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Asynchronous Bidirectional Replication of Polyoma Virus DNA†

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The structure of polyoma virus replicative intermediates isolated from infected 3T6 cells was analyzed by two-dimensional agarose gel electrophoresis (Sundin and Varshavsky, Cell 21:103–114, 1980) and quantitative electron microscopy (Krauss and Benbow, J. Virol. 38:815–825, 1981). DNA replication was initiated at a single site (*ori*) in essentially all of the replicative intermediates. Most of the early replicative intermediates were formed by unidirectional synthesis in the direction of early transcription. Most mid- and late replicative intermediates contained two replication forks which had traveled unequal distances from the origin. Asynchronous initiation of the two growing forks was postulated to account for these observations.

DNA replication in papovaviruses was originally reported to proceed bidirectionally from a unique origin (7, 8, 18). Alternative unidirectional (17) or rolling circle (2) mechanisms have also been proposed to account for the infrequent replicative intermediates which were not consistent with bidirectional synthesis from the normal origin. Aberrant replicative intermediates which appear to be inconsistent with bidirectional synthesis can also be generated by unequal replication fork growth rates (3, 7, 10, 24), by "death" of one fork (3), or by shearing of replicative intermediates during isolation (10).

During our studies on replication of polyoma virus in isolated 3T6 cell nuclei (Buckler-White and Pigiet, manuscript in preparation) and on the replication of polyoma virus DNA (1) or polyoma virus minichromosomes (unpublished data) in extracts of unfertilized frog eggs (*Xenopus laevis*), we observed high frequencies of replicative intermediates which were inconsistent with simple bidirectional synthesis proceeding at equal fork rates. This led us to reexamine normal polyoma virus DNA replication in infected 3T6 cells in vivo. Most previous experiments either examined the replication of selected populations of molecules (6) or measured values which were statistically averaged over large numbers of molecules (8).

In this work we directly examined the structures of individual polyoma virus replicative intermediates by a combination of two-dimensional gel electrophoresis (23) and quantitative electron microscopy (13). We present evidence that most of the polyoma virus replicative intermediates isolated directly from infected 3T6 cells were synthesized by a mechanism more complex than simple bidirectional synthesis. An alternative hypothesis which involves asynchronous initiation of the two growing forks is proposed.

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MATERIALS AND METHODS

Preparation of replicating polyoma virus and simian virus 40 (SV40) DNAs. Mouse 3T6 cells were infected as described previously (5) with a large-plaque strain of polyoma virus (12, 25) grown from a single plaque isolate at low multiplicity (0.001) on primary baby mouse kidney cells. [^{3}H]thymidine (Amersham Corp.; 40 to 60 Ci/mmol; 1 mCi in 3 ml of culture medium) was added for 5 or 30 min before the cells were harvested at 27 h after infection. Labeling was stopped by three washes with ice-cold phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.0).

Replicating SV40 DNA was prepared from virus and BSC-40 cells obtained from D. Nathans, Johns Hopkins University School of Medicine. Infected cells were labeled with [^{32}P]orthophosphate (Amersham; carrier-free) from 39 to 40 h after infection.

In vitro replication of polyoma virus DNA. Nuclei were isolated at 4°C (5) by agitating the cells in isotonic HEPES buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 220 mM sucrose, pH 7.8) with 0.5% (wt/vol) nonionic detergent Nonidet P-40 (Sigma Chemical Co.). After centrifugation (600 × *g*, 5 min), the nuclei were incubated for 30 min at 25°C in 1 ml of reaction mixture containing 40 mM NaCl, 5 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid), 5 mM $MgCl_2$, 4 mM phosphoenolpyruvate, 2 mM ATP, 70 μM each dATP, dCTP, and dTTP (Sigma), 2 μM [α-^{32}P]dGTP (Amersham; 400 Ci/mmol), and 15 U of pyruvate kinase (Calbiochem). The reaction was stopped by 10-fold dilution in isotonic HEPES buffer, and the nuclei were recovered by centrifugation (600 × *g*, 5 min).

Restriction endonuclease digestion. Viral DNA was selectively extracted by the procedure of Hirt (11) and digested with RNase A (25 μg/ml; Sigma type XII-A) for 30 min at 37°C and with proteinase K (25 μg/ml; EM Labs) for 60 min at 37°C. The DNA was desalted by chromatography on Bio-Gel P-60 (Bio-Rad Laboratories), precipitated with 3 volumes of ethanol, and dissolved in appropriate buffers for subsequent digestion. Restriction endonucleases *Bgl*I and *Eco*RI were obtained from Bethesda Research Laboratories and used as specified by the manufacturer.

Two-dimensional agarose gel electrophoresis. Digestion with *Bgl*I was terminated by addition of one-fourth volume of 50% glycerol containing 0.05% bromphenol blue, 0.05% xylene cyanol, 1% sodium dodecyl sulfate, and 50 mM EDTA. Samples were electrophoresed at 4°C on submerged agarose gels (4 mm thick; 1.2%) at 5 V/cm for 18 h in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.8).

Individual tracks were excised, soaked in alkaline buffer (30 mM NaOH, 2 mM EDTA, 1 mM EGTA), cast into 1.5% alkaline agarose gels, and electrophoresed submerged at 2.5 V/cm for 20 h at 4°C (23). For samples labeled with ^{32}P, gels were dried onto filter paper and autoradiographs were made with Kodak X-Omat R X-ray film at room temperature. For samples labeled with [^{3}H]thymidine, gels were impregnated for 3 h with Enhance (New England Nuclear Corp.), washed in water for 3 h, dried, and exposed at −70°C.

Electron microscopy. Viral DNA was precipitated with ethanol and either suspended in 10 mM Tris–1 mM EDTA (pH 7.8) or digested with *Bgl*I or *Eco*RI as described above. DNA was spread for electron microscopy on Parlodion-coated 3-mm copper grids by the aqueous procedure of Davis et al. (9). Grids were stained with uranyl acetate, rotary shadowed with platinum:palladium (80:20), and photographed on a Japan Electron Optics Laboratory JEM 100B electron microscope at 20,000× magnification. Contour lengths were traced at 10× magnification on a Nikon model 6 shadowgraph and measured with a Keufel and Esser map measurer. Electron micrographs were printed directly on Ilfospeed 5.1 M paper without further contrast enhancement. Quantitation of the frequencies of replicative intermediate structures was carried out as described by Krauss and Benbow (13).

For intermediates cleaved with *Bgl*I, length was defined as the sum of A + B + D (see the legend to Fig. 6), where A is the length of the unreplicated portion, B is the length of the longer arm of the longer fork, and D is the length of the longer arm of the shorter fork. The conventional definition of length, A + (B + C)/2 + (D + E)/2 (7) was not used because extensive single-stranded regions have been reported in replicating polyoma virus DNA molecules (13). These single-stranded regions collapse under aqueous spreading conditions and artifactually shorten the calculated contour length of some arms. However, formamide spreading conditions which extend single-stranded DNA cannot be used because of substantially greater variations in contour length measurements (9; see below). To establish the validity of our definition of length, we analyzed the measurements (shown in Fig. 4–6) with the University of Minnesota SPSS program (McGraw-Hill Book Co., New York, 2nd ed., 1975). For polyoma virus replicating in vitro (n = 103 molecules), contour length (L) calculated as L = A + B + D yielded a mean value of 1.689 μm (1.695 μm is the actual contour length of monomeric polyoma virus DNA of this strain), σ = 0.055 μm, kurtosis = 0.028. By contrast, if L had been calculated as L = A + (B + C)/2 + (D + E)/2, then the mean value would have been 1.671 μm, σ = 0.115 μm, kurtosis = 3.456. Note that both the standard deviation (σ) and the kurtosis (a measure of the normality of the distribution) increased when the latter equation was used for length calculations. Moreover, the skewness for this latter distribution was −0.402, confirming the excess of shortened arms consistent with the collapsed single-stranded regions observed by Krauss and Benbow (13). Intermolecular variations in contour lengths in aqueous spreads calculated by L = A + B + D were about 150 base pairs, and intramolecular variations (see Fig. 4–6) were substantially less than 100 base pairs.

Percent replicated was defined as 100 − %A, where %A is defined as A/L × 100. The asymmetry index was defined as 5,365 × (%B − %D), where 5,365 is the number of base pairs per genome of the strain of polyoma virus used in these experiments. %B is defined as B/L × 100, and %D is defined as D/L × 100.

Calculations for structures resulting from *Eco*RI cleavage of polyoma virus replicative intermediates were done as follows. The length for Y and H structures was defined as above. Length for linear structures containing eyes was defined as A + B + D (see the legend to Fig. 5). Percent replicated was defined as B/L × 100 for linear structures with eyes and as described above for Y and H structures. Percent A, for linear structures with eyes, was defined as A/L × 100 and corresponds to L1 used by Crawford et al. (7).

RESULTS

Frequency of replicative intermediates. Direct quantitative electron microscopy (13) of deproteinized viral DNA was used to establish the frequency and conformation of putative replicative intermediates. It should be emphasized that we examined total extracted DNA, not molecules selected by velocity or equilibrium sedimentation. All preparations contained 2 to 5% putative replicative intermediate structures (Table 1). About 90% of these were θ structures and about 10% were σ structures. σ structures with tails equal to or less than a full genome length could have been generated either by breakage of

TABLE 1. Relative frequency of θ and σ structures in replicating polyoma virus and SV40 DNAs

DNA prepn	No. of Cairns (θ) structures	No. of σ structures[a]		Total molecules examined[b]
		Tail ≥ 1	Tail < 1	
In vivo 1	51	1	5	2,500
In vitro	54	1	6	2,500
SV40	88		10	5,000
In vivo 2	491	13	27	11,000

[a] A tail length of 1 is a full genome length.

[b] Includes only structures containing circular polyoma virus DNA; linear DNA molecules (host, cleaved polyoma virus, etc.) were not counted.

θ structures or as rolling circle intermediates. Very few σ structures with tails greater than unit length (unambiguous rolling circle structures) were observed, although we cannot rule out trapping of some high-molecular-weight viral DNA species in the precipitated fraction (11). Both the frequency of total replicating molecules and the individual frequencies of θ and σ structures (Table 1) were consistent with previous reports (2, 4, 10). There was no evidence for excessive breakage of θ structures or extensive rolling circle synthesis. Similar frequencies of θ and σ structures were also obtained by different isolation procedures, with several independent isolates of different strains of polyoma virus, and with several different incubation protocols (data not shown).

Two-dimensional gel electrophoresis of cleaved replicative intermediates. The restriction endonuclease *BglI* cleaves both SV40 and polyoma virus DNAs within 100 base pairs of the origin (20, 21). Replicating polyoma virus and SV40 DNAs were digested with *BglI* and analyzed by two-dimensional gel electrophoresis (23). In this procedure, replicative intermediates are separated in the first dimension as a function of the extent of replication. Early replicative intermediates of a given conformational class usually migrate more rapidly than do late replicative intermediates. Electrophoresis in the second dimension under alkaline denaturing conditions then separates the labeled daughter strands as a function of single-strand length (i.e., distance of the fork from the *BglI* cleavage site).

Autoradiographs of two-dimensional gels of replicating polyoma virus or SV40 DNA are shown in Fig. 1. In each autoradiograph, a prominent diagonal band (labeled H; see below) extends from approximately 50% of the genome length (2,680 base pairs) to a position corresponding to the migration of small oligonucleotides. This band was broader than was expected from the band width in the first dimension, indicating that the labeled daughter strands were heterogeneous in length. This length heterogeneity was even greater in molecules replicated in vitro (compare Fig. 1A, B, and D with C). A fainter second diagonal band (labeled Y; see below) is observed parallel to the major band and curving back to the position of full-length linear molecules.

In all four gels a substantial fraction of the labeled molecules migrated as full-length linear molecules in the first dimension, but as molecules of approximately 50% of the genome length in the second dimension. We assume that these correspond to the termination intermediates described in SV40 by Sundin and Varshavsky (23) and Laipis et al. (14) which accumulate during in vitro synthesis (19, 22).

To identify the structures of the replicative intermediates which comprise the prominent and faint bands, we excised the regions indicated in Fig. 1B from a duplicate first-dimension gel, dissolved them in potassium iodide, and examined them by quantitative electron microscopy (13). The first region contained almost exclusively H structures (27 out of 29 molecules), with arm lengths characteristic of late replicative intermediates. The second region contained a mixture of Y and H structures (25 Y and 10 H structures out of 35 molecules). The H structures exhibited arm lengths characteristic of early or mid-replicative intermediates. Although we cannot rigorously prove that the molecules seen in the electron microscope correspond to the labeled species observed on the autoradiographs, it is reasonable to assume that the major diagonal bands shown in Fig. 1 correspond to H structures and that the minor bands correspond to Y structures. The anomalous migration of Y structures with labeled daughter strands greater than 50% of the genome length was a result of optimization of gel electrophoresis for maximal resolution of H structures. At low voltages (1 V/cm, 90 h) in 0.8% agarose gels, the Y structures migrated as a nearly straight diagonal, but H structures were less well resolved.

The data in this section were consistent with previous reports that most polyoma virus and SV40 replicative intermediates are formed by bidirectional synthesis, although a small percentage replicate unidirectionally (2, 17). However, the width of the band of labeled H structures suggested that replication was not entirely symmetric or that growth of the two forks was asynchronous.

Quantitation of Y and H structures by electron microscopy. Quantitative electron microscopy was used to analyze the H (Fig. 2) and Y (Fig. 3) structures obtained from *BglI* digestion of DNA molecules replicated in vivo and in vitro. For polyoma virus in vivo, polyoma virus in vitro, and SV40 in vivo, 32, 41, and 35%, respectively, of the cleaved replicative intermediates were Y structures. The SV40 percentage (35%) was higher than that previously reported for SV40 replicative intermediates selected by fractionation techniques (15) and considerably higher than the percentage of possible rolling circle intermediates (Table 1). It is not possible to rule out artifactual generation of some Y structures (e.g., by breakage of θ structures). Nevertheless, the electron microscopic analyses (Fig. 4–6) suggested that most of these structures were formed during the normal course of replication.

We repeated the in vivo experiment with another plaque-purified isolate of our polyoma virus strain and counted more molecules to

improve the accuracy of our data. This preparation contained few σ structures (91.9% of the 531 putative replicative intermediates observed were θ structures). Nevertheless, over 41% of the replicative intermediates (out of 500 measured) were converted to Y structures during digestion with *Bgl*I. The frequency of replicative intermediates (θ plus σ structures) in this preparation was 0.048, in excellent agreement with the frequency of H and Y structures (0.049) observed after cleavage with *Bgl*I. This confirmed that few if any H and Y structures branch-migrated away during the digestion with *Bgl*I (26). Similarly high levels of Y structures were observed after *Bgl*I cleavage of replicative intermediates of six additional strains of polyoma virus obtained from other laboratories (data not shown).

The extent of replication for Y and H structures was determined by electron microscopy (Fig. 4). Most of the Y structures isolated in vivo (77%) or in vitro (69%) were early replicative intermediates. By contrast, most of the H structures were mid- or late replicative intermediates. A disproportionate number of H structures were greater than 90% replicated, as reported previously for polyoma virus (4) and SV40 (16, 19, 24). Out of over 300 cleaved replicative intermediates photographed, only one was a linear molecule containing an eye. This indicated that essentially all replication was initiated at the normal physiological origin (6, 7).

Electron microscopic analysis of early replicative intermediates. Y structures could have been generated from replicative intermediates either by *Bgl*I cleavage of σ structures (putative rolling

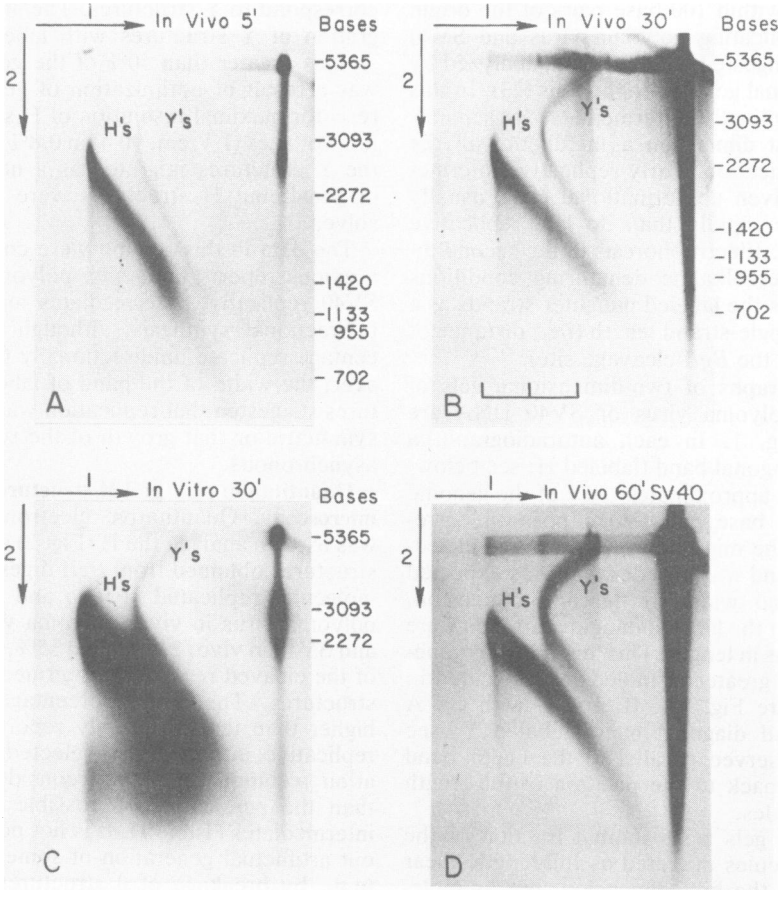

FIG. 1. Two-dimensional gel electrophoresis of replicating polyoma virus and SV40 DNA digested with *Bgl*I. Polyoma virus DNA labeled (A) in vivo with [^{3}H]thymidine for 5 min, (B) in vivo with [^{3}H]thymidine for 30 min, (C) in vitro with [α-^{32}P]dGTP for 30 min; (D) SV40 DNA labeled in vivo with [^{32}P]orthophosphate for 60 min. The migration of linear molecular weight markers in the second dimension is indicated. The bracketed areas in panel B correspond to regions of a duplicate first-dimension gel which were excised and examined by electron microscopy.

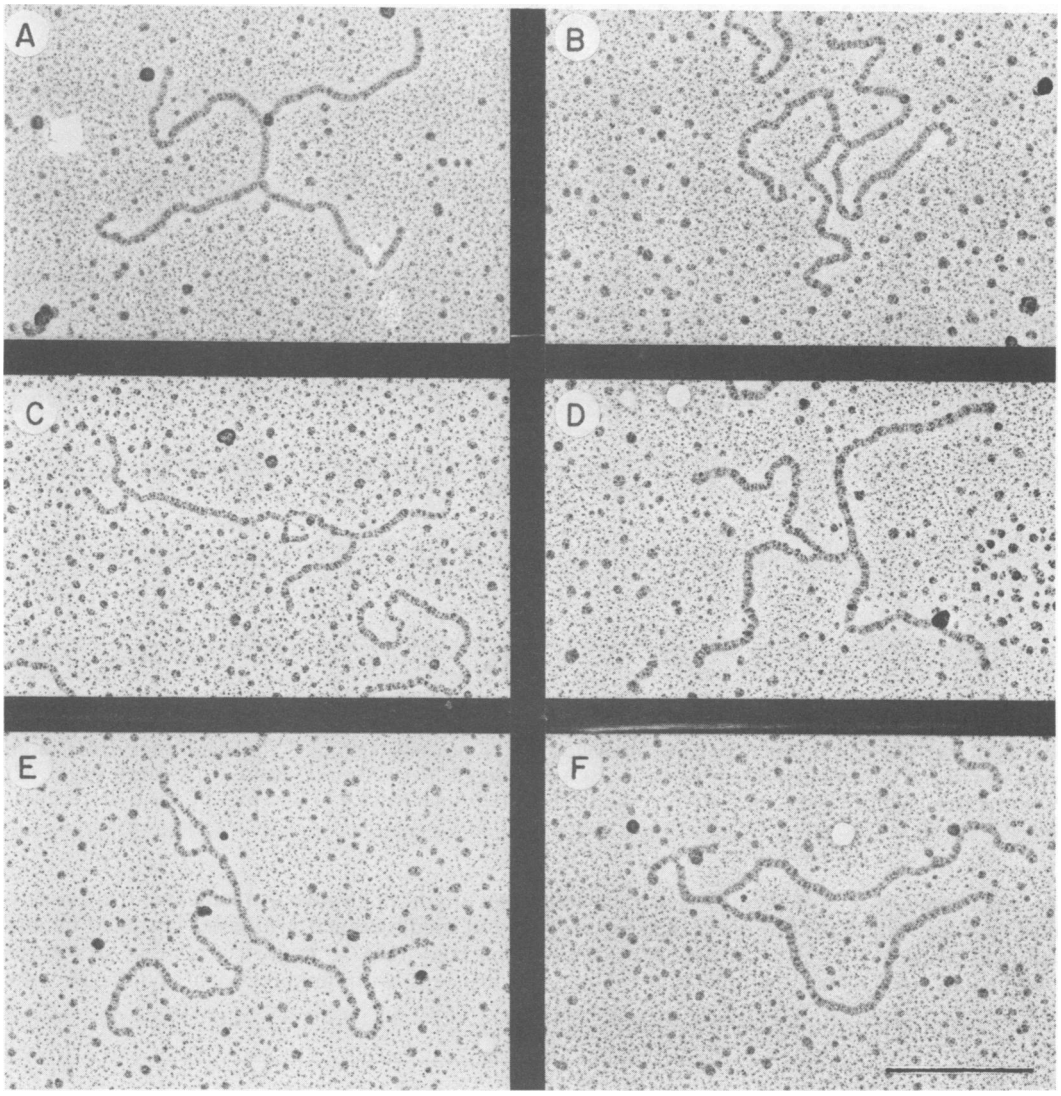

FIG. 2. Polyoma virus H structures. Putative intermediates of polyoma virus DNA replication when cleaved with *BglI*. (A) Symmetric H structure. The region presumed to be unreplicated (A) measures 0.2 μm. The regions presumed to be replicated (B, C, D, and E) measure 0.75, 0.74, 0.70, and 0.70 μm (see diagram in Fig. 5). (B) Symmetric H structure: A measures 0.04 μm. B, C, D, and E measure 0.85, 0.80, 0.80, and 0.80 μm. (C) Asymmetric H structure: A measures 1.05 μm. B, C, D, and E measure 0.40, 0.40, 0.23, and 0.21 μm. (D) Asymmetric H structure: A measures 0.10 μm. B, C, D, and E measure 0.88, 0.85, 0.79, and 0.76 μm. (E) Asymmetric H structure: A measures 0.20 μm. B, C, D, and E measure 1.22, 1.21, 0.30, and 0.29 μm. (F) Asymmetric H structure: A measures 0.25 μm. B, C, D, and E measure 1.35, 1.34, 0.15, and 0.15 μm. Bar, 0.5 μm.

circle intermediates) or by cleavage of θ structures replicating unidirectionally from the normal origin. To distinguish between these two alternatives, we digested the replicative intermediates with *Eco*RI, a restriction endonuclease which cleaves 1,560 base pairs from the origin in the early transcription region. Three classes of structures were formed: linear DNA molecules with eyes (Fig. 7), Y structures, and H structures. A diagram of 63 randomly selected cleaved intermediates is shown in Fig. 5. One branch point was located 1,560 base pairs from the *Eco*RI cleavage site (i.e., at *ori*) in 44% of these molecules. This value was consistent with

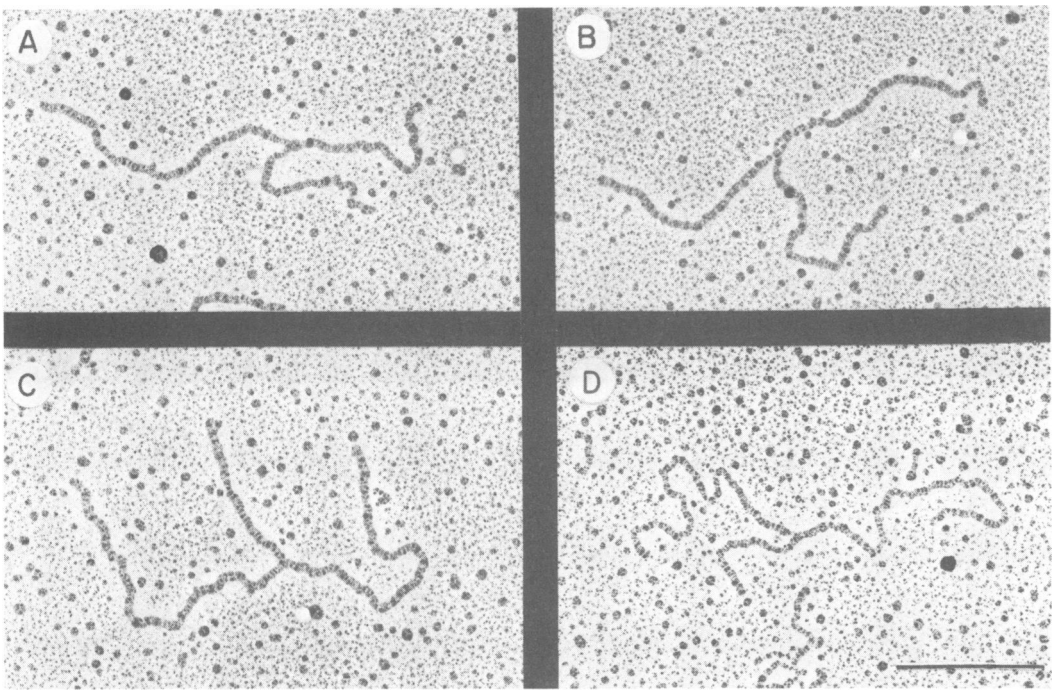

FIG. 3. Polyoma virus Y structures. Putative intermediates of polyoma virus DNA replication when cleaved with *BglI*. (A) The region presumed to be unreplicated (A) measures 1.10 μm. The regions presumed to be replicated, B and C, measure 0.69 and 0.64 μm. (B) A measures 0.76 μm, B and C measure 0.89 and 0.86 μm. (C) A measures 0.56 μm, B and C measure 1.16 and 1.15 μm. (D) A measures 0.45 μm, B and C measure 1.25 μm. Bar, 0.5 μm.

the percentage of Y structures (41%) observed after cleavage with *BglI*. Moreover, since essentially all (309 out of 310) of the replicative intermediates commenced synthesis at the normal origin, this value also indicated the frequency of unidirectional synthesis. It should be emphasized that a majority of the structures consistent with unidirectional synthesis were

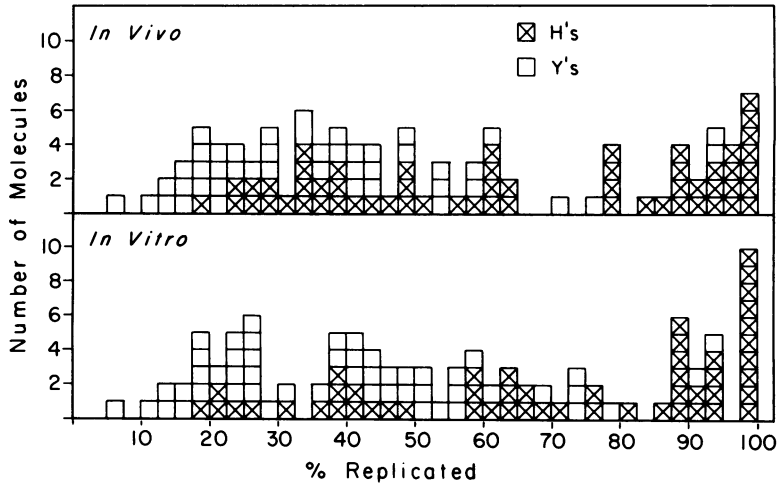

FIG. 4. The extent of replication of *BglI*-cleaved polyoma virus replicative intermediates quantitated as described in the text.

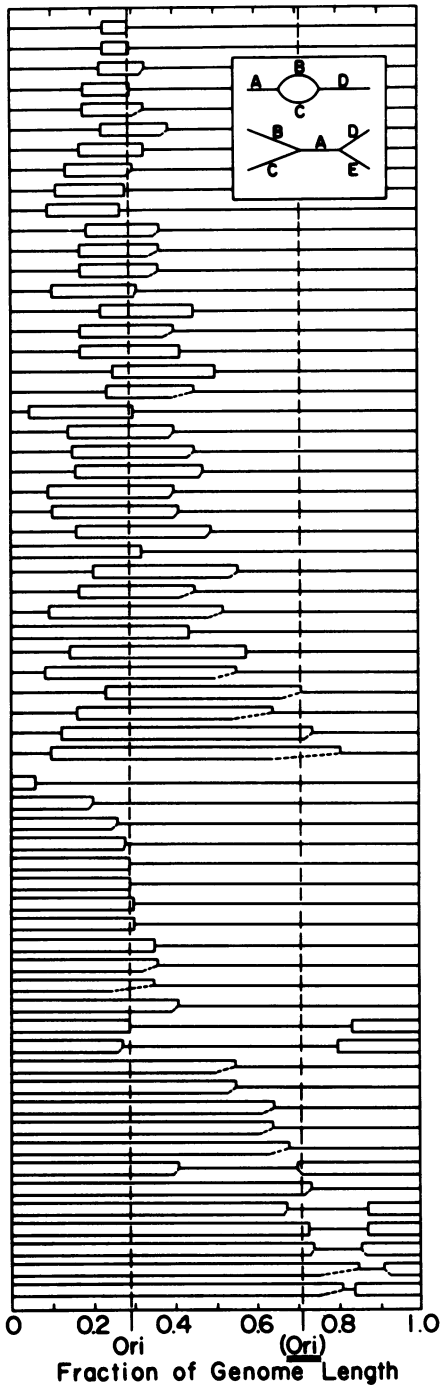

FIG. 5. Schematic diagram of polyoma virus replicative intermediates digested with *Eco*RI. Replication eyes and forks were arbitrarily oriented with respect to the origin (Ori) 1,560 base pairs from the *Eco*RI cleavage site. In some late replicative intermediates the orientation cannot be determined unambiguously. The alternative orientation of the origin with respect to the *Eco*RI site is indicated by (*Ori*). Molecules with only one fork located near to the origin were considered to be replicating by a unidirectional mechanism.

linear molecules containing eyes. This suggested that unidirectional synthesis takes place in intermediates with the morphology of θ structures and that most of the Y structures observed after *Bgl*I cleavage were not generated from classic rolling circle intermediates. Obviously, we cannot rule out rolling circle synthesis in which the tail remains attached to the origin throughout isolation, extraction, and spreading for electron microscopy.

It was potentially of great interest that most of the earliest replicative intermediates were oriented so that the fork proceeded in the direction of early transcription (Fig. 5). This suggests that fork growth may be initiated asynchronously, with one fork preferentially synthesized earlier. This hypothesis predicts that most replicative intermediates should be asymmetric, with the two replication forks unequal distances from the origin. In accord with this prediction, only 24% of the molecules (Fig. 5) were replicating by a clearly symmetric bidirectional mechanism.

Electron microscopic analysis of asymmetry. Contour length measurements of Y and H structures generated by *Bgl*I cleavage of polyoma virus replicative intermediates formed in vivo or in vitro revealed that the growing forks usually had proceeded unequal distances from the normal origin (Fig. 6). A similar conclusion is implicit in the excessive scatter found by Crawford et al. (Fig. 1 in reference 6). Examples of both symmetric and asymmetric H structures are shown in Fig. 2. In more than 50% of the molecules, one fork had proceeded 200 to 800 base pairs further, and in 19% of the molecules the difference was more than 800 base pairs. Extensive asymmetry was observed even in early replicative intermediates, consistent with the hypothesis of asynchronous initiation of the two growing forks. The *Bgl*I cleavage site may be located as far as 35 bases from the origin of replication in SV40 (15, 20, 24) and 85 bases from the origin in polyoma virus (21). We did not correct the data shown in Fig. 6 for this small difference. Since the *Bgl*I site in polyoma virus is located between the *Eco*RI site and the origin, and since most early replicative intermediates were oriented such that the fork proceeded towards the *Eco*RI site, the asymmetry may have been slightly underestimated. The uncorrected median asymmetry was 350 base pairs for polyoma virus preparation 1 in vivo and 440 base pairs for polyoma virus preparation 2 in vivo (the measurement variation was less than 100 base pairs; see above). In SV40 replicative intermediates the degree of asymmetry was substantially less, with a median asymmetry of 190 base pairs (data not shown).

After incubation of nuclei in vitro the extent of asymmetry increased, with 29% of the mole-

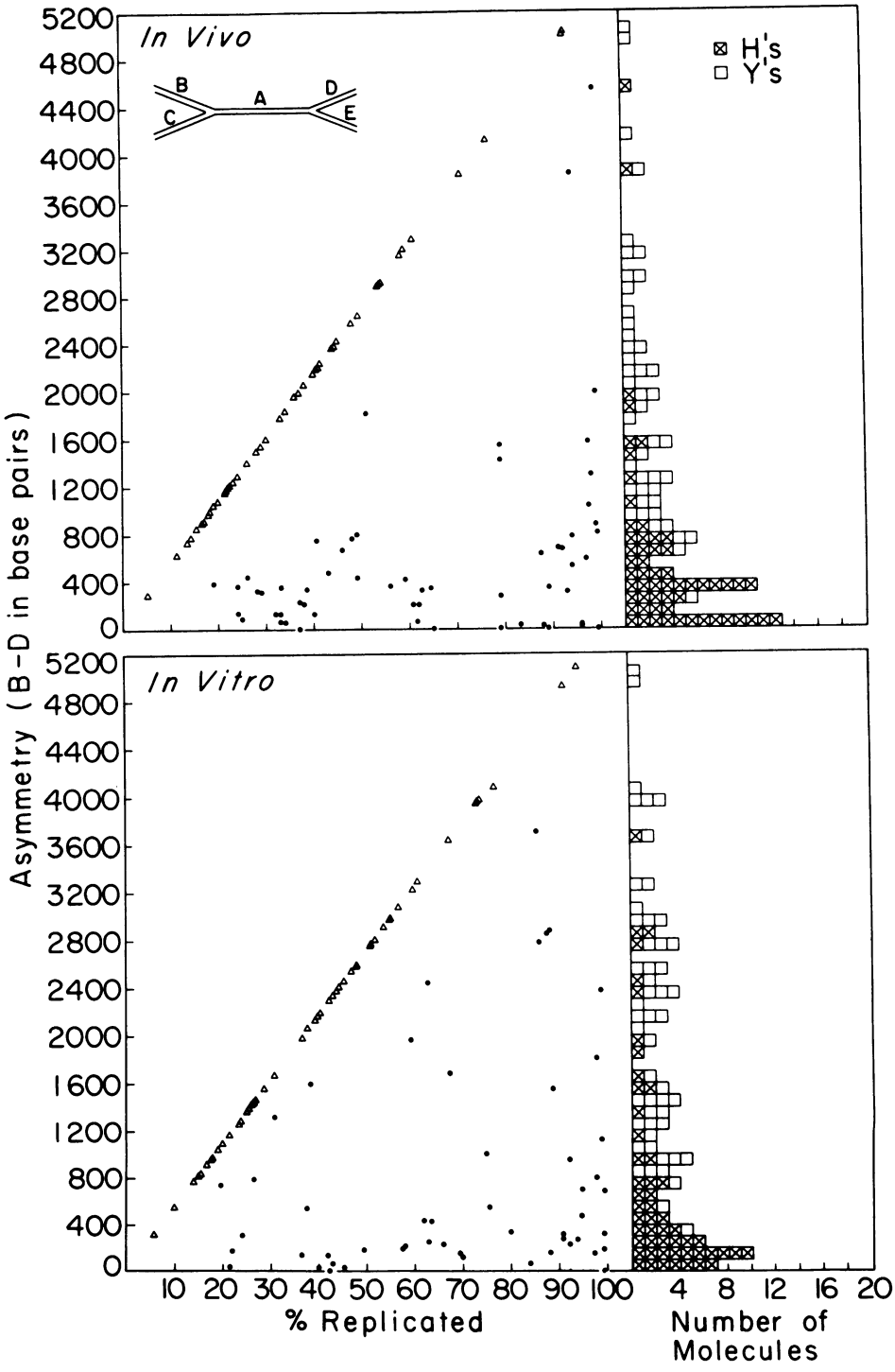

FIG. 6. The asymmetry of polyoma virus replicative intermediates after digestion with *BglI*. The asymmetry measured the difference in distance traveled from the origin between the two replication forks on the same molecule and was quantitated as described in the text. (Top) Polyoma virus replicating in vivo. (Bottom) Polyoma virus replicating in vitro.

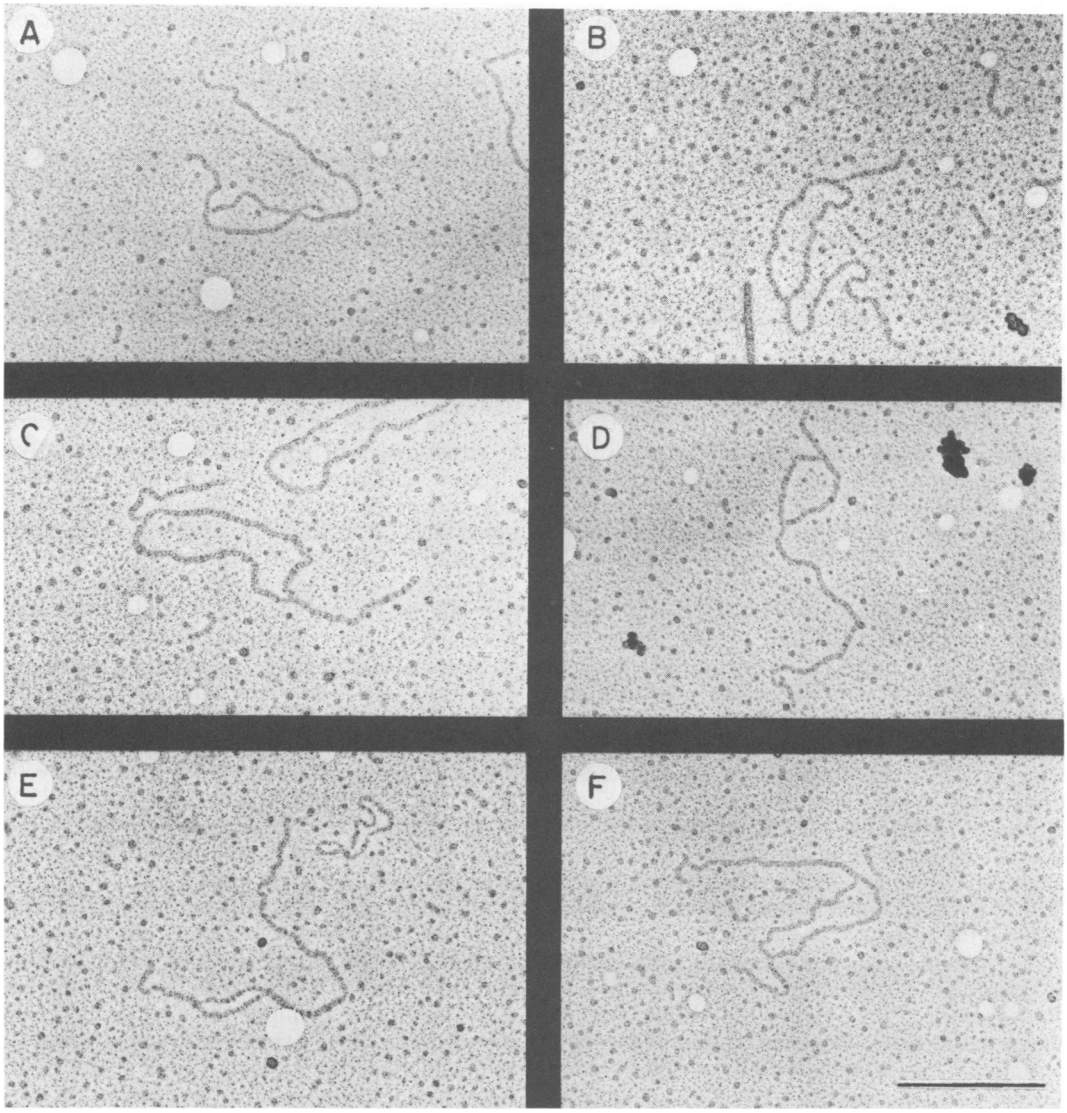

FIG. 7. Putative intermediates of polyoma virus DNA replication after cleavage with *Eco*RI. All panels show linear structures with replication eyes. (A) The regions presumed to be unreplicated, A and D (see Fig. 5), measure 0.25 and 0.85 μm. The regions presumed to be replicated, B and C, measure 0.35 μm. This structure was consistent with bidirectional synthesis from the origin. (B) A and D measure 0.27 and 0.88 μm, B and C measure 0.54 and 0.52 μm. This structure was also consistent with bidirectional synthesis from the origin. (C) A and D measure 0.38 and 0.48 μm, B and C measure 0.79 and 0.72 μm. This structure was consistent with unidirectional synthesis from the origin into the late region. (D) A and D measure 0.13 and 1.02 μm, B and C measure 0.33 μm. This structure was consistent with unidirectional synthesis from the origin into the early region. (E) A and D measure 0.29 and 1.10 μm, B and C measure 0.26 μm. This structure was consistent with asynchronous synthesis from the origin. (F) A and D measure 0.32 and 0.72 μm, B and C measure 0.56 and 0.45 μm. This structure was consistent with asynchronous synthesis from the origin. Bar, 0.5 μm.

cules having fork lengths differing by more than 800 base pairs. Death of one fork in vitro (3) may account for this increase in asymmetry.

DISCUSSION

To reconcile the high levels of molecules replicating unidirectionally with the high levels of replicative intermediates containing forks that had proceeded unequal distances from the origin, we propose that the two replication forks are initiated asynchronously. It should be emphasized that the polyoma virus and SV40 stocks used in these studies were prepared from well-characterized plaque-purified virus grown

at very low multiplicities. Furthermore, the experiments were carried out early in the period of active DNA replication to avoid the anomalous species often seen late in replication. Viral DNA was isolated by standard techniques, with no selection or fractionation of molecules. Replicative intermediates comprised 2 to 5% of the total polyoma virus DNA and were predominantly θ structures, in agreement with previous studies (2, 4, 10).

Although some of the molecules examined were consistent with an alternative origin (<1% of replicating molecules) or with artifactual generation of aberrant structures, neither mechanism could account for the level of unidirectional synthesis we observed or the substantial asymmetry found within molecules replicating bidirectionally. Unidirectional replication was initially measured as the percentage of Y structures generated by *BglI* digestion of replicative intermediates. These Y structures conceivably could have been formed by branch migration of H structures; however, the incubation conditions were such that little branch migration should have occurred (26). Moreover, the frequency of linear structures with unidirectional eyes observed after *Eco*RI cleavage (Fig. 5) was more consistent with unidirectional synthesis in θ structures. Quantitatively, the amount of unidirectional synthesis determined after *Eco*RI cleavage (44%) was in agreement with the amount of unidirectional synthesis determined after *BglI* cleavage (41%). This argues against both extensive branch migration and breakage of arms at the fork.

Y structures conceivably could also be formed by cleavage of rolling circle intermediates by *BglI*. However, the putative replicative intermediates in this study consisted of more than 90% θ structures, based on morphology as observed in the electron microscope; σ structures comprised only about 10% of the replicating molecules. Furthermore, the pattern of Y structures observed after *Eco*RI cleavage (Fig. 5) was not consistent with extensive rolling circle synthesis.

Although we propose that unidirectional replication is the result of initiation of only one fork, we obviously cannot determine whether some of the Y structures we observed in the electron microscope represented dead-end molecules. Nonetheless, they must have been formed at some time by unidirectional synthesis in θ structures. Fork death has been suggested as one method of increasing asymmetry during in vitro synthesis of polyoma virus DNA (3) and may explain the differences between the in vivo and in vitro intermediates (Fig. 6). However, both H and Y structures were labeled both in vitro and in vivo with short (5 min) pulses. Therefore, the Y structures we observed in the electron microscope cannot represent simply the accumulation of molecules with dead forks. In addition, essentially all of the labeled Y structures could subsequently be chased into mature (form I) DNA molecules (Buckler-White and Pigiet, unpublished data). Thus, for the hypothesis of fork death to account for most of our observed Y structures, many dead forks would subsequently have to be resurrected.

Although both H and Y structures were labeled during short pulses, the autoradiographic data (Fig. 1) would seem to suggest a large excess of H (bidirectional) structures over Y (unidirectional) structures. By contrast, however, about 40% of all cleaved intermediates were Y structures when measured by quantitative electron microscopy. This apparent contradiction can be reconciled by noting several additional facts. Y structures contain only one replication fork, whereas H structures contain two. Moreover, most Y structures were early replicative intermediates, whereas most H structures were mid- to late replicative intermediates (Fig. 4); short-labeled regions will not show up in the exposure range used. Confirmation that the ratio of Y to H structures was greater than would be anticipated visually from the autoradiographs (Fig. 1) comes from the direct microscopy of DNA (Fig. 1B, region 2) in which a ratio of 25 Y to 10 H structures was seen, even though the band of H structures was considerably darker than the band of Y structures.

Although more complicated hypotheses can be envisioned, all of the results above can be explained simply by postulating asynchronous initiation of the two forks, with the first initiation event usually occurring in the direction of early transcription. This asynchrony would seem to be much greater in polyoma virus than in SV40. Asynchrony would result in an apparent asymmetry in the distances the two forks have proceeded from the origin. The fork hesitation sites near the termination region described by Tapper and DePamphilis (24), however, provide an obvious mechanism for synchronizing apparent fork growth before termination. In accord with this latter idea, we have noted that a large fraction of Y and asymmetric H structures contained one fork which was located between 2,500 and 2,900 base pairs from the *BglI* cleavage site (i.e., near the normal termination site) and that many of the full-length linear molecules had nicks or gaps in the labeled daughter strands in this region.

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