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Two-Dimensional Gel Electrophoretic Method for Mapping DNA Replicons

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We describe in detail a method which allows determination of the directions of replication fork movement through segments of DNA for which cloned probes are available. The method uses two-dimensional neutral-alkaline agarose gel electrophoresis followed by hybridization with short probe sequences. The nascent strands of replicating molecules form an arc separated from parental and nonreplicating strands. The closer a probe is to its replication origin or to the origin-proximal end of its restriction fragment, the shorter the nascent strands that are detected by the probe. The use of multiple probes allows determination of directions of replication fork movement, as well as locations of origins and termini. In this study, we used simian virus 40 as a model to demonstrate the feasibility of the method, and we discuss its applicability to other systems.

Although considerable indirect evidence suggests that initiation of eucaryotic DNA replication occurs at specific nucleotide sequences, or origins (2), only recently have direct tests for initiation sites become available. Both Huberman et al. (7) and Brewer and Fangman (1) recently reported the use of two-dimensional (2D) gel electrophoretic methods which allowed localization of the replication origins and termini (sites where replication forks stop) used in vivo by *Saccharomyces cerevisiae* plasmids. Both methods show promise for application to eucaryotic chromosomal DNA. This report provides additional details of the method of Huberman et al. (7) and a current assessment of its applicability to higher eucaryotic organisms.

A hypothetical stretch of chromosomal DNA containing a replication origin and two sites for a restriction endonuclease (R) is shown in Fig. 1. The short segments labeled 1, 2, and 3 are assumed to be available for use as hybridization probes, and the entire restriction fragment is assumed to be replicated by forks traveling from left to right. DNA is isolated from cells (which must be growing but need not be synchronized), cut with restriction enzyme R, and enriched for fork-containing molecules by chromatography through benzoylated, naphthoylated DEAE-cellulose (BND-cellulose). The planar, aromatic benzoyl and naphthoyl groups can interact (in a manner similar to base stacking in native DNA) with the unpaired bases present in single-stranded DNA. Replicating molecules contain small single-stranded regions at their forks. These and other molecules with single-stranded regions are eluted from BND-cellulose with caffeine, which competes with the unpaired bases for the aromatic groups on BND-cellulose.

The replication-fork-enriched DNA molecules are then electrophoresed through a neutral agarose gel (Fig. 1D). Nonreplicating molecules form a sharp band (closed vertical bar), whereas the replicating molecules form a trailing smear. The position of a replicating molecule in the smear is determined by its extent of replication: molecules beginning replication migrate just behind the nonreplicating molecules, whereas almost fully replicated molecules are the most retarded.

The gel lane containing replicating DNA is rotated 90° and another gel with a higher percentage of agarose is poured around it. The entire second-dimension gel is then soaked in alkaline electrophoresis buffer to denature the DNA. During second-dimension electrophoresis, the four strands of each replicating molecule separate to produce two lines: a horizontal line composed of parental strands (which are the same size in all replicating molecules) and an arc containing nascent strands of increasing size. The DNA is then transferred onto a nylon membrane and hybridized with probes 1, 2, and 3 (Fig. 1E). The probe closest to the origin (probe 1) detects all but the shortest nascent strands, and the probe farthest away (probe 3) detects only the longest nascent strands.

If the results shown in Fig. 1E were obtained, it could be immediately concluded that replication forks travel through this restriction fragment from left to right and that the origin must be located at the left end of the fragment or outside the fragment to its left. Similar analysis of restriction fragments to the left would allow localization of the origin. The results shown in Fig. 1E are different from the results that can easily be predicted if this restriction fragment was replicated from randomly located origins rather than from a specific origin.

MATERIALS AND METHODS

Viral infection and DNA isolation. Simian virus 40 (SV40) was propagated in CV-1 cells (4) grown in Dulbecco modified Eagle medium containing 5% calf serum and 480 μg of gentamicin per ml. Viral DNA was extracted by the method of Hirt (6) at 42 h postinfection, treated with RNase A at a concentration of 2 mg/ml for 30 min, extracted with an equal volume of 90% (vol/vol) phenol–10% (vol/vol) 100 mM Tris (pH 8) at 4°C, and precipitated with ethanol. The DNA pellet was suspended in standard saline citrate (1× SSC; 150 mM NaCl, 15 mM sodium citrate). We now recommend that the DNA pellet be suspended in TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0).

Digestion with restriction endonucleases and enrichment for replicating DNA. For small DNA molecules (such as SV40) similar results are obtained regardless of the order in which restriction digestion and BND-cellulose fractionation are carried out. However, for large DNA molecules derived from eucaryotic chromosomes, we recommend that restric-

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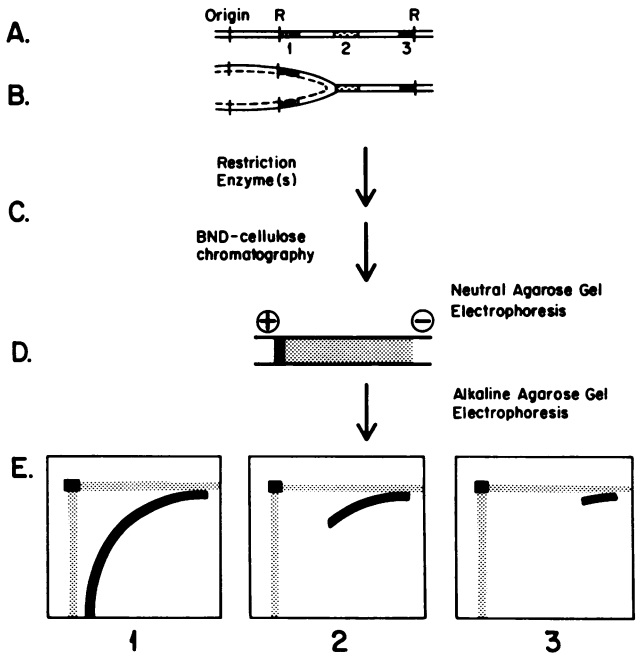

FIG. 1. Outline of basic replicon mapping procedure. Details are given in the text. (A) R, Restriction endonuclease cut sites in the area of interest; 1, 2, and 3, cloned sequences to be used as hybridization probes. (B) The same region being replicated by a replication fork traveling from left to right. (C) Cleavage by the appropriate restriction enzyme(s) and enrichment for replicating intermediates by BND-cellulose chromatography. (D) Neutral agarose gel electrophoresis in the first dimension (toward the positive electrode). (E) Alkaline agarose gel electrophoresis in the second dimension (from top to bottom). The three diagrams represent autoradiograms of the same gel blotted onto a nylon membrane and hybridized with the indicated probes. The closed box at the top left represents nonreplicating DNA, with randomly nicked strands extending downward (stipples) and the parental strand line extending horizontally (stipples). The closed arcs represent the nascent strands detected by the indicated probes.

tion digestion precede BND-cellulose fractionation. The restriction digestion should be carried out with a large excess of enzyme so that the time of incubation can be minimized, thus minimizing the possibility of branch migration. We have found that digestions shorter than 4 h at 37°C show no evidence of significant branch migration. For the experiments reported here, replicating SV40 DNA molecules were enriched by adsorption to BND-cellulose by a previously described column procedure (10). However, we have found that our recently described batch adsorption method (7) provides superior enrichment of replicating molecules (20- to 50-fold) with considerably less effort.

Electrophoresis. Although we did not do so in the experiments reported here, we recommend that appropriate size markers be run in lanes adjacent to the experimental samples in both the first and, especially, second dimensions. For the experiments reported here, between 20 and 50 ng of caffeine wash SV40 DNA (enriched for replicating molecules) and 1 μg of *Eco*RI-restricted calf thymus DNA as a carrier were used for each gel. The first-dimension gel (1% agarose) was run in TAE electrophoresis buffer (40 mM Tris-acetate, 1 mM EDTA; pH 8) at 1.5 V/cm for 16 h. The lane of interest was cut out and placed horizontally at the top of a second gel tray, and a 1.5% agarose (in water) gel was cast around it. The entire second-dimension gel was soaked in alkaline electrophoresis buffer {40 mM NaOH, 2 mM EDTA [ethylene glycol-bis(β-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid]} for 1 h and run at 1.5 V/cm for 24 h in the same buffer. Buffer recirculation and cooling by tap water may be useful during alkaline electrophoresis because the alkaline electrophoresis buffer has high conductivity and low buffering capacity.

Alkaline transfer and hybridization. After electrophoresis, the gels were depurinated in 0.25 N HCl for 30 min at room temperature, rinsed twice with distilled water, and blotted to Zetabind (Cuno) nylon membranes overnight by the alkaline transfer method (9). SV40 DNA restriction fragments (25 to 30 ng) used for hybridization probes (Fig. 2A) were labeled with [^{32}P]dTTP in low-gelling-temperature agarose by the random-oligonucleotide-primed synthesis method (3) to an activity of $>10^8$ cpm/μg of DNA. Hybridization was carried out at 42°C as described previously (8) except that the first two washes were at room temperature and the final wash was at 60°C. After autoradiography at −80°C, the hybridization probe was stripped from the membranes by three washes (1 liter each) with boiling 0.1× SSC–0.1% sodium dodecyl sulfate for 15 min. We now find that the probe can

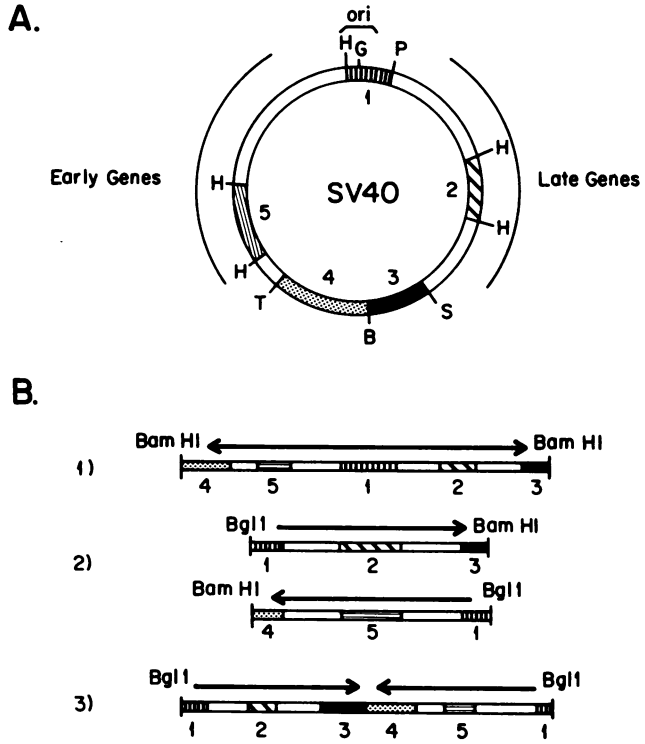

FIG. 2. (A) Restriction map of SV40 DNA, indicating regions used as hybridization probes. The nucleotide boundaries of each probe are as follows: 1, 5172 to 273; 2, 1047 to 1494; 3, 2138 to 2534; 4, 2535 to 3209; 5, 3447 to 4003. Abbreviations: B, *Bam*HI; G, *Bgl*I; H, *Hin*dIII; P, *Pvu*II; S, *Sau*3AI; T, *Pst*I. (B) Strategies used in this work to test the replicon mapping method. The arrows represent directions of replication fork movement. The strategies correspond to a replication origin in the region of interest, giving rise to bubble-containing restriction fragments (1); a replication origin either 5′ or 3′ to the region of interest, giving rise to restriction fragments traversed by replication forks from one end to the other (2); and a replication terminus in the region of interest, producing a restriction fragment with two replication forks moving inward from the ends (3).

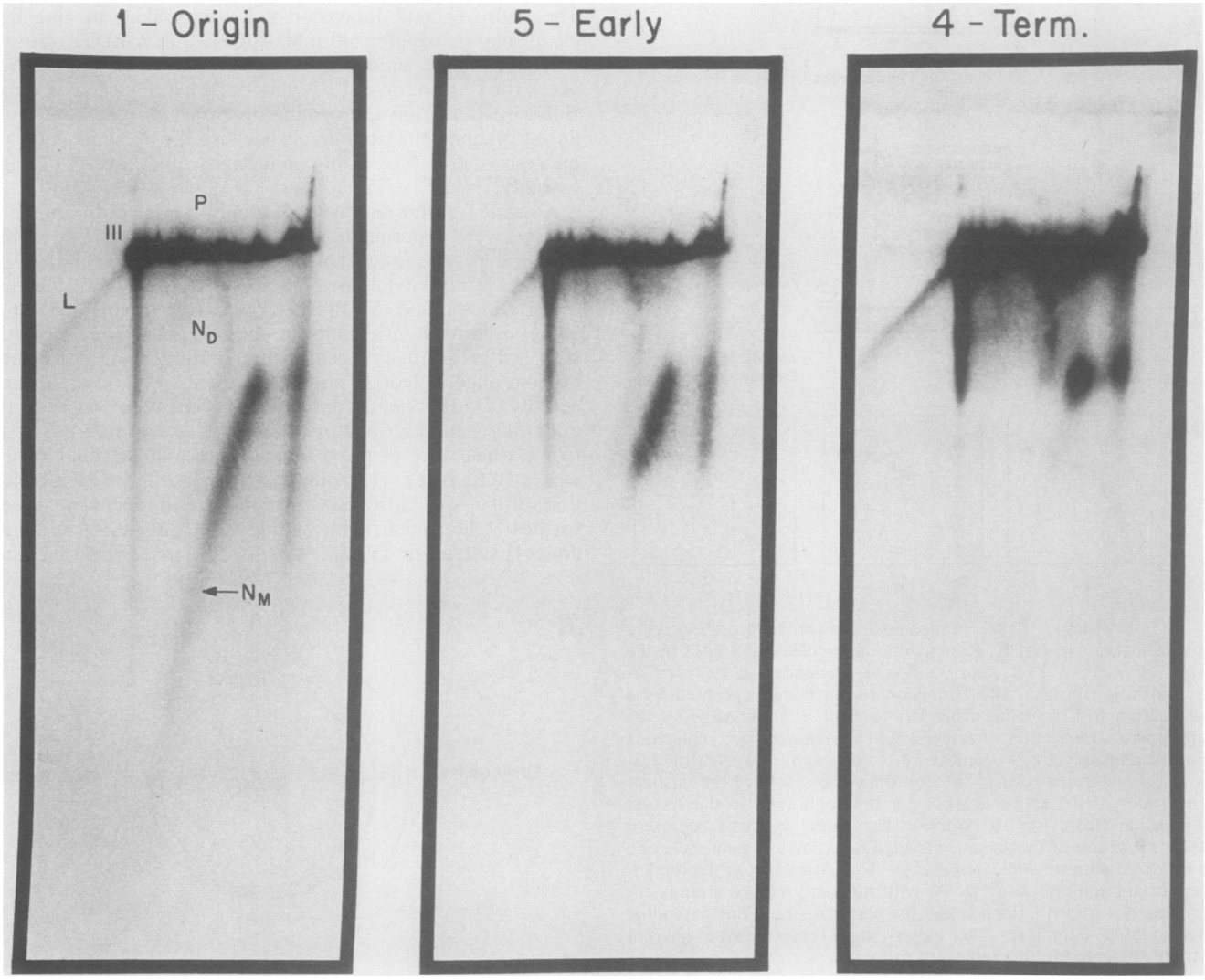

FIG. 3. *BglI*-restricted replicating SV40 DNA separated by 2D gel electrophoresis. First-dimension neutral gel electrophoresis was from right to left, and second-dimension alkaline electrophoresis was from top to bottom. The 2D gel was blotted to a nylon membrane and then hybridized sequentially with probe 4 from the terminus region (Term) (right), probe 5 from the early region (center), and probe 1 from the origin region (left). See Fig. 2 for probe positions. The larger total radioactive signals in the right and center panels than in the left panel here and in Fig. 4 are due to intentional longer exposure of the probe 5 and probe 4 autoradiograms to accurately assess the level of hybridization of the origin-distal probes to the shortest nascent strands. Abbreviations: L, linear molecules formed by random cleavage before first-dimension electrophoresis; III, nonreplicating form III molecules produced by cleavage with *BglI*; P, parental strand line; N_M, nascent strand arc formed by monomer molecules; N_D, nascent strand arc formed by dimer molecules.

be stripped from the membranes more simply and more effectively by washing the membranes twice with 0.2 N NaOH (30 min each at room temperature) followed by one wash of 20 min in 0.1 N Tris buffer fully titrated with HCl (Trizma hydrochloride; Sigma Chemical Co.)–1 mM EDTA.

RESULTS AND DISCUSSION

We used SV40 DNA to characterize our 2D gel origin mapping method because the unique SV40 DNA replication origin has been mapped with single-nucleotide precision (5). In addition, because the nucleotide sequence of the SV40 DNA molecule is known, we were able to select restriction enzyme combinations which allowed us to simulate results that might be obtained with a chromosomal restriction fragment containing an origin (Fig. 2B, strategy 1) or terminus (Fig. 2B, strategy 3) or traversed by a replication fork from one end to the other (Fig. 2B, strategy 2).

All of the experiments suggested in Fig. 2 were carried out. Because all of them yielded results consistent with the known location of the SV40 origin, only one experiment will be presented in detail.

***BglI*-digested SV40 DNA.** If replicating SV40 DNA is cut near the origin with *BglI*, double-fork-containing fragments are produced (Fig. 2B, strategy 3). These fragments are analogous to chromosomal fragments containing replication termini. For the SV40 *BglI* fragment, the terminus is located at the midpoint of the fragment. Therefore, nascent strands

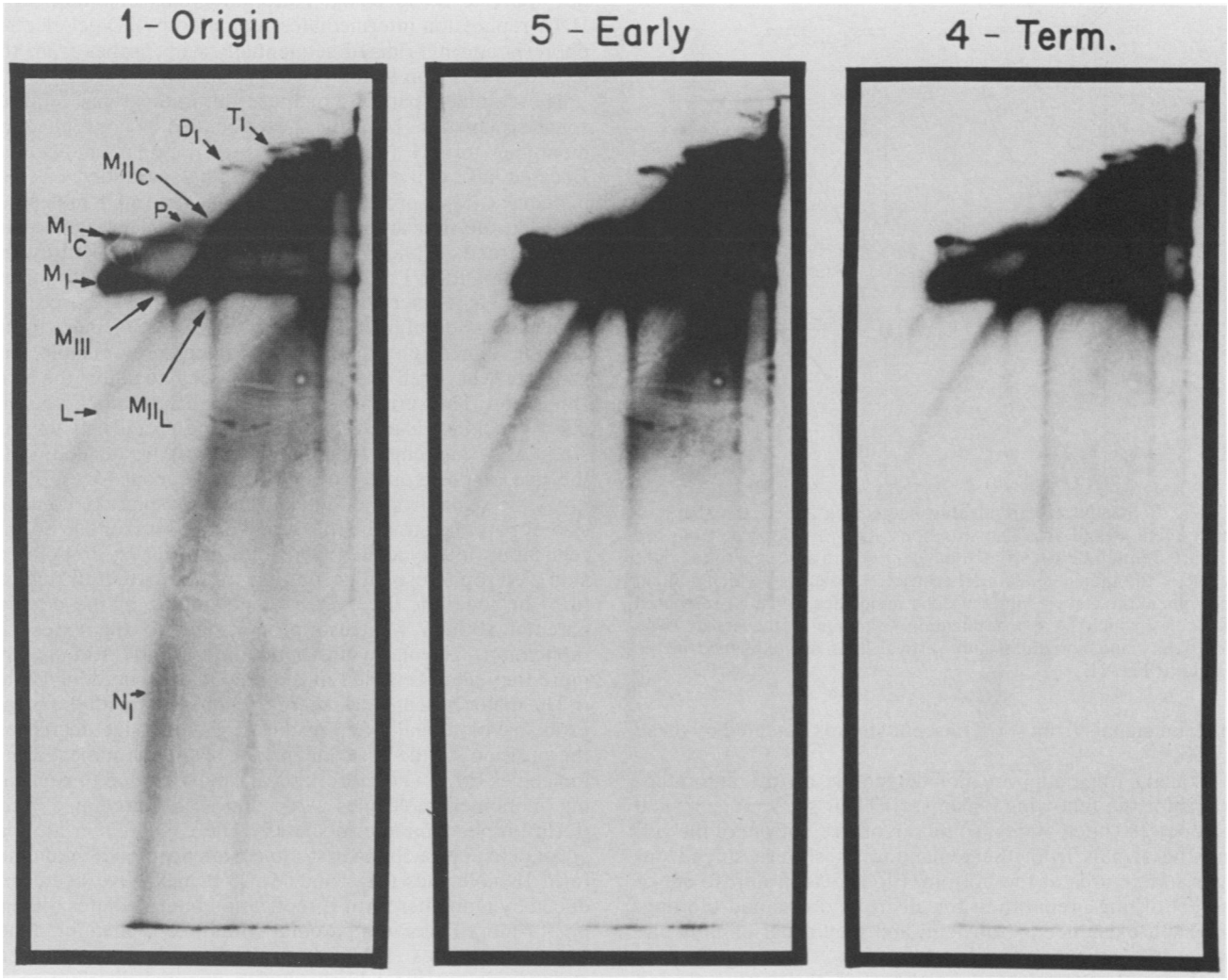

FIG. 4. Intact replicating SV40 DNA separated by 2D gel electrophoresis. The probes and directions of electrophoresis are described in the legend to Fig. 3. Abbreviations: T_I, D_I, and M_I, trimer, dimer, and monomer nonreplicating form I molecules, respectively; M_{II_C} and M_{II_L}, single-stranded circular and linear monomer molecules, respectively, arising from denaturation of monomer form II molecules; P, parental strand line; M_{I_C}, single-stranded circles arising from the denaturation of monomer form I molecules that were nicked between the first and second dimension; M_{III}, linear monomer molecules; L, random-size linear molecules formed by double-strand breakage before first-dimension electrophoresis; N_I, nascent strand arc derived from replicating form I molecules.

grow to a maximum size of one-half the genome length. The nascent strands attain full genome length by ligation during the process of termination. Autoradiograms of a blot of a 2D gel of *BglI*-cut replicating SV40 DNA sequentially hybridized with the indicated probes (Fig. 2A) are shown in Fig. 3. In addition to the major spot due to linearized nonreplicating DNA (III), a diagonal line (L) terminating at this spot is evident. This line is due to the linear molecules present in the original population. When cut with *BglI*, these molecules formed a heterogeneous-size collection of linear molecules, all having the *BglI* site at one end. The horizontal line due to parental strands is labeled P. The vertical streaks located on the right-hand sides of the autoradiograms are most likely due to replication intermediates trapped in the well during first-dimension electrophoresis and released in the second dimension, while the vertical streak descending from the major spot (III) is due to occasional randomly located nicks in the nonreplicating DNA.

The arc of nascent strands from replicating SV40 monomer molecules (N_M) is evident in the left panel of Fig. 3 (probe 1). The longest strands in this arc are about half the genome size, as expected for termination in the middle of the SV40 *BglI* fragment. Consistent with the known location of the SV40 origin, the probe covering the origin region (probe 1) detected nascent strands of all sizes, whereas probe 5 (early region) detected strands only of intermediate and longer sizes and probe 4 (termination region) detected only the longest nascent strands. The probe 4 autoradiogram was intentionally overexposed to detect possible faint signals from shorter nascent strands, and indeed, a faint signal (visible in the original autoradiogram) is detectable from the entire nascent strand arc. This faint signal was also detected with probe 3 (results not shown), and in other experiments both probes 3 and 4 (but not probe 2 or 5) detected faint signals from short nascent strands (results not shown). Although we could not find any significant homology between the probe 3 or 4 region and the origin region by computer search, we did detect minor cross-hybridization between these probes and SV40 restriction fragments containing the origin region (results not shown), thus explaining

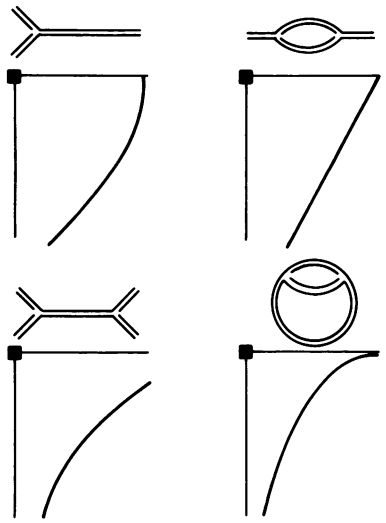

FIG. 5. Structure of replicating molecules affects the shape of the nascent strand arc. The situations shown, clockwise from top left, are *Bam*HI-*Bgl*I-restricted molecules with an origin to the right or left of the molecule, *Bam*HI-restricted molecules which contain an origin at the center, intact circular molecules, and *Bgl*I-restricted molecules which have a replication terminus at the center. The closed box and horizontal and vertical lines are explained in the legend to Fig. 1E.

the faint signals from short nascent strands detected by these probes.

Okazaki fragments are not detectable on the autoradiograms for the following reasons. (i) Their sizes range from 0 to about 200 nucleotides; some ran off the bottom of the gel, and the signals from the remaining ones were spread out over a large area at the bottom. (ii) They can diffuse out of the gel during preparation for electrophoresis and blotting. (iii) Their existence is transient, and they occur on only one side of the replication fork; consequently, they are present in much smaller amounts than those of the longer ligated nascent strands.

Dimer replication. There is also a minor arc (N_D) shown in Fig. 3 extending sharply downward from the parental strand line to the left of and parallel to the monomer nascent strand arc (N_M). As for the monomer nascent strand arc, probe 1 detected the shortest strands in N_D, with only increasingly longer strands detected by probe 5 and probe 4. We think that the near vertical portion of N_D is due to size-independent migration of large Y-shaped molecules in the first dimension (see below). Because the maximum nascent strand length in N_D is full genome size, this arc could be due to bidirectional replication from one of the two origins of dimer molecules or to unidirectional replication of monomer molecules. The latter possibility was ruled out, however, by the observation that probe 5 (Fig. 3) and probe 2 (results not shown) each detected nascent strands of about one-fourth to full genome length. There was no significant increase in signal intensity with either probe for nascent strands longer than three-fourths of the genome length. Thus, this arc (N_D) arose from replicating dimers. As far as we are aware, the experiments reported here are the first to provide evidence that circular dimers of SV40 replicate bidirectionally from one of the two origins.

Intact SV40 molecules. Although intact eucaryotic chromosomal DNA molecules are so large that they are difficult to obtain and to resolve by electrophoresis, these limitations do not apply to SV40 DNA. Autoradiograms of intact SV40 DNA replication intermediates separated by 2D gel electrophoresis and hybridized sequentially with probes from the termination region to the origin are shown in Fig. 4.

The complex patterns in these autoradiograms requires some explanation. M_I is the spot produced by nonreplicating monomer form I (covalently closed circular) molecules. Located just above this spot are single-stranded circular molecules (M_{I_C}) produced by nicking of form I molecules between the first and second dimensions. The signal from linear strands produced at the same time is masked by the comigrating form I molecules. M_{II_L} (monomer-length linear) and M_{II_C} (monomer-length circular) strands were produced by denaturation of form II (nicked circular) molecules in the alkaline dimension. Dimer and trimer form I molecules generated the small spots (D_I and T_I) located above the form II position. The vertical streaks on these autoradiograms are due to nicking, leading to linear single strands of various sizes. M_{III} (monomer form III molecules) are located on L, the line of linear molecules of heterogeneous sizes. P denotes the signal from parental strands. Despite the constant size of parental strands, this signal is not horizontal. Form I replication intermediates were retarded during first-dimension electrophoresis in proportion to their extent of replication. In addition, the degree of unwinding of the circular parental strands was also proportional to the extent of replication. The more unwound the parental strands, the more they were retarded in the second alkaline dimension.

The major nascent strand arc (N_I) obtained with the origin probe asymptotically approaches (as strand size decreases) the position, in the first dimension, of the monomer form I molecules (M_I). Therefore, this arc must be due to replicating monomer molecules with unnicked parental strands (form I replication intermediates). There is a fainter arc that appears to approach the first-dimension position of monomer form II molecules (M_{II_L} and M_{II_C}). It may have been produced by monomer form II replication intermediates (having one or more nicks in a parental strand) or replicating dimer form I molecules (D_I) or both. The swirls in the nascent strand arcs are most likely due to unequal distribution of charge during manufacture of the nylon membrane (a problem we have seen with several lots of membranes from various manufacturers). As in previous experiments, probe 1 detected nascent strands of all sizes, probe 5 detected nascent strands of intermediate to full size, and probe 4 detected only the longest nascent strands (except for a faint signal from shorter strands due to cross-hybridization).

Quantitation of dimer replication. By combining data from Fig. 4 and 3, we were able to determine whether dimers replicate in proportion to their abundance. First, we used densitometry to determine the ratio of form I dimers to form I monomers in Fig. 4. The ratio is 4.1%. We also used densitometry to integrate the signals from the nascent strand arcs produced by dimers and monomers in Fig. 3. The ratio of dimer nascent strands to monomer nascent strands is 6.8%. Thus, dimers replicate approximately in proportion to their abundance.

Shapes of nascent strand arcs. An unanticipated conclusion of the experiments discussed (Figs. 3 and 4) and of other experiments in which SV40 DNA was cut with *Bam*HI or *Bam*HI plus *Bgl*I to generate restriction fragments with internal bubbles or Y-shaped forks (results not shown) is that the structures of replicating molecules affect the shapes of the nascent strand arcs produced by 2D neutral-alkaline gel electrophoresis (Fig. 5). Because in all instances second-dimension alkaline electrophoresis separates single strands

according to the logarithm of their size, we conclude that the differences in arc shape shown in Fig. 5 derive from differences in the relationship between the extent of replication and mobility in the first dimension. This relationship is approximately logarithmic when SV40 DNA is cut with *Bam*HI (Fig. 5, top right), producing a linear nascent strand arc. The relationship is closer to linear in intact and *Bgl*I-cut molecules, producing arcs which curve to the right (Fig. 5, bottom). Beyond 50% replication, the mobility of *Bam*HI-*Bgl*I-cut molecules is not significantly affected by the extent of replication, leading to a nascent strand arc which curves upward (Fig. 5, top left). Similar shape-dependent differences between the extent of replication and mobility have been detected by Brewer and Fangman (1).

Accuracy of origin mapping. For the trial experiments reported here, no size markers were included in the second dimension. When size markers are included so that the lengths of nascent strands can be measured, origins and termini may be mapped with an accuracy which depends on probe size and on probe proximity to the origin or terminus. We have used this method to map the replication origin of the *S. cerevisiae* 2μm plasmid with an accuracy of ±100 base pairs (7).

A complementary method. Brewer and Fangman (1) have independently developed a 2D gel method for replicon mapping. The two methods appear to complement each other, and both methods yield the same information about the replication properties of the *S. cerevisiae* 2μm plasmid (1, 7). Both methods offer a significant advantage over most previous methods for mapping replicons: they allow direct determination of replicon properties in normal cells undergoing normal growth. The enrichment for replicating DNA provided by adsorption to BND-cellulose makes synchronization unnecessary with either method.

Application to chromosomal DNA molecules. We are applying this technique to stretches of unique *S. cerevisiae* chromosomal DNA and have obtained results of greater clarity than those obtained with SV40 (Fig. 3 and 4) or the *S. cerevisiae* 2μm plasmid (7). This increased clarity is due simply to the absence of multimeric and isomeric forms of unique chromosomal DNA. We are now starting with 100-μg quantities of yeast chromosomal DNA, cutting the DNA with appropriate restriction endonucleases, fractionating it by adsorption to BND-cellulose, and obtaining yields of about 1 μg in the caffeine wash. The entire 1 μg is then subjected to 2D gel electrophoresis in a minigel format (7) and blotted to a nylon membrane. Hybridization with probe DNAs of about 500 base pairs yields clear signals from nascent strands after an overnight exposure.

The human genome is about 200-fold more complex than the *S. cerevisiae* genome. Direct translation of our current conditions to the human genome would require subjecting about 200 μg of caffeine wash DNA from growing cells to 2D gel electrophoresis. Although these amounts of DNA are not impossible to obtain or electrophorese, several simple technical modifications would allow the use of smaller amounts. These possible modifications include the use of longer exposure times, larger probes, four ^{32}P-deoxynucleoside triphosphates rather than a single one during probe labeling, and a smaller 2D gel format. It is our hope that numerous laboratories will begin to use this technique and that of Brewer and Fangman (1) to explore the replicon organization of the human and other higher eucaryotic genomes.

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