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Method for Mapping DNA Replication Origins

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We have developed a method which allows determination of the direction in which replication forks move through segments of chromosomal DNA for which cloned probes are available. The method is based on the facts that DNA restriction fragments containing replication forks migrate more slowly through agarose gels than do non-fork-containing fragments and that the extent of retardation of the fork-containing fragments is a function of the extent of replication. The procedure allows the identification of DNA replication origins as sites from which replication forks diverge. In this paper we demonstrate the feasibility of this procedure, with simian virus 40 DNA as a model, and we discuss its applicability to other systems.

During the eucaryotic S phase, DNA replication initiates at multiple discrete sites spaced irregularly along chromosomal DNA (23). These sites are termed origins because replication proceeds bidirectionally away from them (23). The question of whether chromosomal replication origins are located at specific nucleotide sequences or are located randomly has been intensively studied (1–4, 6–8, 12, 13, 18, 19, 21, 25, 28–30, 37–39, 42, 43, 45, 47), but so far no definitive answer has been obtained.

The clearest evidence for specific origins has been obtained in studies of certain extrachromosomal DNAs: the extrachromosomal rDNA in *Physarum polycephalum* and *Tetrahymena thermophilia* (6, 42, 43) and the 2μ plasmid of yeasts (30). Evidence that chromosomal origins may occur nonrandomly comes from studies with certain repeated DNAs: the rDNAs of several species (2, 3, 28) and the amplified dihydrofolate reductase genes of a Chinese hamster cell line (18, 19); however, the resolution of these studies has not been sufficient to determine whether initiation occurs at a specific nucleotide sequence or anywhere within a relatively long stretch of DNA. For instance, the early labeling restriction fragments of the amplified dihydrofolate reductase gene are 6.1 and 11.5 kilobases (kb) in length (19).

The discovery that certain specific nucleotide sequences (autonomously replicating sequences [*ars*'s]) permit extrachromosomal DNAs to replicate autonomously in yeast cells (1, 4, 7, 8, 12, 13, 25, 37–39, 47) suggests the possibility that *ars*'s may correspond to chromosomal replication origins, but such a correspondence has not yet been directly demonstrated.

Other studies suggest that particular nucleotide sequences alone are insufficient to precisely determine the site and timing of initiation of DNA replication. (i) The two copies of the mammalian female X chromosome are replicated at different times in S phase, although their nucleotide sequences are probably identical (46). (ii) Sequences used as origins in early embryos of insects and amphibians cannot be used reproducibly in adults because replicons are smaller and more closely spaced in embryos than in adults (reviewed in reference 11). (iii) Viral or plasmid DNAs, when injected into *Xenopus laevis* eggs, initiate efficiently at multiple, apparently random sequences (16, 29). Further examples of the complexity of replication initiation are reviewed by Hand (14).

From this brief review it is clear that more information is needed on the location of sequences which function as origins in the chromosome. We describe here a method which should allow the examination of origin location for many different repeated- and single-copy eucaryotic chromosomal DNAs. This method allows the immediate determination of the direction(s) of replication fork movement through defined segments of chromosomal DNA. Origin locations can be deduced by mapping the direction(s) of replication fork movement through long contiguous stretches of chromosomal DNA.

Mapping direction(s) of replication fork movement. The rationale of this procedure can best be understood by referring to the diagram in Fig. 1.

Consider a hypothetical stretch of chromosomal DNA, ACB, which can be cut by restriction enzymes 1 and 2 into three fragments of different lengths A, B, and C. Assume that ACB has been cloned and is available for use as a hybridization probe. If total chromosomal DNA is isolated from cells undergoing DNA replication, digested with restriction enzyme 1, and electrophoresed in an agarose gel, then fragments ACB which do not contain replication forks will migrate as a sharp band. The replication fork-containing fragments ACB will migrate as a smear. Fragments with small forks will migrate further than those with large forks. If the DNA fragments in each fraction of this first-dimension agarose gel are treated with restriction enzyme 2, electrophoresed in a second-dimension agarose gel, transferred to nitrocellulose, and hybridized to radioactively labeled, cloned sequence ACB, one of several possible patterns will be seen after autoradiography. If the origin of replication is located outside and to the left of sequence ACB, then the pattern in Fig. 1B will be obtained. Nonreplicating ACB from the first gel will give rise to unit-size subfragments A, B, and C in the second dimension. Small, replication fork-containing fragments from the first gel will give rise to unit-size B and C but larger-than-unit-size A in the second dimension. Increasingly larger, fork-containing fragments from the first-dimension gel will yield increasingly larger A subfragments in the second gel. When fragments from the first gel are encountered, in which replication forks have passed the A/C boundary, subfragment C will begin to increase in size, whereas subfragment A will return to its unit size. Subfragment C will increase in size until fragments containing replication forks which have passed the C/B boundary are encountered. At this point, subfragment C will

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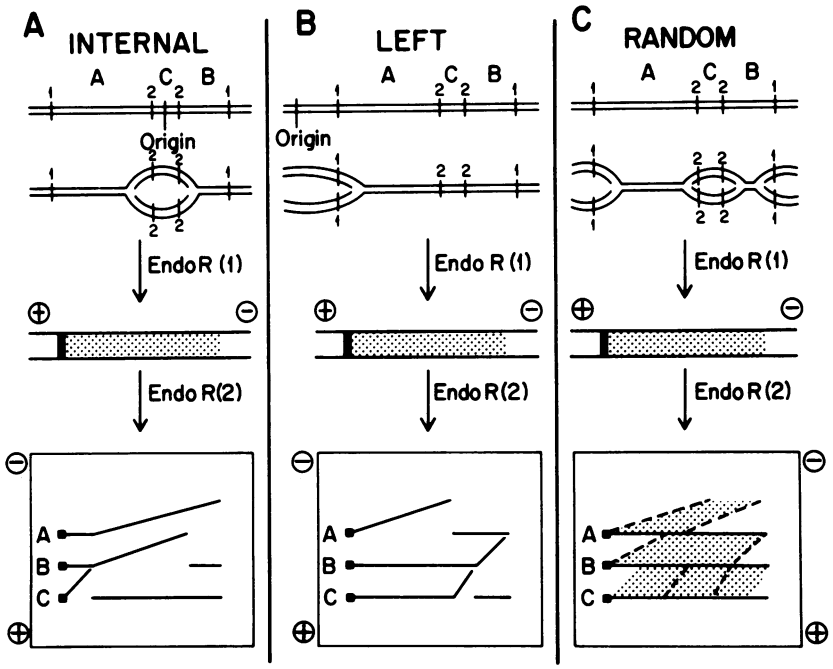

FIG. 1. Diagrammatic representation of the procedure for mapping the direction(s) of replication fork movement. See the text for explanation.

return to unit size and subfragment B will begin to increase in size.

The diagonals in the lower portion of Fig. 1 represent the positions of replication fork-containing subfragments. The diagonals have been drawn at different angles; for instance, the diagonal depicting replication fork-containing subfragment C is steeper than the one depicting replication fork-containing subfragment B (Fig. 1A). The reason for this is that the relationship between molecular weight and migration distance (mobility) of DNA molecules in an agarose gel is logarithmic. This means that the distance between two DNA molecules which differ in size by a constant amount (i.e., 500 base pairs) is greater for smaller DNA molecules than it is for larger DNA molecules.

An analogous scheme can be drawn for origins located to the right of the segment of interest or for an origin located within the segment (Fig. 1A). However, if the origin from which the segment is replicated is not specific (e.g., is located in different sequences in different cells), then the pattern in Fig. 1C will be found. The predominant feature of the random-location pattern will be that of unit-size subfragments from all regions of the first agarose gel. In addition, diagonals will arise from all subfragments simultaneously (stippled area of bottom panel of Fig. 1C). Most copies of this sequence will be replicated from origins outside the sequence because the average size of the eucaryotic replicon (30 to 100 kb) usually will be greater than the size of ACB (ideally 5 to 10 kb). Therefore, both the diagonals predicted for outside origins to the left of ACB (as in Fig. 1B) and the diagonals predicted for outside origins to the right of ACB should be observed in relative abundance, as shown by the dashed lines in Fig. 1C.

The examples (Fig. 1) serve to illustrate that, from the pattern of diagonals and unit-size fragments observed in the second dimension, one can determine the direction(s) of replication fork movement through the sequence ACB. From the direction(s) of fork movement, one can infer the location(s) of origin(s), that is, outside ACB to the left or right, within ACB, or at multiple positions.

MATERIALS AND METHODS

Cell culture and viral infection. BSC-1 cells, a continuous line of African green monkey kidney cells, were infected with simian virus 40 (SV40), small plaque strain 776, at a multiplicity of 1 to 10 PFU per cell. (Cells and virus were a gift from M. Woodworth.) Cells were propagated in Eagle minimal essential medium containing 2% fetal calf serum and 8% calf serum. Infection was carried out in Eagle minimal essential medium with 2% calf serum.

DNA isolation and purification. At 36- to 40-h postinfection, cells were labeled for 5 min with 100 μCi of [^{3}H]thymidine (42 Ci/mmol; Amersham Corp.) per 75-cm^2 plate. Label incorporation was stopped by washing cells in ice-cold phosphate-buffered saline. Viral DNA was then selectively extracted by the method of Hirt (22). In some experiments, cells were labeled overnight with [^{14}C]thymidine (40 mCi/mmol; Amersham Corp.) at 0.1 μCi per plate.

The Hirt supernatant, containing viral DNA, was treated with proteinase K (Boehringer Mannheim Biochemicals) at 50 μg/ml for 2 h at 37°C. It then was dialyzed for 4 to 6 h against 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.8; TE buffer) to reduce NaCl and sodium dodecyl sulfate concentrations. The DNA was ethanol precipitated, resuspended in TE buffer, and brought to a refractive index of 1.392 with CsCl. Hoechst dye 33258 (5 mg/ml) was added at 0.1 ml/5 ml of CsCl solution. Gradients were then centrifuged at 80,000 × *g* for 40 h at 20°C in a Beckman 50.1 Ti rotor. DNA was visualized as a fluorescent band with long-wave UV light and removed by a Pasteur pipette. The Hoechst dye was removed by two treatments with CsCl-saturated isopropanol,

CsCl was removed by dialysis, and the DNA was ethanol precipitated.

Benzoylated, naphthoylated DEAE-cellulose (BND-cellulose; Serva Fein Biochemica) was used to enrich for replication intermediates as in references 20 and 27, with the exception that DNA was applied to the column in 0.1 M NaCl–10 mM Tris-hydrochloride–1 mM EDTA (pH 7.4). The column was washed with 0.3 M NaCl–10 mM Tris–1 mM EDTA (pH 7.4), and the bulk of nonreplicating material was eluted from the column with 0.8 M NaCl–10 mM Tris–1 mM EDTA. Single-strand-containing molecules were then eluted with 2% caffeine in the 0.8 M NaCl buffer. For 200 μg of SV40 DNA, a column (height, 4 cm; diameter, 0.9 cm) was used. The flow rate was ca. 0.5 ml/min.

After enrichment on BND-cellulose, the replication intermediate-containing caffeine eluate was dialyzed against TE buffer and ethanol precipitated before digestion with restriction endonucleases. Restriction enzymes were obtained from Bethesda Research Laboratories, International Biotechnology, Inc., and New England BioLabs and were used according to the instructions of the manufacturers.

First-dimension agarose gel electrophoresis. The restriction digest, containing both replicating and nonreplicating fragments, was separated by size on an agarose gel. This was accomplished with an electroelution gel apparatus from Bethesda Research Laboratories (model no. 1100PG). Fractions were eluted from a 0.6 to 0.8% agarose tube gel (height, 2.5 to 4 cm) with a 3× concentration of electrophoresis buffer (1× = 30 mM Tris, 10 mM sodium acetate, 5 mM EDTA [pH 8.0]) and collected continuously when the bromophenol blue dye marker reached the bottom of the gel. The voltage applied to the gel was 40 V, and the flow rate of the elution buffer was 3 to 4 ml/h. The fraction volume was 0.25 to 0.35 ml. The M_r of the fragment of interest was determined by analysis on a slab gel of similar composition and running conditions, and from this the elution position of the fragment from the electroelution gel could be predicted. A small sample of each fraction from the electroelution gel was electrophoresed on a slab gel to verify the elution position of the fragments of interest. ^{3}H radioactivity was determined in another small sample of each fraction to determine the recovery of replicating DNA.

Second-dimension agarose gel and autoradiography. The appropriate fractions from the first-dimension gel were ethanol precipitated with 20 μg of tRNA per ml as carrier. The fractions were resuspended in a buffer appropriate for the second retriction enzyme. Up to a 10-fold excess of enzyme was used, and incubation never exceeded 2 h. The products of the restriction digestion were applied to a 1% agarose slab gel and electrophoresed at 25 to 30 V for 16 to 20 h. The DNA was transferred out of the gel onto nitrocellulose filters by the procedure of Southern (36). Plasmid p49, containing the large *Pst*I fragment of SV40 cloned into the *Pst*I site of pBR322 (R. Saavedra and R. Hill, unpublished data), was labeled by nick translation with [^{32}P]dTTP (34) and then used as probe. Specific activities ranged from 0.5×10^8 to 2×10^8 cpm/μg. Hybridization (in the absence of dextran sulfate) and autoradiography were performed by established procedures (references 44 and 26, respectively).

Selective transfer of replicating DNA to nitrocellulose filters. The second-dimension agarose slab gel was soaked in a high-salt buffer (1 M Tris [pH 8.0], 1.5 M NaCl) for 45 min with gentle agitation. No denaturing buffer was used. After transfer in the normal manner (36) and baking the filter at 80°C under vacuum, the filter was treated at 65°C with 0.01× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate)–0.1× Denhardt solution (10) for 2 h with several changes of solution. The filter then was drained of excess liquid, and prehybridization was performed as noted above.

RESULTS

We have used replicating DNA of SV40 to test our procedure and to optimize experimental conditions. SV40 DNA contains a single, specific replication origin whose location is well known (9, 17, 48), and replicating SV40 DNA can easily be prepared in quantities sufficient for these studies (40).

BND-cellulose chromatography. The origin location procedure requires that diagonals on the autoradiograms (as diagrammed in Fig. 1) be clearly visible and not obscured by a vast excess of nonreplicating subfragments. It is necessary, therefore, to obtain replicating DNA as free of nonreplicating DNA as possible. This problem was addressed in two ways: (i) selection for replicating DNA on a column of BND-cellulose and (ii) selective transfer of replicating DNA to a nitrocellulose filter before hybridization with the cloned SV40-specific probe, p49. Here we discuss enrichment for replicating DNA by BND-cellulose chromatography; selective transfer to nitrocellulose is discussed later.

BND-cellulose has been shown to select for DNA molecules which contain regions of single-stranded character (20), and has been used by others to enrich for replicating SV40 DNA molecules (17, 27, 29, 41). To quantitate the extent of enrichment in this study, nonreplicating and replicating SV40 DNA were selectively labeled with [^{14}C]- and [^{3}H]thymidine, respectively. ^{14}C and ^{3}H radioactivity were then measured in fractions from the BND-cellulose column. In addition, the proportion of replicating molecules in the caffeine eluate was analyzed by electron microscopy. The results of both analyses were in agreement. The caffeine eluate from the BND-cellulose column contained 90% of the ^{3}H radioactivity but only 10% of the ^{14}C radioactivity. Of the SV40 molecules in the caffeine eluate, 10% contained replication structures visible in the electron microscope, whereas only 1% of the starting material did (data not shown; L. Spotila, Ph.D. thesis, State University of New York, Buffalo, 1985). Thus BND-cellulose chromatography provided a ca. 10-fold enrichment for replicating DNA.

First-dimension agarose gel electrophoresis. Buckler-White and Pigiet (5) have shown that restriction fragments of polyoma DNA which contain replication forks can be resolved by agarose gel electrophoresis. Similarly, Tapper and DePamphilis (40) observed that unrestricted circular SV40 DNA molecules can be displayed as a linear function of extent of replication by agarose gel electrophoresis. The procedure described here requires that the ordering of replication intermediates achieved by the first-dimension agarose gel be maintained in the second-dimension analysis. In addition, the DNA from the first dimension must be successfully cut by restriction enzymes.

To achieve two-dimensional analysis of replication intermediates several methods were tried. (i) The first-dimension gel, consisting of low gelling temperature agarose, was sliced, the slices were heated to melt the agarose, and the second-dimension restriction enzyme was added (32). This method did not provide an adequate signal from replicating DNA molecules in the second dimension, presumably because high temperature promoted branch migration (49). (ii)

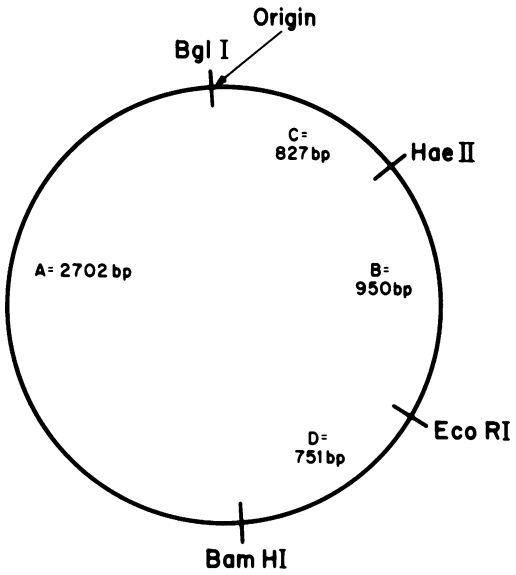

FIG. 2. SV40 restriction map showing the position of the origin of replication and the restriction enzyme sites relevant to this study.

We also tried to restrict the DNA displayed in the first-dimension gel in situ with the second-dimension restriction enzyme (35). However, in our hands, successful cutting by the second restriction enzyme could not be achieved. (iii) The third method was electroelution of DNA molecules from the first-dimension gel, followed by restriction enzyme treatment of each fraction (33). This method proved satisfactory; electroelution of replication intermediates is described above. DNA molecules in alternate fractions from an electroelution gel were viewed with the electron microscope to analyze the extent of replication. We concluded that the electroelution gel did fractionate replication intermediates according to the extent of replication with reasonable success (data not shown; Spotila, Ph.D. thesis).

Second-dimension analysis of replicating DNA. To test the origin location procedure with SV40 DNA, three sets of restriction enzymes were used (Fig. 2). In the first experiment (Fig. 3), the enriched population of replicating DNA molecules from the BND-cellulose column was cut with *Eco*RI (Fig. 2) and then electrophoresed on an electroelution gel, and each fraction was restricted with *Bgl*I (Fig. 2). Since the location of the SV40 origin is very close to the *Bgl*I site, it was predicted that early replication intermediates would give rise to two fork-containing subfragments after *Bgl*I digestion (Fig. 3a). Similarly, replication intermediates in which the replication fork had passed the *Eco*RI site would result in structures containing two forks (corresponding to AD in Fig. 2) and unit-size molecules (corresponding to BC in Fig. 2). The products of the *Bgl*I digestion were sequentially applied to an agarose slab gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled SV40-specific probe (Fig. 3b). The most prominent feature of the autoradiogram is the intense signal in the position of nonreplicating unit-size molecules (1.8 and 3.4 kb). However, increasingly larger-than-unit-size subfragments also can be detected as progressively more advanced replication intermediates are encountered in fractions 18 to 32. In fraction 30 there is an increase in the amount of unit-size, nonreplicating subfragment CB, which arises from replication intermediates in which the fork has passed the *Eco*RI site.

In the second experiment (Fig. 4), the replication intermediate-enriched population of molecules was incubated with *Bgl*I and *Bam*HI. The products were fractionated on an electroelution gel and then incubated with *Hae*II. Knowing the location of the origin with respect to these restriction sites, the following could be predicted (Fig. 4a). The 2.7-kb *Bgl*I-*Bam*HI fragment (A of Fig. 2) should contain a replication fork from the earliest position of the first gel, and the fork size should increase as later fractions are encountered, giving rise to a simple diagonal. The 2.5-kb *Bgl*I-*Bam*HI fragment (CBD of Fig. 2) is cut by *Hae*II into 0.8 (C) and 1.7 (BD) kb subfragments. Subfragment C should contain forked structures starting at the earliest position, whereas subfragment BD should remain unit size until forked structures from the first gel are encountered in which the fork has passed the *Hae*II site. At this point, subfragment BD will begin to increase in size, but subfragment C will return to

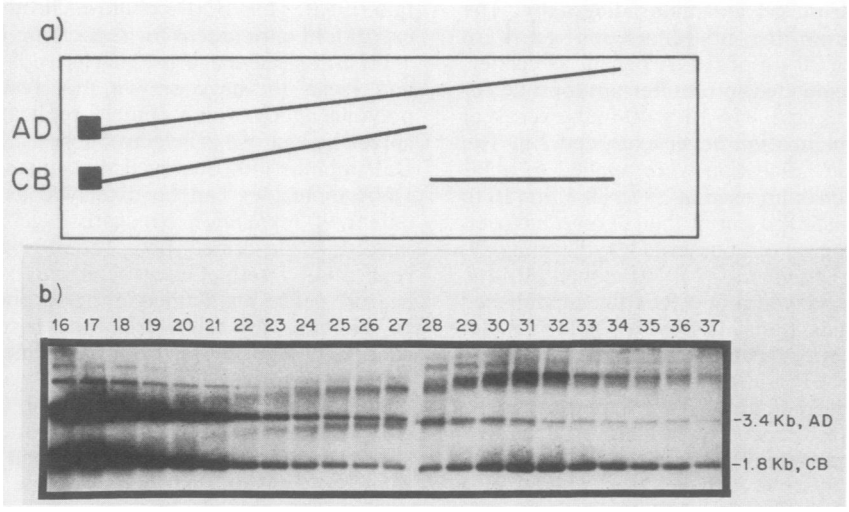

FIG. 3. The caffeine eluate from a BND-cellulose column was incubated with *Eco*RI and electrophoresed in the first dimension on an agarose electroelution gel. Fractions containing SV40 DNA were restricted with *Bgl*I and sequentially applied to an agarose slab gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled p49. a) Predicted pattern of nonreplicating and replicating DNA subfragments. DNA subfragments which migrate to the unit-size position do not contain replication forks, whereas subfragments which migrate more slowly do contain replication forks. b) Autoradiogram; Exposure was 20 h.

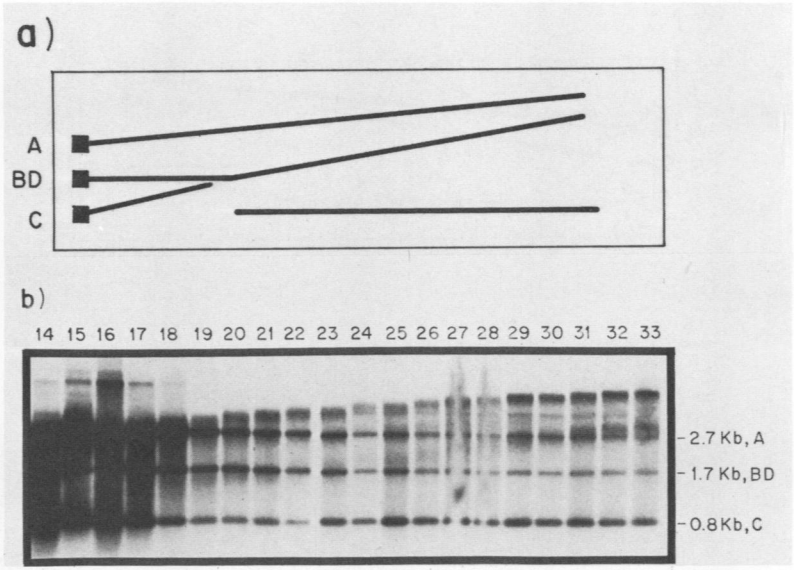

FIG. 4. Replication intermediate-enriched SV40 DNA was cut with *Bgl*I and *Bam*HI, and the products were electrophoresed on a 0.6% agarose electroelution gel. Fractions containing the SV40 fragments were restricted with *Hae*II and applied sequentially to an agarose slab gel, transferred, and hybridized. a) Predicted pattern of nonreplicating and replicating DNA subfragments. b) Autoradiogram; exposure was 20 h.

unit size. Although the diagonals from subfragments A and BD can be discerned in Fig. 4b, the region of the autoradiogram in which the diagonal from subfragment C should be displayed is obscured by the vast excess of unit-size, nonreplicating subfragments. From this it was concluded that additional enrichment for replicating molecules was essential.

Several methods of enrichment were tried (data not shown). (i) The DNA was cross-linked with psoralen and UV light in vivo (15, 24) to prevent putative branch migration (49); (ii) the replication intermediates were gel purified before the two-dimensional analysis; and (iii) the replication intermediate-enriched DNA from the caffeine eluate of the BND-cellulose column was applied to a second BND-cellulose column. None of these procedures was successful (Spotila, Ph.D. thesis). The method which did prove successful was the selective transfer of replication intermediates to nitrocellulose filters.

Selective transfer. Binding of DNA to nitrocellulose requires that the DNA have some single-stranded character (31). Thus the usual procedure (36) when transferring DNA from an agarose gel to nitrocellulose is to denature the DNA by soaking the gel in 0.5 N NaOH–1.5 M NaCl (denaturing buffer), followed by soaking in 3.0 M NaCl–1.0 M Tris (pH 5) (neutralizing buffer). It occurred to us that the replication intermediates already might have sufficient single-stranded character to allow transfer to nitrocellulose without prior denaturation. If so, then additional enrichment for replication intermediates could be achieved by selective transfer. Therefore, the gel was soaked only in a neutral-pH, high-salt buffer, blotted to nitrocellulose, and baked in an 80°C vacuum oven for 2 h. Experiments with ^{14}C- and ^{3}H-labeled DNA had indicated the following: (i) nondenatured DNA was efficiently transferred out of the gel, but only ca. 10% bound to the nitrocellulose filter (the remainder probably passed through the filter); (ii) although only ca. 10% of the ^{14}C-labeled material bound to the filter, most of the replicating material did so. However, the DNA that bound to the nitrocellulose under selective conditions did not hybridize efficiently with the labeled probe. Therefore, the DNA bound to the filter had to be denatured after baking to permit its subsequent detection. This was accomplished by incubating the baked blot at 65°C in 0.01× SSC–0.1× Denhardt solution for 2 h. Compared to a conventional transfer, there was an overall 3- to 5-fold loss of signal from replicating DNA (diagonals), but the ratio of diagonal to unit-size subfragments was increased 10- to 20-fold.

By using the selective transfer procedure, it was possible to detect small fragments containing replication forks which were obscured by excess nonreplicating DNA in a normal transfer (Fig. 5). The same sequence of restriction enzymes was used as shown in Fig. 4. The top autoradiogram is a 19-h exposure to film in which the A and BD replication interme-

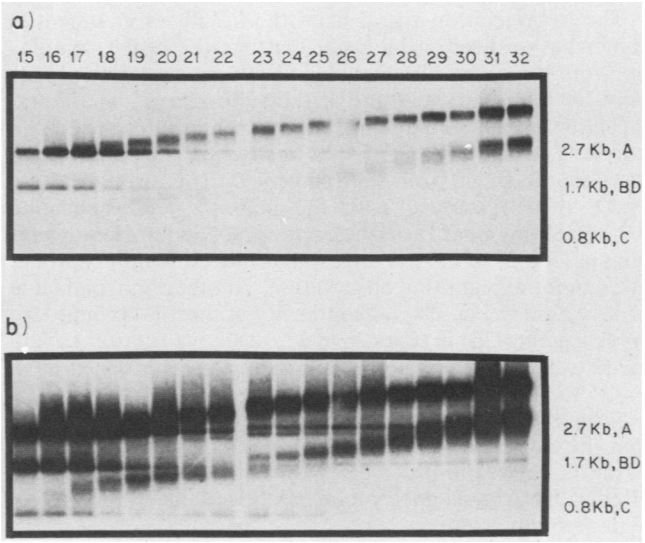

FIG. 5. Selective transfer of replicating DNA to nitrocellulose. The DNA in this experiment was restricted with the same enzymes used in Fig. 4. The second-dimension agarose gel was transferred to nitrocellulose by the selective transfer procedure (see the text). a) Exposure was 19 h. b) Exposure was 5 days.

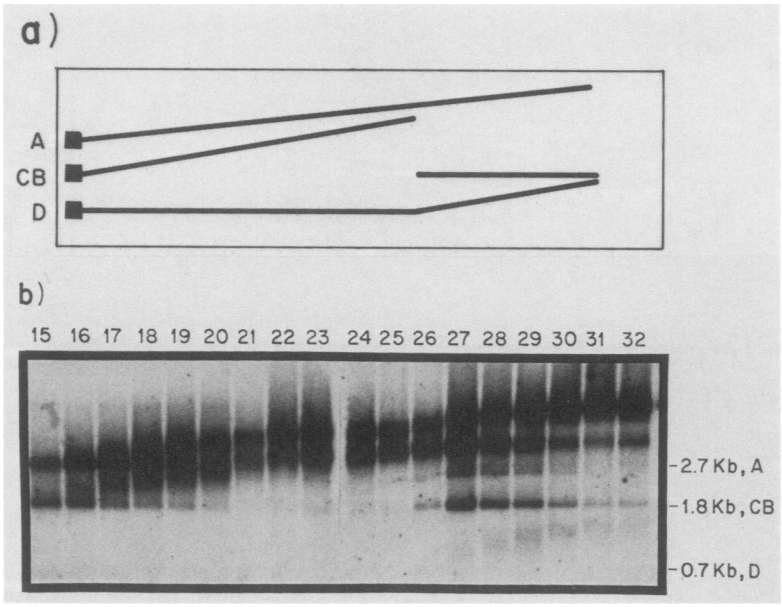

FIG. 6. Replication intermediate-enriched SV40 DNA was cut with *Bgl*I and *Bam*HI as in Fig. 4 and 5. After the first-dimension 0.8% agarose electroelution gel, each fraction was restricted with *Eco*RI, applied to the second-dimension gel, transferred by the selective transfer procedure, and hybridized. a) Diagrammatic representation of the predicted result. b) Autoradiogram; exposure was 5 days.

diates can be seen. The lower panel of Fig. 5 is a 5-day exposure of the same filter in which the diagonal from fragment C is obvious. Figure 4a depicts subfragment C returning to unit size when subfragment BD increases. This is not observed in Fig. 5, presumably because unit-size C fails to bind to nitrocellulose during selective transfer.

To further demonstrate the procedure, one more set of restriction enzymes was used (Fig. 6). As in Fig. 5, the replication intermediates were restricted with *Bgl*I and *Bam*HI before the first dimension. Fractions from this gel then were restricted with *Eco*RI. Under these circumstances, the 1.8-kb subfragment BC was predicted (Fig. 6a) to give rise to replication fork-containing fragments before the 0.7-kb subfragment D. Figure 6b shows that this is indeed the case.

The hybridization signal from the 0.7-kb subfragment is somewhat reduced in intensity because the probe contains only the large *Pst*I fragment of SV40 and therefore shares only 100 base pairs with the 0.7-kb subfragment. Therefore, the filter used to obtain Fig. 6 was rehybridized with the purified, nick-translated SV40 restriction fragment D to increase the signal from subfragment D. The result (Spotila, Ph.D. thesis) showed both the unit-size and replicating 0.7-kb subfragment D very clearly. The band present beginning in lane 27 of ca. 2.6 kb also hybridized with this probe. We cannot explain this observation. Another unexplainable observation in Fig. 6b is the intensity of the 1.8-kb, unit-size subfragment (CB) in lanes 26 to 32. Selective transfer should mediate against transfer of this fragment, as was observed in Fig. 5 for the analogous subfragment C.

From the experiments presented in Fig. 3 to 6 we conclude that the rationale on which the origin location procedure is based is valid; the procedure is feasible, at least for small circular DNA molecules which can be obtained in relatively high amounts.

DISCUSSION

In this paper we have described a method for determining the direction(s) of replication fork movement through any cloned segment of DNA. From the direction(s) of fork movement, the location(s) of the replication origin(s) can be inferred. We have demonstrated that the approach is technically feasible with replicating SV40 DNA.

The advantages of the approach described here are several. (i) The cells need not be synchronized in S phase. Selection for replicating molecules is accomplished biochemically by BND-cellulose chromatography and selective transfer to nitrocellulose. Ordering of DNA molecules at various stages of replication is achieved physically by agarose gel electrophoresis. However, although synchronization to S phase is not a necessary part of the procedure, its use would maximize the yield of replicating DNA.

(ii) This approach can be applied to any segment of DNA for which a cloned probe with known restriction sites is available. It is not dependent on physically separating the segment of interest from the remainder of the DNA because specificity is accomplished by the probe. Previous origin location methods have relied on selective labeling of a repeated DNA sequence in vivo (3, 18) or analysis of replicating molecules identifiable by electron microscopy such as plasmids (30), extrachromosomal DNA (6, 42, 43), and purified ribosomal DNA repeats (2, 3, 28). If enough replicating DNA can be obtained, the method described in this paper will allow location of origins in stretches of unique chromosomal DNA.

(iii) The method will allow the determination of origins directly. That is, it will provide information on the location and specificity of chromosomal origins in vivo. It does not rely on model systems (16, 21, 29) or on the assumption that a sequence which promotes plasmid replication (an *ars* sequence) corresponds to a chromosomal replication origin (1, 7, 8, 37–39, 45, 47). Two questions regarding *ars* function can be investigated with this procedure. Does an *ars* sequence in a plasmid actually serve as the origin of replication of the plasmid? Does a particular *ars* function as a replication origin in the chromosome?

There are, however, some potential problems to this approach. (i) Resolution is limited by the frequency of the usable restriction sites in the DNA of interest and by

reduced signal intensity from smaller subfragments. However, once an origin is located to a particular restriction fragment by this procedure, it may be possible to locate it more exactly by determination of the site of transition from discontinuous to continuous DNA synthesis for each direction of synthesis within the origin region (17).

(ii) Branch migration may result in some loss of replication intermediates and in some loss of resolution. We attempted to prevent putative branch migration by photochemical cross-linking but found that the cross-linked DNA was difficult to recover, gave rise to abnormal structures, and did not hybridize efficiently (Spotila, Ph.D. thesis). Fortunately, if branch migration did occur in our studies with uncross-linked DNA, the level was low enough that it did not prevent our obtaining clear results. The likelihood of branch migration can be reduced by minimizing the exposure of DNA to low-salt buffers and to elevated temperatures.

(iii) Appoximately 5 to 50 ng of replicating DNA of the sequence of interest is needed. This estimate is based on the sensitivity of the selective transfer and hybridization procedures in our hands (10 pg per band in an overnight exposure), the number of bands on the second-dimension autoradiogram (ca. 50 bands), and overall recovery of replication intermediates (1 to 10%). Although it is relatively simple to obtain adequate quantities of replicating SV40 DNA, it may be more difficult to obtain sufficient replicating DNA in other systems.

Because the yeast *Saccharomyces cerevisiae* has an unusually small genome and is easily synchronized, it is a choice organism for further application of this technique. We are currently locating the in vivo origin(s) of 2μ plasmid, a multicopy plasmid found in many strains of yeasts, and in the near future we shall apply the origin location procedure to single-copy segments of yeast chromosomal DNA.

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LITERATURE CITED

1. **Beach, D., M. Piper, and S. Shall.** 1980. Isolation of chromosomal origins of replication in yeast. Nature (London) **248**:185–187.
2. **Botchan, P. M., and A. I. Dayton.** 1982. A specific replication origin in the chromosomal rDNA of *Lytechinus variegatus*. Nature (London) **299**:453–456.
3. **Bozzoni, I., C. T. Baldari, F. Amaldi, and M. Buongiorno-Nardelli.** 1981. Replication of ribosomal DNA in *Xenopus laevis*. Eur. J. Biochem. **118**:585–590.
4. **Broach, J. R., and J. B. Hicks.** 1980. Replication and recombination functions associated with the yeast 2μ circle. Cell **21**:501–508.
5. **Buckler-White, A. J., and V. Pigiet.** 1982. The isolation and characterization of replication forks from discrete regions of the polyoma genome. J. Virol. **44**:499–508.
6. **Cech, T. R., and S. L. Brehm.** 1981. Replication of the extrachromosomal ribosomal RNA genes of *Tetrahymena thermophilia*. Nucleic Acids Res. **9**:3531–3543.
7. **Chan, C. S., and B.-K. Tye.** 1980. Autonomously replicating sequences in *S. cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **77**:6329–6333.
8. **Chan, C. S., and B.-K. Tye.** 1983. Organization of DNA sequences and replication origins of yeast telomeres. Cell **33**:563–573.
9. **Danna, K. J., and D. Nathans.** 1972. Bidirectional replication of SV40 DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:3097–3100.
10. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
11. **Edenberg, H. J., and J. A. Huberman.** 1975. Eukaryotic chromosome replication. Annu. Rev. Genet. **9**:245–284.
12. **Fangman, W. L., R. H. Hice, and E. Chlebowicz-Sledziewska.** 1983. ARS replication during the yeast S phase. Cell **32**:831–838.
13. **Gorman, J. A., W. F. Dove, and N. Warren.** 1981. Isolation of *Physarum* DNA segments that support autonomous replication in yeast. Mol. Gen. Genet. **183**:306–313.
14. **Hand, R.** 1978. Eucaryotic DNA organization of the genome for replication. Cell **15**:317–325.
15. **Hanson, C. V., C.-K. J. Shen, and J. E. Hearst.** 1976. Cross-linking of DNA *in situ* as a probe for chromatin structure. Science **193**:62–64.
16. **Harland, R. M., and R. A. Laskey.** 1980. Regulated replication of DNA microinjected into eggs of *Xenopus laevis*. Cell **21**:761–771.
17. **Hay, R. T., and M. L. DePamphilis.** 1982. Initiation of SV40 replication *in vivo*: location and structure of 5′ ends of DNA synthesized in the ori region. Cell **28**:767–779.
18. **Heintz, N. H., and J. L. Hamlin.** 1982. An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. U.S.A. **79**:4083–4087.
19. **Heintz, N. H., J. D. Milbrandt, K. S. Greisen, and J. L. Hamlin.** 1983. Cloning of the initiation region of a mammalian chromosomal replicon. Nature (London) **302**:439–441.
20. **Henson, P.** 1978. The presence of single-stranded regions in mammalian DNA. J. Mol. Biol. **119**:487–506.
21. **Hines, P. J., and R. M. Benbow.** 1982. Initiation of replication at specific origins in DNA molecules microinjected into unfertilized eggs of the frog *Xenopus laevis*. Cell **30**:459–468.
22. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
23. **Huberman, J. A., and A. D. Riggs.** 1968. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. **32**:327–341.
24. **Isaacs, S. T., C.-K. J. Shen, J. E. Hearst, and H. Rapoport.** 1977. Synthesis and characterization of new psoralen derivatives with superior photoreactivity with DNA and RNA. Biochemistry **16**:1058–1064.
25. **Kiss, G. B., A. A. Amin, and R. E. Pearlman.** 1981. Two separate regions of the extrachromosomal ribosomal DNA of *Tetrahymena thermophila* enable autonomous replication of plasmids in *S. cerevisiae*. Mol. Cell. Biol. **1**:535–543.
26. **Laskey, R.** 1980. The use of intensifying screens or organic scintillators for visualizing radioactive molecules resolved by gel electrophoresis. Methods Enzymol. **65**:363–371.
27. **Levine, A. J., H. S. Kang, and F. E. Billheimer.** 1970. DNA replication in SV40 infected cells. I. Analysis of replicating SV40 DNA. J. Mol. Biol. **50**:549–568.
28. **McKnight, S. L., M. Bustin, and O. L. Miller, Jr.** 1977. Electron microscopic analysis of chromosome metabolism in the *Drosophila melanogaster* embryo. Cold Spring Harbor Symp. Quant. Biol. **42**:741–754.
29. **McTiernan, C. F., and P. J. Stambrook.** 1984. Initiation of SV40 DNA replication after microinjection into *Xenopus* eggs. Biochem. Biophys. Acta **782**:295–303.
30. **Newlon, C. S., R. J. Devenish, P. A. Suci, and C. J. Roffis.** 1981. Replication origins used *in vivo* in yeast. ICN-UCLA Symp. Mol. Cell. Biol. **21**:501–516.
31. **Nygaard, A. P., and B. D. Hall.** 1963. A method for the detection of RNA-DNA duplexes. Biochem. Biophys. Res. Commun. **12**:98–104.
32. **Parker, R. C., and B. Seed.** 1980. Two-dimensional agarose gel electrophoresis: "SeaPlaque" agarose dimension. Methods Enzymol. **65**:358–363.
33. **Potter, S. S., and C. A. Thomas.** 1977. The two-dimensional fractionation of *Drosophila* DNA. Cold Spring Harbor Symp.

Quant. Biol. **42**:1023–1031.
34. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
35. **Rosenvold, E. C., and A. Honigman.** 1977. Mapping of AvaI and XmaI cleavage sites in bacteriophage DNA including a new technique of DNA digestion in agarose gels. Gene **2**:273–288.
36. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
37. **Stinchcomb, D. T., K. Struhl, and R. W. Davis.** 1979. Isolation and characterization of a yeast chromosomal replicator. Nature (London) **282**:39–43.
38. **Stinchcomb, D. T., M. Thomas, J. Kelly, E. Selker, and R. W. Davis.** 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. U.S.A. **77**:4559–4563.
39. **Struhl, K., D. T. Stinchcomb, S. Sherer, and R. W. Davis.** 1979. High frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. U.S.A. **76**:1035–1039.
40. **Tapper, D. P., and M. L. DePamphilis.** 1978. Discontinuous DNA replication: accumulation of simian virus 40 DNA at specific stages in its replication. J. Mol. Biol. **120**:401–422.
41. **Tapper, D. P., and M. L. DePamphilis.** 1980. Preferred DNA sites are involved in the arrest and initiation of DNA synthesis during replication of SV40 DNA. Cell **22**:97–108.
42. **Truett, M. A., and J. G. Gall.** 1977. The replication of ribosomal DNA in the macronucleus of *Tetrahymena*. Chromosoma **64**:295–303.
43. **Vogt, V. M., and R. Braun.** 1977. The replication of ribosomal DNA in *Physarum polycephalum*. Eur. J. Biochem. **80**:557–566.
44. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzloxymethyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
45. **Watanabe, S., and J. H. Taylor.** 1980. Cloning of an origin of DNA replication of *Xenopus laevis*. Proc. Natl. Acad. Sci. U.S.A. **77**:5292–5296.
46. **Willard, H. F., and S. A. Latt.** 1976. Analysis of DNA replication in human X chromosomes by fluorescence microscopy. Am. J. Hum. Genet. **28**:213–227.
47. **Zakian, V. A.** 1981. Origin of replication from *Xenopus laevis* mitochondrial DNA promotes high-frequency transformation of yeast. Proc. Natl. Acad. Sci. U.S.A. **78**:3128–3132.
48. **Zannis-Hadjopoulos, M., A. B. Chepelinsky, and R. G. Martin.** 1983. Mapping of the 3'-end positions of SV40 nascent strands. J. Mol. Biol. **165**:599–607.
49. **Zannis-Hadjopoulos, M., M. Persico, and R. G. Martin.** 1981. The remarkable instability of replication loops provides a general method for the isolation of origins of DNA replication. Cell **27**:155–163.