25). In all of these reports, replicating minichromosomes were presumed to coincide with the peak of incorporated radioactive pulse label, but no quantitative statement could be made about the number of replicating DNA molecules. This number would be useful to calculate the number of enzyme molecules (15) and T-antigen molecules (G. Humphrey, B. B. Gourlie, M. R. Krauss, R. M. Benbow, and V. Pigiet, manuscript in preparation) associated with each replicative intermediate (RI) minichromosome and to calculate the extent of DNA replication on replicating molecules in the above *in vitro* systems.

In this paper, we classify the DNA structures and quantitate RI DNA molecules found in fractions of the polyoma minichromosome gradients of Gourlie et al. (14). To determine the absolute concentrations of replicating and nonreplicating polyoma DNA molecules, we used the technique of quantitative electron microscopy (3, 5, 19, 21). Nonparametric statistical analysis was used to demonstrate the reproducibility of the technique and the validity of the calculated concentrations.

Quantitative electron microscopy provided information which was not obvious by other methods. For example, nearly as high concentrations of unambiguous RI DNA structures were present in the mature region of polyoma minichromosome sucrose gradients as in the RI region. However, virtually all of the replicative inter-

mediates in the mature region were early and would be expected to contain little pulse label (14). Almost all late replicative intermediates were found in the RI region. A second major finding was that asymmetrical and unusual replicating molecules were observed at higher levels than symmetrical θ (RI) structures (7, 8, 16). These structures raise questions about the mechanism of replication of isolated polyoma minichromosomes.

MATERIALS AND METHODS

Polyoma minichromosomes (nucleoprotein complexes). Polyoma minichromosomes were harvested from infected cells and purified as described in the companion paper (14). The individual fractions of the sucrose gradient were adjusted to a final concentration of 0.3 M NaCl and then either mixed with a known volume of plasmid pXlr101 DNA (6) or prepared without plasmid for electron microscopy. We selected 0.3 M NaCl because this salt concentration was used when replicative enzymes were assayed in fractions of the gradient (15). Similar results were obtained with 2 M NaCl. The total ionic strength during spreading was 0.75 M ammonium acetate and 0.03 M NaCl, high enough to remove most proteins.

Electron microscopy. The aqueous Kleinschmidt method, as modified by Davis et al. (9), was used to visualize the DNA. Samples were not dialyzed to remove sucrose or otherwise treated. The ability to observe DNA without added manipulations such as dialysis, phenol or other extractions, or ethanol precipitation minimized selective losses during sample preparation for microscopy. The sample remained on the hypophase for 3 min to relax the DNA (see below). For detailed characterizations, the polyoma DNA was photographed either at $\times 30,000$ magnification with a

Siemens Elmiskop 1A or at $\times 20,000$ with a JEOL JEM 100B electron microscope. For quantitative electron microscopy with plasmid pXlr101 DNA, molecules were counted at $\times 20,000$ magnification. Photographs were printed on Ilfospeed 5.1 M glossy paper without further contrast enhancement.

Quantitative electron microscopy: total polyoma DNA concentration. Five microliters of standard plasmid pXlr101 (5.2 $\mu\text{g}/\text{ml}$; 16.15 kb/molecule) was added with polyoma DNA to the hyperphase (just before spreading) for quantitation of polyoma DNA molecules. The plasmid was isolated as a single peak on a high-salt isokinetic sucrose gradient, shown to be a single band on an agarose gel, and judged to be more than 95% pure (M. Bayne, unpublished data). Nine samplings of 100 molecules from nonoverlapping regions were counted for each fraction. For statistical reasons, the median value was used for further calculations (see below). All nonlinear molecules were classified as either large or small. Since large polyoma structures could be misclassified as pXlr101 molecules, a correction was made. The frequency of pXlr101 molecules was set equal to the median frequency of observed large structures minus the median frequency of large polyoma structures determined in the absence of pXlr101. Similarly, the frequency of polyoma molecules was set equal to the median frequency of observed small structures plus the median frequency of large polyoma structures determined in the absence of pXlr101. From these values and the known concentration of pXlr101 DNA, we calculated the concentration of total polyoma DNA as follows: (median frequency of polyoma/median frequency of pXlr101) $\times$ [pXlr101], where concentration is expressed as molecules per milliliter.

Polyoma DNA replicative intermediates. Polyoma DNA was spread for microscopy in the absence of pXlr101, and at least 9 samplings were counted as above. Using these numbers and the concentration of total nonlinear polyoma DNA calculated above, we calculated the concentration of total RI DNA molecules as follows: (median frequency of total RI DNA/median frequency of total polyoma) $\times$ [total polyoma], where concentration is expressed as molecules per milliliter. The concentrations of the individual classes of replicative intermediates were computed similarly. This equation implicitly assumes that molecules identified as replicative intermediates are not products of recombination or other reactions which join together (or break apart) initially monomeric structures. In support of this assumption, only 2 of 9,000 molecules were unambiguous recombinant molecules (i.e., figure-eight structures [4]).

Complex DNA populations for statistical analysis. Since the replicative intermediates of polyoma DNA were analyzed from a complex population of molecules, as a control we carried out a statistical analysis of a sample of pXlr101 DNA which contained at least 20 different structures. To simplify analysis, the structures were grouped into six categories: supercoiled; relaxed and nicked; linear; σ ; multimeric; and ambiguous. The frequencies ranged from 49% relaxed and nicked structures to 1% σ structures. Extensive statistical analyses of this population as well as of a simpler mixed population consisting of monomeric

ColE1 and pXlr101 molecules are given in Results.

Statistical methods. To evaluate the accuracy and reproducibility of quantitative electron microscopy, we performed a nonparametric statistical analysis (22). Nonparametric methods were used because the integral numbers of molecules in each morphological category were not necessarily normally distributed. The χ^2 test was applied in two ways. First, to test whether the distribution of types of molecules was consistent from one population to the next, all data for each structure were summed, and then all sums from one population were paired with the same structures from another population. Second, to test whether the distribution of frequencies (molecules per 100) was consistent from one population to the next, the raw data of N sets of 100 molecules for each structure were paired with the data for the same structure from a second population. By pairing in the order in which the data were taken, we sought to randomize any ordering of the actual observations. The test was also used to evaluate the difference between raw data and expected values (22).

The χ^2 tests were interpreted as follows. Assuming that the data were the same, the probability associated with a particular χ^2 , with n = degrees of freedom, was obtained from standard tables. When $P \geq 0.10$, the χ^2 was relatively small, and the data were said to be not significantly different. This criterion was extremely stringent for evaluating the similarity of two populations.

To show that values from two sets of data had the same median or that the median from one set of data was the same as an expected value, the Mann-Whitney U test was used. This test was interpreted as follows: when $\alpha = 0.10$, the expected maximum and minimum values for U are obtained from standard tables. If the observed U is between the expected values, then the medians are not significantly different. We used $\alpha = 0.10$ as a stringent test for the similarity of medians.

RESULTS

Quantitation of polyoma DNA in each fraction of the minichromosome sucrose gradient. Fractions were taken directly from the sucrose gradient of Gourlie et al. (14) and spread simultaneously with a plasmid pXlr101 DNA standard. The concentration of total polyoma DNA per fraction was calculated as described in Materials and Methods (Table 1). The peak value of 4.1×10^{11} molecules per ml in fraction 11 coincided with the peak of [^{14}C]thymidine incorporation and corresponded to the center of the region of the gradient containing labeled mature forms of polyoma DNA. Similarly, the skewed region of the DNA concentration curve, extending to fraction 7, coincided with the skewed region of the [^{14}C]thymidine incorporation curve and corresponded to the region of the gradient which contained labeled RI DNA (see Fig. 1).

Accuracy of quantitative determinations of DNA concentration. Different ratios of purified plasmid pXlr101 and ColE1 DNA mole-

cules were mixed and spread for electron microscopy to see if the observed number of molecules would be different from the predicted number. Nine observations of 100 molecules were made at each dilution. The expected values of 81, 15, 3.4, and 0.7 ColE1 DNA molecules per 100 molecules observed were not significantly different from the observed median values as judged by the Mann-Whitney *U* test. However, χ^2 analysis showed the data to be significantly different. This was due to one data point (1 set of 100 molecules) observed for the 1:2,500 dilution (3.4 ColE1 DNA molecules per 100, expected value). This data point was so wrong that it made the composite data for this dilution significantly different from the expected value. This emphasized the importance of using median values for calculating DNA concentrations, since stray data points are deemphasized in the final calculation.

Identification of replicative intermediates in each fraction of the minichromosomes sucrose gradient. Fractions taken directly from the sucrose gradient of Gourlie et al. (14) were also prepared for electron microscopy without a plasmid standard. For each fraction, 1,000 molecules were classified (Table 2) based on their morphology (3–5). All structures which conceivably could be replicative intermediates were assigned to one of three classes (putative, probable, or possible replicative intermediates) depending on how easily the structure could be reconciled with previously described intermediates in polyoma DNA replication (7, 12, 16, 18). All structures with two branch points and three arms, two of which were equal (Fig. 8 in 14), were classified as putative replicative intermediates. All structures with obviously more than one genome equivalent of DNA were classified as either probable or possible replicative intermediates (see Fig. 1 for examples), as shown in Table 2.

Some of the structures classified as probable or possible replicative intermediates had amorphous material (residual nuclear matrix?) associated with the DNA (Fig. 2). Although these molecules were placed in separate categories, there was no evidence that they differed from molecules without amorphous material. Molecules with amorphous material were observed even after treatment with 2 M NaCl. As a control, a portion of fraction 12 was extracted twice with buffer-saturated phenol and twice with anhydrous ether and was then spread for electron microscopy. The number of molecules with amorphous material was reduced, but the kinds and percentages of putative replicative intermediates were not significantly altered (except

TABLE 1. *Determination of DNA concentration^a in fractions of a polyoma minichromosome sucrose gradient*

Fraction no.	Median frequency ^b		Maximum relative error (%) ^c	Polyoma concn ($\times 10^{11}$ molecules/ml)
	pXlr101	Polyoma		
6	0.91	0.09	10.8	0.3
7	0.88	0.12	9.9	0.4
8	0.79	0.21	7.0	0.8
9	0.71	0.29	5.5	1.2
10	0.59	0.41	4.1	2.0
11	0.42	0.58	2.9	4.1
12	0.59	0.41	4.2	2.0
13	0.70 ^d	0.31 ^d	5.0	1.3
14	0.93	0.07	11.8	0.2
16	0.98	0.02	22.1	0.1

^a Equal volumes of plasmid pXlr101 (5.2 μ g/ml) and sucrose gradient fraction (in 0.3 M NaCl) were simultaneously spread for electron microscopy. For minimum error, the concentration of the standard plasmid was adjusted to give ~50:50 median frequency ratios in the mature region of the gradient. Fraction 15 was not determined.

^b Nine samplings of 100 molecules were made for each fraction. The median values were selected and used for calculation as described in Materials and Methods.

^c Calculated (see Table 4) for the total frequency of polyoma molecules.

^d The median frequencies for pXlr101 and polyoma are independent and do not necessarily sum to 1.0.

for the transfers to categories without amorphous material). The possibility that the amorphous material corresponds to nuclear matrix is under investigation.

Replicative intermediates were not limited to gradient fractions 7, 8, and 9, previously referred to as the RI region based on radioactive pulse-labeling experiments (14). Our data (and additional unpublished data) showed that late replicative intermediates were found preferentially in the RI region, but that early replicative intermediates were found in both the RI and nature regions.

Characterization of unusual or asymmetric DNA replication intermediates. Previous characterizations of polyoma RI DNA (7, 16) emphasized the symmetry of the observed θ structures and the paucity of extensive single-stranded regions (which would collapse to form bushes under the spreading conditions used, and thus shorten the measured contour lengths of one or more arms of the θ structures). As a control to establish that the high percentage of asymmetric structures we observed were not artifacts either of our direct spreading procedure or of our isolated polyoma minichromosomes, we examined *in vivo* replicating polyoma DNA

FIG. 1. RI structures. (A) Putative replicative intermediate: late θ structure. The replicated region consists of two unequal-length arms of 1.3- and 1.5- μm contour length. The unreplicated region is 0.11- μm contour length. (B-D) Probable replicative intermediates: plus structures. The contour lengths are 2.0, 2.5, and 2.2 μm , respectively. It is unlikely that the unusual DNA molecules in each panel result from spreading artifacts since they were also seen in phenol-extracted DNA and were not seen in the added standard DNA molecules. In addition, similar molecules were seen in the Hirt supernatant of polyoma DNA replicating *in vivo* (see Fig. 3). The scale marker equals 0.5 μm . All photographs are the same magnification. From the uneven appearance of the background in Fig. 1 and 2, it seems likely that the minichromosome proteins are distributed uniformly throughout the field. Extensive control experiments established that these contaminating proteins do not significantly interfere with either identification or quantitation of the replicative intermediates.

isolated by the Hirt procedure (16) from infected 3T6 cells. Asymmetric and unusual structures similar to those shown in Fig. 1 were seen and were abundant (Fig. 3). We postulate that these structures were not observed previously (7, 16) because sedimentation procedures were used to isolate the DNA which inadvertently selected for particular classes of replicative intermediates. To confirm the existence of extensive single-stranded regions, the *in vivo* replicating DNA was treated with the single-strand nuclease S1 and reexamined by electron microscopy. The percentage of asymmetric (plus)

structures was reduced from ~ 40 to $\sim 5\%$, and the percentage of σ or two-tailed structures increased to $\sim 30\%$.

Quantitation of replicative intermediates in each fraction of the minichromosome sucrose gradient. The frequency of total replicative intermediates in the mature region was too low to permit quantitation to the desired degree of accuracy based on medians of sets of 100 molecules (see Table 4). We therefore counted additional molecules and used medians based on sets of 1,000 molecules to more accurately determine the percentages of replicative

TABLE 2. *Distribution of molecular forms of polyoma DNA^a*

Structure	No. of molecules in given fraction ^b									
	6	7	8	9	10	11	12	13	14	
Simple ^c										
Monomeric	285	394	391	515	609	795	763	592	596	
Monomeric with amorphous material	108	109	117	88	75	94	110	128	58	
σ	3	9	12	7		8	6	9	2	
σ with tail(s) ^d		2	7	2	8	2		6	2	
α^e	3		1	3				1	2	
Mini monomeric	15	11	15	5	13	2	5		157	
Mini- σ	19	21	12	5	5				6	
Mini- α			1					27		
Linear										
Linear, polyoma length	222	161	81	124	90	34	19	153	73	
Linear, >polyoma length ^f	202	137	104	115	87	11	11	38	56	
Y ^g	19	21	17	13	19	5		4	5	
X and H ^h	6	4	6	4	5	2			3	
Putative replication										
θ (early) ^{c,i}	11	6	31	9	8	6	4	3	5	
θ (mid) ^c	5	6	19	4	1		1			
θ (late) ^k	4	4	8	6		1		2		
Dimeric (ambiguous linkage) ^{j,k}	7	16	12	17	15	6	9	3	2	
Catenated ^k	4	1					2			
Probable replication										
Plus ^{c,l}	30	42	94	37	29	19	28	21	18	
Circular dimeric ^k		6	6	2				1		
Other dimeric ^{k,m}	1	2	2		1		2		1	
Dimeric (ambiguous linkage) with amorphous material ^k	27	24	16	21	13	4	21	3	7	
Trimeric ^{k,n}	3		3	4	2					
Tetrameric ^k		4	1							
Possible replication										
θ^c (early and mid) with amorphous material	6	1	5	6	1					
Multimeric with amorphous material ^k	24	20	31	11	21	7	14	1	6	
Other possible replication ^{k,o}	3		4	2	1			2		
Ambiguous	2	1	3	5		2	2	3	1	

^a Sucrose gradient fractions (in 0.3 M NaCl) were spread for electron microscopy. About 1000 molecules were classified for each fraction to obtain a statistically significant sample of each population.

^b Fractions 6, 8, and 13 were observed to contain large numbers of small DNA fragments.

^c Small structures.

^d Also includes σ structures with amorphous material: 2 molecules of the 29 observed.

^e Also includes α structures with amorphous material: 1 molecule of the 14 observed.

^f Also includes linear structures with amorphous material: 2 molecules of the 761 observed.

^g Also includes Y structures with terminal loop: 2 molecules of the 103 observed.

^h Also includes complex branched structures: 1 molecule of the 34 observed.

ⁱ Also includes θ (early) with whisker: 1 molecule of the 83 observed.

^j Also includes figure-eight structures: 2 molecules of the 87 observed.

^k Large structures.

^l Also includes plus structures with tail: 1 molecule of the 317 observed.

^m This category includes dimeric structures (ambiguous linkage) with tail (1 molecule), dimeric structures (ambiguous linkage) with eye (2 molecules) and dimeric structures (dumbbell linkage) (6 molecules).

ⁿ Also includes trimeric structures with amorphous material: 2 molecules of the 12 observed.

^o Includes circular tetrameric structures with tail (large structures; one molecule), mini- θ (small) structures (early; two molecules), Y structures with eye (three molecules) and linear structures (with eye) of greater than polyoma length (six molecules).

FIG. 2. RI structures with amorphous material. (A) Possible replicative intermediate: θ structure with amorphous material. The replicated region consists of two unequal arms of approximately 0.34- and 0.24- μm contour lengths. The unreplicated region is 0.94 μm . (B) Probable replicative intermediate: late θ structure with amorphous material. The replicated region consists of two unequal arms of 0.86 and 1.5 μm . The unreplicated region is 0.05- μm contour length. (C) Possible replicative intermediate: multimeric structure with amorphous material. Contour lengths were not determined. Scale marker equals 0.5 μm . All photographs are the same magnification.

intermediates shown in Table 3. The net effect of this was to give each of the frequencies for total replicative intermediates a maximum error of $\pm 10\%$.

Using these ratios, we then calculated the number concentration (molecules per milliliter) of the RI structures in each fraction as described in Materials and Methods. Figure 4 shows the

concentrations of putative replicative intermediates and total replicative intermediates along with the [^{14}C]thymidine profile (14). The shoulder of radioactive label which centered on fraction 8 coincided with the fraction containing the highest concentration of replicative intermediates. However, high levels of replicative intermediates were found in a broad peak ranging

from fractions 8 through 12.

Statistical analysis. The validity of our quantitation of replicative intermediates depends entirely on the reproducibility of quantitative electron microscopy. As one test of reproducibility, 4 sets of 100 molecules from a complex population of pXlr101 were observed by the same investigator on two different occasions. With the procedure outlined in Materials and Methods, χ^2 values ranged from $\chi^2_3 = 1.14$ ($P > 0.70$, σ structures) to $\chi^2_3 = 4.37$ ($P > 0.20$, linear

structures). When the data were summed, $\chi^2_6 = 4.9$ with $P > 0.25$. The U values ranged from 5 to 11.5, and all were within the acceptable limits at $\alpha = 0.10$.

When 9 sets of 100 molecules were identified on two different grids touched at 1-min intervals to the same hypophase, the χ^2 values ranged from $\chi^2_8 = 5.25$ ($P > 0.70$, supercoiled structures) to $\chi^2_8 = 17.13$ ($P > 0.02$, linear structures). When the data were summed, $\chi^2_6 = 13.87$ ($P > 0.025$). The U values ranged from 27.5 to 69.9. Only

FIG. 3. Unusual and asymmetric RI structures. Polyoma DNA replicating *in vivo* in infected 3T6 cells was isolated by the Hirt procedure (16) and examined by the aqueous spreading procedure of Davis *et al.* (9). Twenty-five to 30% of the structures containing more than one genome length of DNA were asymmetric or otherwise unusual. Probable replicative intermediate (plus) structures. (A) The unreplicated region is 0.95 μm ; the replicated regions are 0.79 and 0.65 μm . The shortened contour length of one arm and the apparent bushes could be accounted for by the collapse of a single-stranded region(s) of 432 base pairs. (B) The unreplicated region is 0.11 μm ; the replicated regions are 1.59 and 1.24 μm . The shortened contour length of one arm and the apparent bushes could be accounted for by the collapse of a single-stranded region of 1,444 base pairs. (C) The unreplicated region is 0.29 μm ; the replicated regions are 1.31 and 0.72 μm . The shortened contour length of one arm and the obvious bush could be accounted for by the collapse of a single-stranded region of 1,967 base pairs. (D) The unreplicated region is 0.15 μm ; the replicated regions are 1.46 and 0.74 μm . The shortened contour length of one arm and the obvious bushes could be accounted for by the collapse of single-stranded regions of 2,388 base pairs. The scale marker equals 0.5 μm . All photographs are at the same magnification.

TABLE 3. Ratio of median frequency of RI structures to median frequency of total polyoma structures

Fraction no.	Frequency ratio				Maximum relative error (%) ^a
	Putative RI	Probable RI	Possible RI	Total RI ^b	
6 ^c	0.058	0.115	0.096	0.250	9.1
7 ^c	0.045	0.119	0.015	0.194	8.5
8 ^c	0.088	0.163	0.050	0.288	5.8
9 ^c	0.038	0.103	0.026	0.141	8.7
10 ^d	0.010	0.045	0.014	0.068	6.7
11 ^c	0.010	0.020	0.005	0.033	6.4
12 ^f	0.012	0.041	0.007	0.061	5.6
13 ^c	0.012	0.012	0	0.036	17.8
14 ^c	0.011	0.023	0	0.034	16.1

^a Calculated for the total frequency of total replicative intermediates.

^b Median of the sums, not sum of the medians.

^c Data were determined from 9 independent observations of 100 molecules.

^d Data were determined from 3 independent observations of 1,000 molecules.

^e Data were determined from 7 independent observations of 1,000 molecules.

^f Data were determined from 5 independent observations of 1,000 molecules.

supercoiled structures ($U = 69.5$) were beyond the acceptable limits at $\alpha = 0.10$. When two independent sets of molecules were observed from the same grid and the data were summed, $\chi^2 = 10.3$, $P > 0.10$.

Three conclusions were drawn from this analysis. First, a single investigator could reproducibly classify the structures of this complex population. Second, multiple observations of 900 molecules from the same grid reproducibly exhibited the same distribution of structures and raw data. Third, individual samples of 100 molecules from two different grids did not reproducibly exhibit the same data distribution for linear, multimer, or ambiguous structures, whereas median values did reproducibly measure all except for supercoiled structures. Thus, grids touched sequentially to the same hypophase appeared to sample populations with different frequencies of supercoiled structures. We subsequently found that supercoiled DNA assumed the morphology of relaxed or nicked DNA, depending on the length of time the sample remained on the hypophase (Fig. 5).

To further test the reproducibility of our analysis, a mixture of ColE1 DNA (6.45 kilobases) and pXlr101 DNA (16.15 kilobases) was observed by two different investigators. Each counted 5 sets of 100 molecules. The molecules were identified as being either large or small. χ^2 values ranged from $\chi^2 = 0.827$ ($P > 0.90$, small

structures) to $\chi^2 = 4.07$ ($P > 0.30$, large structures). When the data were summed, $\chi^2 = 1.22$ with $P > 0.50$. The U values were 6.5 and 19 with upper and lower limits of 21 and 4, respectively.

The DNA mixture was also analyzed on two different days. This experimental design not only tested day-to-day variation but also the nuisance variables of using different grids, using different Parlodion films, and repeatedly spreading the same sample. The χ^2 values ranged from $\chi^2 = 1.33$ ($P > 0.99$, large structures) to $\chi^2 = 2.16$ ($P > 0.95$, small structures). When the data were summed, $\chi^2 = 2.46$ with $P > 0.25$. The U values were 48.5 and 52 with upper and lower limits of 60 and 21, respectively. In both of the experiments above, the χ^2 values were low enough to conclude that the data were not significantly different, and the U values were acceptable with the limits at $\alpha = 0.10$.

FIG. 4. Nuclear extract from polyoma-infected 3T6 cells was applied to a low-salt, 5 to 30% linear sucrose gradient and centrifuged as described by Gourlie et al. (14). Fractions 6 through 14 were prepared for electron microscopy in the presence and absence of pXlr101 DNA (standard at 5.2 $\mu\text{g/ml}$). The concentrations of total polyoma DNA, total RI DNA, and putative RI DNA were determined in molecules per milliliter. The [^{14}C]thymidine profile was taken from Gourlie et al. (14).

DISCUSSION

Using a refinement of the method of quantitative electron microscopy (3, 5, 19, 21), we analyzed the population of DNA molecules associated with polyoma minichromosomes separated by sucrose gradient sedimentation (14). Four important features of our analysis were: (i) the use of DNA spread directly from the sucrose gradient to eliminate extractions or manipulations which might bias the analysis; (ii) the relaxation of supercoiled DNA structures by permitting them to remain on the hypophase, thus eliminating the need to use nucleases; (iii) the use of nonparametric statistical analysis to show that our spreading techniques were reproducible and that DNA concentrations were accurately calculated for molecules appearing as infrequently as 7 in 1,000; and (iv) the use of ultrapure DNA standards to accurately calculate unknown DNA concentrations.

In all of the quantitative determinations above, 9 samplings of 100 molecules resulted in a population of molecules with $\pm 10\%$ error for frequencies as low as 0.10. When determining the concentration of total RI structures in fractions of the mature region, the frequency was very low and a population of 900 molecules was too small to achieve the desired 10% maximum relative error. As a guide for other investigators who wish to use quantitative electron microscopy, in Table 4 we have calculated the minimum population size and minimum sample size necessary to obtain particular relative errors for several molecular frequencies.

Our electron microscope analysis led to the following conclusions. The peak concentration of total polyoma DNA was present in the gradient fraction which contained the maximum *in vivo* [^{14}C]thymidine incorporation. The concentration of replicating DNA was the highest in the gradient fraction which corresponded with the accepted position of replicating minichromosomes (11, 14, 20, 23–25). However, we observed nearly as high concentrations of early RI DNA in gradient fractions traditionally referred to as mature DNA (11, 14, 20, 23–25). Table 2 showed that there were clearly more early than late RI structures in the mature region and that almost all late replicative intermediates and complex multimeric structures were found in the RI region.

All previous studies on replicating versus mature polyoma minichromosomes have implicitly or explicitly assumed that substantially higher numbers of replicative intermediates are found in the RI region (11, 20, 23–25). Our data showed this not to be the case. Moreover, our data underestimated the number of early RI struc-

FIG. 5. Apparent relaxation of supercoiled DNA during preparation for electron microscopy. Plasmid ColE1 DNA was isolated and purified by benzoylated naphthoylated DEAE-cellulose chromatography followed by high-salt isokinetic sucrose gradient centrifugation. The peak fraction containing supercoiled DNA (>95% pure by agarose gel electrophoresis) was directly spread for electron microscopy without dialysis or intervening steps. The hyperphase was sampled at 0, 1, 2, 3, and 4 min. Time zero is the minimum time to spread the sample, pick up a grid, and touch the hyperphase. This was 20 s or less. Symbols: (x) Supercoiled structures; (O) relaxed structures; (●) relaxed or nicked structures; (Δ) linear structures. When the plasmid was dialyzed into a low-ionic-strength buffer, the time for complete relaxation was approximately 4 min. A control experiment incubating supercoiled DNA in the spreading solution for 30 min showed that the DNA was not nicked or relaxed when analyzed by agarose gel electrophoresis. Control experiments using purified supercoiled and nicked DNA molecules showed that >90% of the simple structures could be properly identified by sampling the hyperphase at time zero. However, we adopted $t = 3$ or 4 min for the classification of polyoma DNA on Table 2, since most molecules were open and more easily classifiable under these conditions.

tures, since those with less than 100 base pairs replicated were not reliably identified (9, 13). Thus, reports of possible initiation using mature minichromosomes (25) must be tempered by the knowledge that a high concentration of early RI structures is found in this region.

Many of the replicating polyoma DNA molecules isolated from polyoma minichromosomes did not look like accepted intermediates of polyoma DNA replication (7, 16). For example, although plus structures (Table 2) had no easily recognizable morphology (Fig. 1), they were classified as probable replicative intermediates be-

TABLE 4. Quantitative electron microscopy: minimum population size^a and minimum sampling size^b

Maximum relative error (%) ^c	Minimum population and sample size for given frequency of molecule observed											
	0.001	0.005	0.01	0.02	0.03	0.04	0.05	0.10	0.20	0.30	0.40	0.50
3.3							17,100 (606)	8,100 (303)	3,600 (152)	2,100 (101)	1,350 (76)	900 (61)
5					12,933 (667)	9,600 (500)	7,600 (400)	3,600 (200)	1,600 (100)	933 (67)	600 (50)	400 (40)
10			9,900 (1,000)	4,900 (500)	3,233 (333)	2,400 (250)	1,900 (200)	900 (100)	400 (50)	233 (33)	150 (25)	100 (20)
25	15,984 (4,000)	3,184 (800)	1,584 (400)	784 (200)	517 (133)	384 (100)	304 (80)	144 (40)				
50	3,996 (2,000)	796 (400)	396 (200)	196 (100)	129 (67)	96 (50)						

^a If the frequency (f) of a molecule equals 0.50, the maximum relative error equals $\frac{1}{\sqrt{n}}$, where n equals the number of molecules observed. To determine lower molecular frequencies ($f < 0.50$) to the same maximum relative error of a frequency of 0.50, a larger number of molecules (n') must be observed, where the minimum population size $= n' = \frac{1-f}{f} \times n$. Similarly, the maximum relative error is $\sqrt{\frac{1-f}{n'(f)}}$. For example, to quantitate a molecule which appears at 0.01 (1%) frequency to $\pm 10\%$ error, no fewer than 9,900 molecules must be observed. If only 1,000 molecules were observed, the error would be between ± 25 and $\pm 50\%$.

^b Since median values should be used to quantitate molecular populations, it was necessary to determine the minimum sampling size (indicated in parentheses). Since 1 standard deviation $= f \times$ maximum relative error and $1/n^* = 1$ standard deviation, the minimum sampling size (n^*) can be determined for all molecular frequencies and maximum relative errors. For example, to quantitate molecules which are found at a frequency of $f = 0.01$ to better than $\pm 10\%$ error, samplings of 1,000 molecules must be made. From the data shown, at least 9,900 total molecules must be observed. Thus, to accurately determine a median value with better than $\pm 10\%$ error, 11 batches of 1,000 molecules must be observed to give the frequency $f \geq 0.01$ better than $\pm 10\%$ error.

^c It is important to note that the maximum relative error is predicted by the properties of numbers and not computed from multiple determinations of the same value.

cause they had substantially more than 5.2 kilobases of DNA. Other molecules had the general morphology of accepted replicative intermediates but had unequal arm lengths in the presumptive replicating strands (Fig. 1-3).

Many of the unusual structures and unequal arm lengths we saw could be explained by the presence of extensive single-stranded regions which would have collapsed under the aqueous spreading conditions used for electron microscopy. There are at least two plausible explanations for the single-stranded regions. Since we looked at DNA prepared from isolated minichromosomes, it is possible that the replicating intermediates became aberrant during minichromosome isolation. In addition, naturally occurring single-stranded regions in replicating eucaryotic DNA have been described, especially in D loops of mitochondrial DNA (17) and in embryonic sea urchin DNA in vivo (1).

We favor a model for polyoma DNA replication with asynchronous leading versus lagging strand synthesis and a mechanism for initiation which uses D loops (such as shown in Fig. 2c of reference (2)). If lagging strand synthesis is delayed at one or both replication forks (as in mitochondrial DNA synthesis), the resulting single-stranded DNA will collapse under aqueous conditions and create asymmetrical structures

consistent with those we observed in the electron microscope. Experiments which strongly support asynchronous unidirectional DNA synthesis in replicating polyoma DNA molecules are described elsewhere (A. Buckler-White, M. R. Krauss, R. M. Benbow, and V. Pigiet, submitted for publication).

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