Two Major Replicating Simian Virus 40 Chromosome Classes

SYNCHRONOUS REPLICATION FORK MOVEMENT IS ASSOCIATED WITH BOUND LARGE T ANTIGEN DURING ELONGATION*

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We have analyzed the asynchronous progression of replication forks through the early (E) and late (L) gene sides in bidirectionally replicating SV40 chromosomes during lytic infection. By cutting purified replicating DNA with an appropriate single-site restriction endonuclease and measuring the contour lengths of replicated and unreplicated segments by electron microscopy, the positions of the two replication forks in each elongating intermediate were determined. Our results indicate that there are at least two major classes of replicating SV40 chromosomes which differ in their relative rate of E and L fork movement, the presence or absence of bound SV40 large T antigen during elongation, and the termination region utilized. These two classes also have altered apparent start sites for initiating bidirectional replication, flanking either side of core ori. The largest group (67%) replicated synchronously associated with T antigen during elongation, appeared to initiate bidirectional elongation at nucleotide 5203 or 41 base pairs (bp) toward the E side of 0/5243, at the junction of T binding site I and ori, and terminated at the typical region centered at 0.5 map units. A second group (24%) replicated asynchronously with the L fork moving 3 times faster than the E fork, was not associated with T antigen during elongation, and terminated at a broad region centered at 0.73 map units. This group appeared to initiate at nucleotide 29 at the junction of the A†-rich region of ori, T binding site I, and the start of the 21-bp repeated transshcriptional control sequences. A third group (9%) appeared to initiate at nucleotide 5148 or 95 bp to the E side of 0/5243 and replicated asynchronously preferentially on the E side at early times. However, this group is related to the synchronous class in that it contains bound T antigen and both forks move synchronously past 30% elongation, terminating at the same region. The association of T antigen with synchronous but not asynchronous DNA molecules indicates that T functions in regulating fork movement during elongation. A synchronization role implies that both forks are closely associated with one another in replicating molecules with bound T. Replicating molecules lacking T not only elongated highly asynchronously but preferential fork progression occurred almost exclusively on the L side. The ori region in asynchronous compared to synchronous intermediates was differentially sensitive to BglI digestion, indicating that nuclease digestion can distinguish between different populations of replicating molecules. We conclude that there are distinct structural classes of replicating SV40 chromosomes which may correlate with differential transcriptional activation of the SV40 genome during productive infection.

During lytic infection, simian virus 40 (SV40) chromosomes replicate bidirectionally via a process requiring large T antigen and a unique origin region (see for reviews DePamphilis et al., 1983; DePamphilis and Wassarman, 1982). This region consists of a genetically required minimal core sequence (ori) of 65 bp, centered at T binding site II, and flanking sequences on both sides of ori (Bergsma et al., 1982; Deb et al., 1986; DeLucia et al., 1986; DiMaio and Nathans, 1980; Jones et al., 1984; Li et al., 1986; Stillman et al., 1985). Upon binding of T, one of several possible Okazaki fragment initiation sites is selected to initiate RNA-primed DNA synthesis (Hay and DePamphilis, 1982). Once DNA polymerase α-mediated bidirectional replication occurs, one fork proceeds through the early (E) gene region while the other moves through the 21- and 72-bp repeats and the late (L) genes. T is bound to essentially all replicating DNA intermediates (RIM) at initiation; however, as replication progresses past 70–80% completion, T rapidly dissociates (Segawa et al., 1980; Tack and DePamphilis, 1983). At about 90% completion, RIM are accumulated 2–3-fold compared to earlier stages of replication (Tack and DePamphilis, 1983; Tapper et al., 1982; Tapper and DePamphilis, 1978). Termination occurs for most molecules when the two forks meet halfway around the genome and does not require a specific DNA sequence. Forks are arrested at several specific sites within a termination region (ter) of about 500 bp prior to merging (Tapper et al., 1982). Pausing of forks within this region is suggested to be important in selecting one of two possible separation pathways, catenated dimer or gapped form II* molecule formation (Weaver et al., 1985).

During elongation of bidirectionally replicating SV40 DNA molecules, about two-thirds of RIM replicate asynchronously; that is, one fork moves significantly farther from ori than the other in a given intermediate (Tapper and DePamphilis, 1980). However, it is not apparent if asynchrony is preferential for one side compared to the other or if there is more than one type of asynchrony. Both E- and L-sided asynchronously replicating polyoma viral DNA molecules have been observed (Buckler-White et al., 1982). In addition, 25% of SV40 DNA molecules replicate bidirectionally via a process requiring large T antigen and a unique origin region (see for reviews DePamphilis et al., 1983; DePamphilis and Wassarman, 1982). This region consists of a genetically required minimal core sequence (ori) of 65 bp, centered at T binding site II, and flanking sequences on both sides of ori (Bergsma et al., 1982; Deb et al., 1986; DeLucia et al., 1986; DiMaio and Nathans, 1980; Jones et al., 1984; Li et al., 1986; Stillman et al., 1985). Upon binding of T, one of several possible Okazaki fragment initiation sites is selected to initiate RNA-primed DNA synthesis (Hay and DePamphilis, 1982). Once DNA polymerase α-mediated bidirectional replication occurs, one fork proceeds through the early (E) gene region while the other moves through the 21- and 72-bp repeats and the late (L) genes. T is bound to essentially all replicating DNA intermediates (RIM) at initiation; however, as replication progresses past 70–80% completion, T rapidly dissociates (Segawa et al., 1980; Tack and DePamphilis, 1983). At about 90% completion, RIM are accumulated 2–3-fold compared to earlier stages of replication (Tack and DePamphilis, 1983; Tapper et al., 1982; Tapper and DePamphilis, 1978). Termination occurs for most molecules when the two forks meet halfway around the genome and does not require a specific DNA sequence. Forks are arrested at several specific sites within a termination region (ter) of about 500 bp prior to merging (Tapper et al., 1982). Pausing of forks within this region is suggested to be important in selecting one of two possible separation pathways, catenated dimer or gapped form II* molecule formation (Weaver et al., 1985).

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molecules elongates asynchronously in the in vitro system of Li and Kelly (1985). One or more of several possible events might account for asynchronous progression of replication forks: (a) preferred (but not unique) DNA sequence signals such as initiation sites for Okazaki fragments or arrest sites for DNA synthesis (see for review, DePamphilis et al., 1983; DePamphilis and Wassarman, 1982); (b) movement of the two forks from ori at different times; (c) binding of a specific factor to one side of the genome but not the other, differentially affecting the elongation rate; (d) aspects of chromatin structure reflecting structural differences between the E and L genes which are differentially regulated during this phase of lytic infection; or (e) other events affecting processivity of DNA polymerase α on its template.

In this paper, we have correlated asynchronous bidirectional replication fork movement through the E compared to the L gene sides of the SV40 genome and the presence or absence of bound T antigen during elongation of SV40 chromosomes with the apparent start and termination sites used. In addition, we have measured the differential sensitivity of ori to BglI digestion in asynchronously and synchronously replicating chromosomes.

EXPERIMENTAL PROCEDURES

Isolation of Mature and Replicating SV40 Chromosomes—SV40, wildtype 800 (Barkan and Mertz, 1981), was propagated in CV-1 African green monkey kidney cells as previously described (Anderson et al., 1977). This SV40 strain has tandem 85-bp repeated elements instead of the typical 72-bp elements (Hay et al., 1984). To prepare replicating SV40 chromosomes, CV-1 cells were infected with sufficient virus to give maximum viral DNA production (10–20 plaque units of enzyme/pg of chromosomal DNA) of either BglI or EcoRI for 24 and 36 h postinfection, respectively, and ethanol precipitation as previously described (Tack et al., 1984). To prepare unreplicating SV40 chromosomes at 24 and 36 h postinfection, cells were harvested, washed, and nuclei were prepared following hypotonic lysis of infected cells (usually 10 150-mm plates). Nuclear extracts were fractionated by sucrose gradient sedimentation into mature 70 S and replicating 90 S SV40 chromosomes with the apparent start and termination sites used. The replicating SV40 DNA was digested with 2 units of enzyme/pg chromosomal DNA and purified, along with the immunosupernatant (-T) fraction, by Dr. E. Gurney, Univ. of Utah. Hamster preimmune serum or NS1 proteinase K in 0.25% SDS, 100 mM NaCl, and 20 mM EDTA for 1 h at 37 °C, followed by extraction with phenol/CHCl3, RNase digestion, proteinase K digestion, phenol/CHCl3 extraction, RNA treatment, and ethanol precipitation as above. Each purified SV40 DNA sample (+T and -T) was then digested with either the restriction endonucleases BglI or EcoRI (New England Biolabs) to completion according to the manufacturer's directions. Generally, 2 units of enzyme were added to 1 µg of DNA in digestion buffer for 1 h at 37 °C and stopped with 20 mM EDTA, 1% SDS. The cut DNA was then purified by phenol/CHCl3 extraction and ethanol precipitation.

To analyze SV40 chromosomes with bound large T antigen, the 90 S pool was immunoprecipitated using saturating amounts of either hamster tumor anti-T serum (National Institutes of Health, Bureau of Logistics and Resources) or monoclonal 101 hybridoma anti-T medium (Gurney et al., 1980) and fixed Staphylococcus aureus (The Enzyme Center) at 0 °C as previously described (Tack and DePamphilis, 1983). The monoclonal medium was generously provided by Dr. E. Gurney, Univ. of Utah. Hamster preimmune serum or NS1 (parent nonsecreter cell line) medium was used to prepare control samples. Viral DNA was released from the immunosorbant with SDS and purified, along with the immunosupernatant (−T) fraction, by proteinase K digestion, phenol/CHCl3 extraction, RNA treatment, and ethanol precipitation as above. Each purified SV40 DNA sample (+T and −T) was then digested to completion with BglI.

For studies on the nuclease sensitivity of SV40 chromosomal DNA, 90 S chromosomes were digested with saturating amounts (usually 50 units of enzyme/µg of chromosomal DNA) of either BglI or EcoRI for 15 min at 37 °C in digestion buffer as previously described (Tack et al., 1981). The samples were then placed in ice and EDTA added to 20 mM. The digested chromosomes were reacted with anti-T as described above; the precipitated and supernatant chromosomal DNA was extracted with SDS and the mixture of cut and uncut SV40 DNA species in each fraction was purified as before.

Analysis of SV40 DNA Species—The purified viral DNA products were analyzed by agarose gel electrophoresis and electron microscopy as previously described (Tack et al., 1981). At least two separate chromatin preparations were analyzed for each set of experimental variables.

For electron microscopic analysis, both the aqueous ammonium acetate and the formamide spreading procedure were used (Davis et al., 1971); samples were analyzed using a Hitachi RM16 electron microscope at between 5000- and 8000-fold magnification. Grids were shadowed with heavy metal (0.8 cm of Pt/Pd wire at a distance of 7.5 cm and a height of 1.0 cm) such that no tungsten carrier wire was vaporized. Electron micrographs were enlarged 10–20-fold using an Omega enlarger and point light source. Tracings were made of all SV40 chromosomes. No contaminating cellular DNA or RNA were involved.

For each RIM identified, contour length measurements (in duplicate) of the replicated and unreplicated segments were made using a Hewlett-Packard model 9800 series computer and digitizing board. Measurements of SV40 circular (relaxed only) and linear DNA molecules were used as internal controls to calculate a mean SV40 genomic length. Repetitive measurements of the same molecule indicated an error of less than 1–2%. The sum of the replicated and unreplicated regions of each RIM was then compared to this; greater than 90% of the molecules scored were within 3% of the unit genome length. Variations were allowed up to 7% of the unit length; RIM in excess of the (less than 2%) was scored as unreplicated. Using this formamide spreading procedure, there was a single-stranded region (130 bp average length; 300 bp maximum length) at one or both replication forks (never on both strands at the same fork) in 75% of the molecules and a single-stranded region at both forks in 34% of the RIM. Using the aqueous spreading procedure, single-stranded regions were less frequent. Average replicated arm pair lengths were within 160 bp of each other (3% of genome length). About 10% of the replicating intermediates had one arm of a pair shorter by 300 bp or more than the other using either spreading procedure. This could be due to collapse of the single-stranded regions at the fork in some cases, preferential compaction of one arm during spreading or possible contamination of endonucleases with exonuclease; calculations were usually made using an average of the paired arm lengths. Only molecules with two distinct arms adjoining a replication bubble (for EcoRI cut early RIM) or two pairs of arms (for BglI cut or EcoRI cut late RIM) were scored. Broken molecules (α or Y structures) representing about 10–20% of the total RIM were not scored unless they could be clearly identified with respect to where and how much DNA was missing (one arm and a tag still remained per arm pair). An arm was scored as a pair if there were two sets of structural types of broken and whole molecules. These were compared to cut samples to ensure that assignments were being correctly made. As RIM greater than 95% complete are difficult to distinguish from interwound catenated dimers, only RIM less than 95% replicated were measured.

For RIM cleaved with EcoRI, the distribution of RIM elongated to varying extents, as well as the relative degree of asynchrony, was determined and then compared to RIM cut with BglI. Data were evaluated using a VAX computer and BMDP statistical software programs (Engelman and Hartigan, 1980). For the nuclelease sensitivity studies, cut and uncut RIM were identified and measured.

RESULTS

Measurements of Fork Lengths in Replicating SV40 DNA Molecules—We wished to determine if replication asynchrony was preferential for either the E or L gene sides of the SV40 genome by measuring the relative position of the two replication forks in each RIM with respect to ori. Previous analysis using electron microscopy to measure the relative arm lengths of SV40 RIM cleaved with BglI indicated that 68% of the RIM had one fork which had traveled at least 3.5% (184 bp) of the genome length farther than the other (Tapper and DePamphilis, 1978). Since BglI cuts the SV40 genome very near ori (Hay and DePamphilis, 1982; Martin and Setlow, 1980; Shortle and Nathans, 1979), assignment of asynchrony to one side or the other was not possible. Inspection of the locations of sites for different single-site restriction endonucleases on the SV40 genome indicated that EcoRI has a staggered cut.
Fig. 1. Synchronously and asynchronously replicating SV40 DNA intermediates elongated to varying extents. Theoretical examples of EcoRI and BglII cut DNA molecules are shown.

asymmetrically with respect to the location of ori and the BglII site. Digestion of RIM with this enzyme would allow us to determine the positions of replication forks, as well as the degree of asynchrony for each RIM analyzed. Replicating 90 S SV40 chromosomes were hypotonically extracted from infected CV-1 cells and fractionated on sucrose gradients (see "Experimental Procedures"). SV40 DNA was then purified and digested with either EcoRI (1782 bp to the L side of 0/5243) or BglII (0/5243). Replicated and unreplicated DNA lengths in each RIM were identified and measured using enlarged electron micrographs.

Fig. 1 identifies synchronous and asynchronous movement of replication forks in RIM following digestion with either BglII or EcoRI. Cleavage with EcoRI generates a complicated but identifiable array of structures. It can be seen from the location of the EcoRI site relative to ori that each fork position can be determined in EcoRI cut molecules. The arm lengths are dependent on the extent of elongation, degree of asynchrony, and side on which the asynchrony occurs. Thus, the distance that the E fork had traveled can be compared to that traveled by the L fork. For RIM with only one EcoRI site/molecule (synchronous and less than 68% complete or asynchronous with replication short of the EcoRI site), the longer arm (a), the longer arm (b), and the replicated portion (c, the average of each of the two strands, c1 and c2, of a replication bubble) were measured. The distal end of the shorter arm, a, is always 0.34 map units away from the BglII site. The extent of elongation is equal to c/(a + b + c). For late RIM (molecules replicated to greater than 68% completion contain two restriction sites/RIM), the shorter pair of arms (a, and aS), the longer pair (b, and bS), and the unreplicated portion (c) were measured. The shorter and longer pair of arms were averaged to give a and b, respectively. Now, the distal ends of the longer pair of arms are 0.34 map units from the BglII site (0). The extent of elongation is (a + b)/(a + b + c). Some EcoRI cut late RIM were ambiguous as to identity: assignment as either nearly unidirectional E-sided or slightly asynchronous L-sided intermediates was possible. The less asynchronous value was always chosen. As the results for the number and degree of asynchronous EcoRI cut RIM were identical to RIM cut with BglII, this assumption appears valid.

For comparison, Fig. 1 also shows the theoretical pattern for RIM cut with BglII. As can be readily seen, cleavage with BglII is predicted to generate only H structures regardless of the extent of replication. This is due to the close proximity of the cutting site to ori so that two restriction sites are generated once replication is initiated. For each molecule, the shorter pair of arms, a, and aS, the longer pair of arms, b, and bS, and the unreplicated portion, c, were measured. Each pair of arms was averaged to give a and b.

Mapping the Positions of Replication Forks: Determination of the Extent of Asynchrony of Fork Movement—Electron micrographs of purified RIM cut with EcoRI are shown in Fig. 2. Synchronous and asynchronous (either E- or L-sided) SV40 DNA molecules elongated to varying extents were apparent following measurement of replicated and unreplicated lengths. For each EcoRI cut RIM, the position of the two replication forks on the physical map of the SV40 genome was determined measuring from the EcoRI site ("Experimental Procedures"). Multiple measurements of linear and circular SV40 DNA molecules (5243 bp) indicated that DNA lengths were accurate to within 75 ± 25 bp, on average. The results are shown in Fig. 3. The distance in map units that each of the two forks had moved from ori is readily apparent. For early RIM, the distance traveled by the L fork, fL, is equal to 0.34 – a, while the distance traveled by the E fork, fE, is equal to 0.66 – b. For late RIM, the distance traveled by the L fork, fL, is equal to 0.34 + a, while the distance traveled by the E fork, fE, is equal to b – 0.34. The difference (e minus f) is a measure of the degree of replication fork asynchrony (shown as a shaded region and representing a fraction of the total genome length, 1.00). The sidedness (preferential movement of either the E or L fork) or fork progression is also apparent. The extent of replication is equal to e plus f. The replicated (arm) pairs a and b and unreplicated (c) segments of each BglII cut RIM were similarly analyzed (see Fig. 1). BglII cut RIM all appeared as H-like structures as predicted. The extent of replication was equal to a + b. The data were not plotted as in Fig. 3, since the sidedness of these asynchronous molecules cannot be determined. The degree of
FIG. 2. Electron micrographs of EcoRI cut synchronous and asynchronous DNA molecules replicated to varying extents. Both E- and L-sided asynchronous replicating intermediates are shown. a, synchronous, 16% replicated; b, L-sided asynchronous, 14% replicated; c, E-sided asynchronous, 24% replicated; d, synchronous, 46% replicated; e, L-sided asynchronous, 55% replicated; f, E-sided asynchronous, 51% replicated; g, synchronous, 83% replicated; h, L-sided asynchronous, 97% replicated; i, E-sided asynchronous, 93% replicated.

asynchrony, b - a, is a measure of the difference in distance that the two forks had progressed.

For BglII cut RIM (data not shown), analysis of the degree of asynchrony with respect to fork movement indicated that 65% were asynchronous (greater than 180 bp or 0.035 map units difference between the two forks); thus, 35% were replicating synchronously. Of the total molecules measured, 40% were slightly asynchronous (greater than 180 bp but less than 500 bp difference) on comparing the lengths of the two pairs of arms while the remaining 25% were highly asynchronous (greater than 500 bp difference). For the latter group, the average degree of asynchrony at late extents of replication was 1635 bp. No unidirectional replication was observed except at very early extents of elongation. For EcoRI cut RIM, 67% were asynchronous. Slightly asynchronous molecules were 44% and highly asynchronous molecules were 23% of the total RIM, respectively. These results using hypotonically extracted chromosomes agree well with those for SV40 DNA purified from Hirt extracts (Tapper and DePamphilis, 1980). Thus, two different methods of viral DNA purification give similar results.

Asynchrony of Replication Fork Movement with Increasing Elongation—For each EcoRI cut RIM, the degree of asynchrony (distance that one fork had moved farther than the other) was analyzed with respect to the extent of elongation (4-95%). The results are shown in Fig. 4. Synchronous RIM (S) were neither E- or L-sided regardless of the extent of elongation. Asynchrony was further defined as either slightly (A) or highly (AA) asynchronous on either the E or L sides (see Fig. 3). For slightly asynchronous RIM (differences of between 180 and 500 bp on comparing the two forks), 88% were asynchronous exclusively on the E side. That is, the E fork had progressed farther than the L fork. This E-sided asynchrony was apparent at early extents of elongation and as these RIM continued to replicate, the degree of asynchrony remained constant. Thus, the preferential rate of fork migration on the E side at early times did not appear to be amplified at later elongation times. Once bidirectional replication was initiated, both forks moved at a constant rate. In contrast, highly asynchronous RIM (greater than 500 bp difference between the two forks) consisted predominantly (84%) of intermediates where the L fork had progressed farther than the E fork. Furthermore, for these L-sided asynchronous molecules, the degree of asynchrony increased as elongation progressed. Thus, these highly asynchronous RIM appeared to terminate within the E gene region rather than at the usual termination region, about 500 bp in size, located around 0.5 map units. One RIM was observed to be highly asynchronous on the E side. One unidirectional (L-sided) molecule was observed for EcoRI cut RIM greater than 10% replicated.

Relationship between Large T Antigen and Replication Fork Asynchrony—The degree and sidedness of replication fork asynchrony was measured for 90 S chromosomes with and without bound T antigen following fractionation by immu-
Replicating SV40 Chromosome Subclasses

FIG. 3. Schematic diagram and orientation of the replicated and unreplicated contour lengths of EcoRI cut SV40 replicating DNA molecules. Replicated (boxed areas) regions for each molecule are shown as a fraction of the unit genome length, 1.0 (5243 bp). Shaded areas indicate the distance that one fork had traveled farther than the other (degree of asynchrony) in a given molecule.

noprecipitation with anti-T ("Experimental Procedures"). About 40% of the short pulse-labeled chromosomal DNA was precipitated by tumor serum, similar to previous results (Tack and DePamphilis, 1983). The DNA contained in the anti-T precipitate (Ip or +T), anti-T supernatant (Isup or −T Ag), and total (before immunosorption) replicating fractions was purified, cut with BglII, and analyzed as before. In this experiment, molecules greater than 50% replicated were preferentially scored because asynchrony is more apparent with increasing elongation (see Fig. 4). Thus, highly asynchronous RIM made up 44% of the total input molecules rather than the usual 25% (see earlier). The results are shown in Fig. 5. There was a corresponding 5-fold enrichment of synchronous, slightly asynchronous, and highly asynchronous DNA fraction which we have shown to be almost exclusively L-sided (see Fig. 4). There was a corresponding 5-fold enrichment of synchronous RIM in the Ip fraction compared to the Isup. Slightly asynchronous molecules were also preferentially found in the Ip. Thus, RIM associated with T appear to be greatly enriched in synchronously replicating DNA. It was not possible to determine if highly asynchronous E-sided RIM (we observed one in Fig. 4) are also associated with T as BglII was used for this analysis. No difference was observed on comparing the monoclonal PAb 101 with polyclonal tumor anti-T serum. While it is possible that highly asynchronous RIM contain

FIG. 4. Relationship between the degree of asynchrony and the extent of replication for EcoRI cut replicating DNA molecules. The degree of asynchrony (L−E) is expressed as a fraction of the genome length, 1.0, and is the difference in distance that the two forks have traveled from the origin, 0/5243 (see Fig. 3). The RIM were divided into three replicating groups: synchronous (S) with forks less than 180 bp apart, slightly asynchronous (A) with forks between 180 and 500 bp apart, and highly asynchronous (AA) with forks greater than 500 bp apart. The RIM were further distinguished as to their forks moving preferentially on the L (AL and AA_L) or E (AE and AA_E) side of the genome.

FIG. 5. The relationship between bound T Ag and the degree of replication asynchrony. The relative distribution of synchronous, slightly asynchronous, and highly asynchronous RIM in the total, anti-T precipitated (+T), and anti-T supernatant (−T) replicating chromosome fractions is shown. DNA was purified from the different fractions following immunoprecipitation, cut to completion with BglII, and analyzed as described under "Experimental Procedures." The degree of replication asynchrony for each RIM was determined as described in the legend to Figs. 3 and 4.
bound T that is unreactive with tumor anti-T, this would require loss of reactivity of all determinants recognized by this polyclonal antiserum.

Cluster Analysis of Asynchronously Replicating Molecules—

The data analyzed in Fig. 4 were plotted so that the degree of asynchrony for a given molecule was expressed as a fraction of the replicated SV40 genome length, rather than as a fraction of the total genome length. In the new plot (Fig. 6), molecules which had replicated asynchronously were more readily classified as such when the extent of replication was small. The results of this analysis indicated that, like the previous diagram, most RIM replicated approximately synchronously; again, most asynchronous RIM were preferentially so on the L side. However, using this new definition of asynchrony, Fig. 6 additionally showed that the few RIM preferentially asynchronous on the E side were also molecules which were replicated to only a small extent.

Molecules represented as data points in Fig. 6 were analyzed and classified as L-sided asynchronous, synchronous, or E-sided asynchronous molecules by the K-means clustering program (Engelman and Hartigan, 1983) of the BMDP statistical package. The data points were partitioned into three clusters, subject to the constraint that the partitioning be according to the degree of asynchrony rather than extent of replication. The numbers corresponding to these clusters rather than data points are shown in Fig. 6. It can be seen that the resulting three clusters correspond to the L-sided asynchronous, synchronous, and E-sided asynchronous RIM groups previously classified in Fig. 4. Note that the RIM in cluster 3 become indistinguishable from those in cluster 2 at later times (greater than 36% complete).

In separate analyses, the same data points were partitioned by the K-means clustering program into four and five clusters to determine if this would also result in clusters which were statistically valid. The standard deviation of the degree of asynchrony was computed. For each cluster, a range of degrees of asynchrony was thereby defined which extended from one standard deviation below the average to one standard deviation above the average. When the data points were partitioned into three clusters (Fig. 6), the corresponding ranges were distinct and did not overlap (data not shown); when the data points were partitioned into four or five clusters, some of the resulting four or five ranges overlapped with others. These results implied that only three statistically discrete clusters were present, corresponding to L-sided asynchronous (24%), synchronous (67–76%), and at early times of elongation, E-sided asynchronous molecules (9%). Thus, these clusters appear both biologically (asynchronous RIM are highly deficient in bound T antigen compared to synchronous RIM) and statistically distinct.

Predicted Start Sites, Termination Sites, and Relative Fork Rates for Subclasses of Bidirectionally Replicating Chromosomes—The data points within each of the three clusters defined above were further analyzed to determine the start site for bidirectional replication for each RIM class. The data were again replotted (Fig. 7) so that the positions of the L and E forks were each plotted against the extent of replication. The data points in such a plot determine two lines: one line represents the position of the L fork and the other represents the position of the E fork at a given extent of replication. The intercept and its associated uncertainty were calculated using the BMDP polynomial curve-fitting program (Jennrich and Mundle, 1983). In such a plot, this value (fork position at 0% replication) is an estimate of the start site for replication. Similarly, the intercept at 100% replication is an estimate of the fork position at termination. The data points within each of the three clusters were also analyzed to determine the ratio of the two fork speeds for each cluster. In a plot of fork position versus extent of replication (Fig. 7), the slope of each line is an estimate of, and therefore approximately equal to, the replication fork speed. Therefore, the ratio of the slopes of the two lines in each plot is an estimate of the ratio of the two fork speeds. The slopes and their associated uncertainties were calculated using the BMDP polynomial curve-fitting program (see above). In the calculations of intercepts, slopes, and their associated uncertainties, the data points were assigned unequal statistical weights to compensate for the unequal uncertainties in their values, intrinsic to data of this type. Unequal uncertainties in the values of the data points were detected and compensated for by previously described procedures (Chatterjee and Price, 1977). Unequal weighting by these procedures results in more precise estimates of intercepts and slopes than those resulting from equal weighting. The results, calculated for each of the three clusters, are shown in Table 1.

Several conclusions are indicated from these analyses. For synchronous RIM (Fig. 7, panel B, cluster 2), both forks appeared to initiate at 41 ± 27 bp on the E side of 0 or near nucleotide 5203 and then progress at the same rate. For E-sided asynchronous RIM (Fig. 7, panel C, cluster 3), both...
FIG. 7. Relationship between the extent of elongation and the distance that each fork had traveled from 0/5243 (Bgl site). RIM were divided into the three classes shown in Fig. 6. Panel A is L-sided asynchronous, panel B is synchronous, and panel C is E-sided asynchronous subclass of RIM. Panel D is the total RIM population. The position of the E and L forks (taken from Fig. 3) was expressed as a percentage of the genome length (made equal to 200 units) and was plotted against the percent replication using the BMDP polynomial regression analysis program. Slopes (apparent rate), intercepts (apparent start site), and Y∞ intercepts (apparent termination site) and their respective standard errors were calculated for each fork. L fork: open circles; E fork: closed circles.

Forks appeared to initiate at 95 ± 24 bp to the E side of 0 or near nucleotide 5148. Once initiated, the E fork moved two times faster than the L fork at early times. As also shown in Fig. 6, after 30% elongation, both forks appeared to progress at the same rate, similar to the synchronous class (cluster 2). For L-sided asynchronous RIM (Fig. 7, panel A, cluster 1), both forks appeared to initiate at 29 ± 59 bp on the L side of 0 or near nucleotide 29. For this class, the L fork moved an average of three times faster than the E fork, consistent with the pattern shown in Fig. 4. Furthermore, when the intercepts of the lines at 100% replication were calculated, L-sided asynchronous RIM appear to terminate at a region centered
at 0.73 map units or 1200 bp from the usual ter region. This is in marked contrast to the synchronous and E-sided asynchronous classes of replicating molecules in which both forks appeared to progress at the same rate after 30% elongation and terminate at the typical ter region at 0.5 map units. The results of analyzing the total EcoRI cut RIM population with increasing extents of elongation, thus averaging together all the data from the three classes of molecules, is also shown in Fig. 7, panel D. Both forks now appear to initiate at 50 ± 64 bp to the E side of 0 or near nucleotide 5193 and progress with approximately equal rates.

**BglI Sensitivity of Replicating Chromosome Subclasses**—We have previously compared the accessibility of several different SV40 chromosomal DNA sequences to their respective single-site restriction endonucleases (Tack et al., 1981). The accessibility for most of the sites was consistent with near random nucleosome phasing. However, the ori region in SV40 chromosomes was hypersensitive to digestion by BglI. Here, we wished to determine if the enhanced cutting of replicating SV40 chromosomes by BglI is related to the degree of replication asynchrony. Native 90 S chromosomes were maximally digested with BglI and then immunoprecipitated with anti-T antigen (see "Experimental Procedures"). SV40 DNA was then purified from the +T and −T fractions and analyzed by electron microscopy. Asynchronous replication, unlike bare SV40 DNA, are partially digested by BglI, a mixture of cut and uncleaved RIM were generated. The degree of asynchrony can be measured only for cut RIM. The difference in the pattern of asynchrony for the partially cut chromosomes was compared to that of totally cut DNA. Differences in the relative number of molecules in the different RIM classes reflect changes in the accessibility of this region to digestion and, thus, possible alterations in chromatin structure at ori.

The distribution of synchronous and asynchronous RIM in the BglI cut chromosome sample compared to BglI cut DNA is shown in Table I. The relative number of synchronous RIM in the Ip compared to the Isup fraction was significantly different for the two samples. For cut chromosomes, there were approximately equal amounts of synchronous RIM in both the Ip and Isup fractions (1.26:1) while for cut DNA, most of the synchronous RIM were in the Ip (4.8:1) rather than the Isup (see Fig. 5). Thus, there was about a 4-fold decrease in the ratio of +T/−T RIM in this fraction upon comparing cut chromosomes with cut DNA. In contrast, the ratio of +T/−T RIM in the highly asynchronous fraction was increased 2.5-fold for cut chromosomes compared to total cut DNA. As a control experiment, a similar analysis was carried out with chromosomes digested with EcoRI and their pattern of asynchrony was compared to that of EcoRI cut DNA. No preferential cutting of synchronous or asynchronous RIM in either the Ip or Isup fractions of chromosomes compared to DNA was observed. It is not possible to distinguish between either increased sensitivity or resistance of one of the two RIM classes to nuclease digestion to account for these differences. However, these results are clearly consistent with there being more than one class of replicating SV40 chromosomes. They also suggest that there are differences in chromatin arrangement near ori but not at the EcoRI site during replication.

**DISCUSSION**

This paper examines the asynchronous replication of SV40 chromosomes during lytic infection. We used a single-site restriction endonuclease that cuts the SV40 genome asymmetrically to position both replication forks in each elongating DNA molecule. SV40 large T antigen-associated chromosomes were fractionated using T-specific antisera. Our results indicate the presence of at least two major classes of replicating SV40 chromosomes which differ in their apparent start sites used for initiating bidirectional replication, the relative elongation rates of the E and L forks, the presence of bound T antigen during elongation, and the termination region utilized. These differences indicate that asynchronous replication fork migration cannot be attributed solely to preferred

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**TABLE I**

Predicted start sites, termination sites, and fork rates for replicating SV40 chromosomes

<table>
<thead>
<tr>
<th>RIM class</th>
<th>Fork</th>
<th>Start site</th>
<th>Relative fork rate</th>
<th>Predicted termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>Late-sided,4 24%</td>
<td>L</td>
<td>+28 ± 62</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>+30 ± 57</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>Synchronous, 67%</td>
<td>L</td>
<td>−41 ± 27</td>
<td>0.49 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>−42 ± 28</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>Cluster 3</td>
<td>Early-sided, 9%</td>
<td>L</td>
<td>−95 ± 24</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>−95 ± 24</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
<td>L</td>
<td>−50 ± 64</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>−65 ± 65</td>
<td>0.49 ± 0.04</td>
</tr>
</tbody>
</table>

* Distance in nucleotides from the BglI site, 0.
4 Late gene side = positive; early gene side = negative.
* Tack and DePamphilis (1983).

<table>
<thead>
<tr>
<th>SV40 fraction</th>
<th>Number of molecules</th>
<th>Type of asynchrony</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Chromosomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>32</td>
</tr>
<tr>
<td>+T</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>−T</td>
<td>21</td>
<td>20</td>
</tr>
</tbody>
</table>

DNA: +T/−T
Chromosomes: +T/−T

4 S = synchronous (less than 180 bp difference); A = slightly asynchronous (180-500 bp difference); AA = highly asynchronous (greater than 500 bp difference).

About 50% of the chromosomes were cut.

From Fig. 5.

**TABLE II**

Sensitivity of synchronously and asynchronously replicating SV40 chromosomes to BglI digestion

<table>
<thead>
<tr>
<th>SV40 fraction</th>
<th>Number of molecules</th>
<th>Type of asynchrony</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
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<tr>
<td>Chromosomes</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>32</td>
</tr>
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<td>30</td>
</tr>
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<td>−T</td>
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</tbody>
</table>

DNA: +T/−T
Chromosomes: +T/−T

4 S = synchronous (less than 180 bp difference); A = slightly asynchronous (180-500 bp difference); AA = highly asynchronous (greater than 500 bp difference).

About 50% of the chromosomes were cut.

From Fig. 5.
that initiate bidirectional DNA synthesis are shown in detail. The major synchronous class (67% of RIM) initiates at nucleotide 5203 (41 bp toward the E side of the ori), at the junction of T binding site I and core ori. This corresponds to the major 2-bp transition site for Okazaki nucleotides of nascent DNA chains.

Multiple Bidirectional Start Sites—The apparent start sites that initiate bidirectional DNA synthesis are shown in detail in Fig. 8. The major synchronous class (67% of RIM) initiates at nucleotide 5203 (41 bp toward the E side of the BglII site, 0) at the junction of T binding site I and core ori. This corresponds to the major 2-bp transition site for Okazaki nucleotides of nascent DNA chains.

The fraction of the total RIM population that initiates at each of these sites (box plus arrow) is given as a percentage. Also shown are the locations of the 5' terminal deoxynucleotides of nascent DNA chains (vertical lines with the length of the line denoting the relative frequency of use) covalently linked to RNA in vivo, the direction of DNA synthesis at these positions (heavy solid arrows), T antigen binding sites I, II, and III (hatched boxes), the minimal 65-bp sequence required for the origin of replication, ori (shaded area), and the proposed transition from continuous to discontinuous DNA synthesis at 3210–3221 nucleotides (gridded box and large white vertical arrow), all taken from Hay and DePamphilis, 1982. DNA sequences highly conserved (double-headed arrows) among all known papovaviruses in this region are also shown (Deb et al., 1986). The six GC-rich repeated DNA sequences are shown as circles.

Fig. 8. Schematic diagram of the three apparent start sites for bidirectional replication within the ori region of replicating SV40 chromosomes. The fraction of the total RIM population that initiates at each of these sites (box plus arrow) is given as a percentage. Also shown are the locations of the 5' terminal deoxynucleotides of nascent DNA chains (vertical lines with the length of the line denoting the relative frequency of use) covalently linked to RNA in vivo, the direction of DNA synthesis at these positions (heavy solid arrows), T antigen binding sites I, II, and III (hatched boxes), the minimal 65-bp sequence required for the origin of replication, ori (shaded area), and the proposed transition from continuous to discontinuous DNA synthesis at 3210–3221 nucleotides (gridded box and large white vertical arrow), all taken from Hay and DePamphilis, 1982. DNA sequences highly conserved (double-headed arrows) among all known papovaviruses in this region are also shown (Deb et al., 1986). The six GC-rich repeated DNA sequences are shown as circles.

Replicating SV40 Chromosome Subclasses

DNA sequence signals such as arrest sites for DNA polymerase α origin initiation sites for Okazaki fragments.

Synchronous RIM initiate just to the E side of ori and elongate synchronously in the presence of bound T. In contrast, asynchronous RIM initiate just to the L side of ori and elongate with preferential fork progression on the L side. The L fork moves 3 times faster than the E fork and is 2410 bp farther, on average, at completion. Asynchronous RIM elongate without bound T, dissociation must occur soon after initiation. Slightly asynchronous RIM also initiate on the E side but outside the limits of ori. This class elongates with the E fork moving twice as fast as the L fork at early times. However, as this class is bound to T and becomes indistinguishable from synchronous RIM at greater than 30% elongation, the two classes are grouped together. The number of synchronous and slightly asynchronous RIM, 76%, is similar to the number of total RIM, 72%, on average, previously shown to bind T antigen (Tack and DePamphilis, 1983). Both synchronous and slightly asynchronous RIM terminate at the preferred region (Tapper et al., 1982; Tapper and DePamphilis, 1980) centered at 0.50 map units. In contrast, highly asynchronous L-sided RIM appear to terminate in the E gene region, centered at 0.73 map units, well outside of ter; pausing of asynchronous RIM at ter was not evident. As shown, two pathways exist for segregation of late SV40 RIM: catenated dimer and gapped SV40(II*) DNA formation (DePamphilis and Wassarman, 1982; Varshavsky et al., 1983).

What is the Role of SV40 Large T Antigen during Viral DNA Replication?—That T-associated RIM are characterized by synchronous progression of their forks indicates a function for T antigen in regulating replication fork movement. This additional role in the replication process, besides ori binding, is consistent with binding of T near some replication forks (Stahl et al., 1985) and inhibition of the in vitro elongation of SV40 chromosomes by antibodies specific for T (Stahl and Knippers, 1983). It may also be related to the discovery of a DNA helicase activity for this protein (Stahl et al., 1986).

Synchronous fork progression implies that one fork can communicate with the other during elongation, in this case, mediated by bound T antigen. Thus, we propose that T, the polymerase-prime complex, and other replication factors may bind simultaneously as a single complex to both forks in synchronous RIM. Such a complex might exist during elongation with the DNA reeling through from both sides of the genome in a highly processive manner until no slack remains (about 80% elongation for the circular SV40 molecule). Dissociation at this point could account for the major loss of T observed for RIM greater than 70% elongated (Tack and DePamphilis, 1983). Synthesis on this template by a now less processive polymerase-prime complex could account for the observed slower rate of fork movement, increased pausing at preferred DNA sequences, and accumulation of late RIM (see for review, DePamphilis and Wassarman, 1982). The replicative properties of SV40 mutants deleted at ter indicate that...
Replicating SV40 Chromosome Subclasses

**FIG. 9.** Major forms of replicating viral DNA intermediates associated with two distinct SV40 replication pathways during lytic infection. The 65-bp core origin (ori) is centered at nucleotide 0/5243; the normal termination (ter) region is centered at 0.5 map units. Initiation of double-stranded supercoiled SV40 DNA (I) templates involves association of T antigen (solid circle), DNA polymerase α (box), and for asynchronous replication only, putative factor X (triangle). Synchronous RIM initiate at bidirectional start site 1 or nucleotide 5203 (BR1) while asynchronous RIM initiate at site 2 or nucleotide 29 (BR2). Separation and termination intermediates include both catenated dimers and gapped SV40 DNA (II*) molecules.

SV40(II*) molecules, but not catenated dimers, are generated (Weaver et al., 1985). Thus, asynchronous RIM lacking T may not form catenated dimers; T bound to synchronously elongating RIM may affect which termination and separation pathway is chosen just prior to its dissociation. A second T binding site near ter (Jessel et al., 1976; Martin and Setlow, 1980) could function in the termination of those RIM with bound T.

Order of Events during Initiation—Sequence analysis of the location of Okazaki fragments synthesized near ori led Hay and DePamphilis (1982) to propose a model where SV40 DNA synthesis first initiates on the E strand and proceeds in the E direction. This becomes the forward arm of the E fork, exposing sites on the opposite DNA strand (see Fig. 8). DNA synthesis then initiates on the L strand and proceeds in the opposite direction, becoming the retrograde arm of this fork and finally the forward arm of the L fork. In short, first the forward arm of the E fork starts followed by that of the L fork. Our results, indicating two major bidirectional start sites at nucleotides 28–30 and 5203 can be integrated into this model using the same in vitro primer sites if one changes either the strand on which the forward arm is first started and/or the location of the sites first utilized for DNA synthesis on the E (nucleotides 5210–107 are available) or L strand (nucleotides 5146–5210 are available). In vitro replication studies using single-stranded SV40 DNA templates (Tseng and Ahlem, 1984) indicate that sites on the L strand within ori and the 21-bp repeats are used in the absence of T; however, these sites are not observed in vivo (Yamaguchi et al., 1985). Thus, utilization of primer sites on the L compared to the E strand within ori is limited in vivo, perhaps by bound T or another factor.

For synchronous RIM, we propose the above order to suffice. In contrast, for asynchronous RIM, the first primer site used may be on the L strand near the transition point with synthesis progressing in the L direction, becoming the forward arm of the L fork, exposing sites on the opposite strand. Initiation on the E strand (on the L side of ori) would proceed in the E direction, becoming the forward arm of the E fork. E-sided slightly asynchronous RIM may represent a subclass of synchronous RIM where the forward arm on the E side progresses farther before sites are exposed on the L strand and/or the forward arm on the L strand may be impeded until it passes through ori. Interestingly, 95 bp corresponds to the average length of spacer DNA between nucleosomes (230-bp repeat length, 146-bp nucleosomal core DNA; see for review, DePamphilis and Wassarman, 1982) in SV40 chromosomes and may reflect the effect of nucleosome phasing on initiation of this subclass. Changes in how T antigen binds at ori, perhaps reflecting the transcriptional state of the chromo-
some, could affect on which side of ori bidirectional initiation occurs (e.g. T bound to the autoregulatory site I may alter which primer sites are utilized).

**Nuclease Sensitivity, Replication Asynony, and Transcriptional Activation**—The distribution of synchronous and asynchronous RIM in BglI cut chromatin compared to BglI cut DNA was different. In contrast, the distribution for EcoRI cut RIM was the same. When the frequency of BglI cutting of the two strands of each replicating chromosome was compared (see Tack et al., 1981, for method), RIM lacking T (predominantly asynchronously) had a cutting pattern consistent with an altered chromatin structure on one compared to the other sibling strand (data not shown). This difference was not observed when total RIM were analyzed (Tack et al., 1981). Together, this indicates that synchronously and asynchronously replicating chromosomes are differentially sensitive to digestion, indicating an altered chromatin structure specifically at ori.

In general, there are several correspondences on analyzing the properties of different SV40 chromosome populations. The number of mature SV40 chromosomes that bind T antigen (25%) appear sensitive to BglI digestion (20–30%) and are nucleosome-free at the ori region (25%) and are all similar (Jakobovits et al., 1980; Saragosti et al., 1980; Tack and Beard, 1984; Tack et al., 1981). The appearance of this nuclease-sensitive site, the initiation of L transcription, and the onset of DNA replication correspond temporally (Cereghini and Yaniv, 1984). These nuclease-sensitive and nucleosome-free mature SV40 chromosomes have been shown to be transcriptionally active (Choder et al., 1984). Here, we show that a 24% subclass of SV40 DNA molecules replicates differently (i.e. asynchronously) and has an altered chromatin structure. This group of asynchronous RIM may represent a differentially transcriptionally active class of DNA molecules. In this way, two different replication pathways may be utilized to propagate an “active” chromatin structure among subsequent generations of viral progeny. Activation may involve just the L genes (transcribed during this phase of the viral lytic cycle) or both the E and L genes. Analysis using SV40 mutants with altered patterns of E and L RNA synthesis and deletions in sequences flanking core ori should clarify the relationship between differential gene activation and replication asynchrony.

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