MOLECULAR AND CELLULAR BIOLOGY, Aug. 1991, p. 3850–3859
0270-7306/91/083850-10$02.00/0

Vol. 11, No. 8

Mapping of Replication Initiation Sites in Mammalian Genomes by Two-Dimensional Gel Analysis: Stabilization and Enrichment of Replication Intermediates by Isolation on the Nuclear Matrix

P. A. DIJKWEL, J. P. VAUGHN, AND J. L. HAMLIN*

Department of Biochemistry and Cell and Molecular Biology Program, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received 7 January 1991/Accepted 29 April 1991

Two complementary two-dimensional gel electrophoretic techniques have recently been developed that allow initiation sites to be mapped with relative precision in eukaryotic genomes at least as complex as those of yeast and *Drosophila melanogaster*. We reported the first application of these mapping methods to a mammalian genome in a study on the amplified dihydrofolate reductase (DHFR) domain of the methotrexate-resistant CHO cell line CHOC 400 (J. P. Vaughn, P. A. Dijkwel, and J. L. Hamlin, Cell 61:1075–1087, 1990). Our results suggested that in this 240-kb domain, initiation of nascent DNA strands occurs at many sites within a 30- to 35-kb zone mapping immediately downstream from the DHFR gene. In the course of these studies, it was necessary to develop methods to stabilize replication intermediates against branch migration and shear. This report describes these stabilization methods in detail and presents a new enrichment protocol that extends the neutral/neutral two-dimensional gel mapping method to single-copy loci in mammalian cells. Preliminary analysis of replication intermediates purified from CHO cells by this method suggests that DNA synthesis may initiate at many sites within a broad zone in the single-copy DHFR locus as well.

In the last 25 years, a wealth of information has been obtained on the mechanisms that regulate DNA replication in microorganisms. Studies on the simple eukaryotic organism *Saccharomyces cerevisiae* have also made enormous progress recently owing to the availability of a phenotypic assay for autonomously replicating sequence (ARS) elements from the yeast genome (26), some of which have been shown to represent bona fide chromosomal origins (16, 20).

However, virtually nothing is known about the regulatory switches for initiation of replication in mammalian chromosomes. The difficulty in studying origins in mammalian cells is due primarily to their very large numbers in the genome (10,000 to 50,000; 15), each one of which could be unique, and to the lack of a reproducible phenotypic assay for the identification and isolation of mammalian ARS elements. Nevertheless, a variety of protocols for following replication fork movement have suggested that origins of replication in mammalian chromosomes may be defined sequence elements (1, 5, 6, 12, 17, 18). To strengthen this proposal, however, higher-resolution approaches are required that can localize replication start sites with great precision so that neighboring *cis*-regulatory control elements can be identified.

Two complementary two-dimensional gel replicon mapping techniques have been developed recently that appear to allow the unambiguous identification of initiation sites in relatively complex genomes (3, 23). Both methods rely on the unique migration patterns of replication intermediates in agarose gels, and both utilize defined molecular probes to examine these intermediates in a genomic region of interest. Both methods also have the advantage that they can be applied to cells in exponential growth, in most cases obviating the need for synchronizing regimens that could introduce artifacts. The two protocols have been used to study replication of the ribosomal DNA locus in yeast cells (4, 20) and have also been employed to show that some (but not all) of the previously identified yeast ARS elements represent legitimate chromosomal initiation sites (27). One of the two-dimensional gel methods has also been used to analyze the amplifying chorion locus in the more complex *Drosophila* genome (13).

The application of these powerful replicon mapping techniques to an analysis of mammalian DNA is limited by the complexity of the mammalian genome and by the small number of initiations occurring at a given origin of replication at any moment in a cell population, even in a synchronized culture. Thus, even when 50 to 100 μg of mammalian DNA is loaded into the well of an agarose gel, the small percentage of any given restriction fragment that actually contains replication intermediates is below the limits of detection by standard Southern blotting and hybridization procedures. Furthermore, both two-dimensional replicon mapping methods are greatly dependent on the ability to avoid destabilization and shear forces during the isolation of DNA.

In our laboratory, we have concentrated on the replication pattern of a defined locus by taking advantage of a methotrexate-resistant CHO cell line (CHOC 400) that has amplified the early-replicating dihydrofolate reductase (DHFR) domain ~1,000 times (21). In an in vivo labeling study on synchronized cells, we showed that replication initiates in only a small subset of restriction fragments in this locus in the early S period (14). These fragments define a 28-kb initiation zone mapping downstream from the DHFR gene (14). In a higher-resolution study in which an in-gel renaturation technique was used to eliminate background labeling from single-copy sequences, there actually appeared to be two rough zones of labeling whose centers lie ~22 kb apart within this 28-kb region (18). Independent evidence for two separate initiation zones was obtained in our laboratory by an approach in which nascent chains centered around origins

* Corresponding author.

were used to probe cloned sequences from the DHFR amplicon (1). Handeli et al. reached the same conclusion in a study that determined the rough locations at which the leading strands of replication switch from one template to the other (11).

In an effort to obtain a higher-resolution picture of the replication pattern of the amplified DHFR domain, we recently applied both two-dimensional gel mapping techniques to an analysis of this locus. By developing methods for stabilizing forked structures against branch migration and shear, we were able to analyze replication intermediates in a defined mammalian chromosomal locus for the first time. Unexpectedly, our data indicated that replication initiates in vivo at many sites distributed throughout the previously defined 28-kb initiation zone in the DHFR locus (although it was not possible to determine whether the number of initiations is higher near the two peaks of early labeling previously identified in this region) (28).

Since this rather heterodoxic result could be unique to amplified DNA, we undertook a study of replication intermediates in the DHFR domain of the parental drug-sensitive CHO cell line. The detailed isolation procedures used in this and the previous study are reported here, as well as a preliminary characterization of replication events in the single copy DHFR domain.

MATERIALS AND METHODS

Cell culture and synchronization protocols. Methotrexate-sensitive CHO cells and the methotrexate-resistant Chinese hamster cell line CHOC 400 (21) were maintained as monolayer cultures in 15-cm dishes in minimal essential medium (MEM) supplemented with nonessential amino acids and 15% fetal calf serum as previously described (14). Cultures were arrested in early G_1 by isoleucine starvation for 45 h and were then released into complete medium containing 10 μg of aphidicolin per ml (14). Twelve hours later, when the cells had collected at the G_1/S boundary, aphidicolin was removed by two successive washes with warm serum-free MEM, and the cultures were incubated in MEM containing serum for 20 to 30 min. The cells were then either harvested immediately or after cross-linking (see below). All tissue culture media and sera were obtained from GIBCO, and aphidicolin was purchased from Sigma Chemical Co.

DNA purification by extraction with organic solvents in low salt (method A). Each 15-cm plate of CHOC 400 cells (three plates of synchronized or six plates of log-phase cells; 2×10^7 to 3×10^7 cells per plate) was washed once with 40 ml of ice-cold PSG (136 mM NaCl, 5.3 mM KCl, 0.6 mM KH_2PO_4, 1 mM Na_2HPO_4; additionally containing 20 mM sodium azide for cross-linked samples), the PSG was drained completely, and 7 ml of lysis buffer was added (100 mM Tris-HCl [pH 8.0], 300 mM NaCl, 25 mM EDTA, 1% sodium lauroyl sarkosine, 2 mg of proteinase K [AMRESCO] per ml). After 3 to 4 h of incubation at room temperature, the viscous lysate was transferred to a 50-ml conical polypropylene tube and extracted gently with an equal volume of phenol saturated with 1 M Tris-HCl, pH 8.0. The phases were separated for 5 min in an IEC bench-top centrifuge at setting 7 at 21°C. The aqueous phase was extracted once more with phenol and twice with chloroform-isoamyl alcohol (25:1), and 2 volumes of ice-cold 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA was added. The DNA was flocculated by the addition of 2 volumes of cold absolute ethanol and gentle inversion of the tube, and the white clump of DNA was removed with a glass hook to a separate tube containing 4 ml of cold TEN buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 10 mM NaCl). After 5 min on ice, the solution of DNA received 0.1 volume of the appropriate 10× restriction buffer, spermidine to 4 mM and, after 10 min of incubation on ice, 1,000 U of the desired restriction enzyme (Bethesda Research Laboratories). After 45 min at 37°C, an additional 1,000 U of restriction enzyme was added; 2.5 h later, pancreatic RNase A (Sigma) was added to 100 μg/ml, and incubation was continued for an additional 30 min. Two volumes of cold ethanol were added to the digest, which was then completely mixed and centrifuged in a Sorvall HB-4 rotor at 0°C and 10,000 rpm for 90 min. The pellet was dissolved in TEN buffer for 5 min at room temperature, NaCl was added to 250 mM, and the mixture was applied to a BND-cellulose column (see below).

DNA purification by extraction with organic solvents at high ionic strength (method B). Cells from one plate were lysed and incubated overnight in 0.5% sodium lauroyl sarkosine–0.5 M EDTA (pH 8.0)–100 μg of proteinase K per ml at room temperature, and organic extractions were carried out without inversion by laying the glass tubes on their sides for 12 h. The DNA was dialyzed at 4°C for 24 h versus two 4-liter changes of 50 mM NaCl–50 mM Tris-HCl (pH 8.0)–0.1 mM EDTA. The sample was made to 10 mM $MgCl_2$, and 4 mM spermidine and 1,000 U of *Xba*I was added. Approximately 2.5 h later, pancreatic RNase was added to 10 μg/ml, and digestion was allowed to continue for 30 min. Digestion was terminated by the addition of EDTA to 10 mM. The DNA was precipitated with 2 volumes of ethanol and was purified as in method A. Alternatively, the DNA was dialyzed against 200 mM NaCl–50 mM Tris-HCl (pH 8.0)–1 mM EDTA and was then precipitated as in method A.

Stabilization of DNA by cross-linking (method C). Each 15-cm plate of CHOC 400 cells (three plates of synchronized or six plates of exponentially growing cells) was washed twice with PSG-azide and drained well. Subsequent operations were performed at 4°C in the dark. To each plate was added 10 ml of PSG-azide and 70 μl of trioxsalen (500 μg/ml of absolute ethanol; Sigma). After the plates were swirled, the cells were allowed to take up the trioxsalen for 2 min in the dark, after which they were irradiated from above with 365-nm light (two model XX15L 15-W lamps; U.V. Products) at a distance of 10 cm for 2 min. The irradiation procedure was repeated a total of five times, with addition of fresh trioxsalen (70 μl) each time. The plates were then washed with 10 ml of PSG, and DNA was isolated as in method A.

Stabilization of replication intermediates by isolation of nuclear matrices (method D). For experiments with exponentially growing and synchronized CHOC 400 cultures, 1.5×10^8 and 0.75×10^8 cells were used (eight and four 15-cm plates, respectively; 2×10^7 cells per plate). For CHO cells, the corresponding numbers were 1×10^9 and 5×10^8 (40 and 20 15-cm plates, respectively; 3×10^7 cells per plate). The preparation of matrices was performed entirely in the cold with the exception of lithium diiodosalicylate (LIS) extraction, which was carried out at room temperature. Plates were rinsed twice with cell wash buffer (CWB; 5 mM Tris-HCl [pH 7.4], 50 mM KCl, 0.5 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, 0.5% thiodiglycol, 0.25 mM phenylmethylsulfonyl fluoride [PMSF]) and were drained well. Then 5 ml of CWB containing 0.1% digitonin (water-soluble form; Sigma) was added to the first of each set of two plates. Cells were immediately scraped off with a rubber policeman and transferred to the second plate. After scraping, the suspension was forced three times through a 21-

gauge hypodermic needle to effect cell lysis and to free nuclei of cytoplasmic tabs. The two plates were rinsed sequentially with an additional 5 ml of CWB-digitonin, which was added to the suspension by forcing it through the needle.

The total nuclear suspension (10 ml) was layered over 4 ml of 12.5% glycerol in CWB-digitonin in a 15-ml conical plastic tube. Nuclei were pelletted in an IEC bench-top centrifuge at setting 4 for 10 min and were then resuspended in 5 ml of CWB-digitonin by one passage through the needle (an aliquot was inspected at this stage by phase-contrast microscopy to ascertain that the nuclei had been released and were largely free of cytoplasmic tabs). Two or three 5-ml suspensions were pooled, and nuclei were again collected by centrifugation. The nuclei were suspended in 2 ml of CWB-digitonin by passage through the needle and ejection into 8 ml of stabilization buffer (5 mM Tris-HCl [pH 7.4], 50 mM KCl, 0.625 mM $CuSO_4$, 0.05 mM spermine, 0.125 mM spermidine, 0.5% thiodiglycol, 0.25 mM PMSF, 0.1% digitonin) and were incubated on ice for 20 min.

The stabilized nuclei were then ejected into 110 ml of LIS buffer (10 mM LIS, 100 mM lithium acetate, 0.05 mM spermine, 0.125 mM spermidine, 0.25 mM PMSF, 0.05% digitonin, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-KOH, pH 7.4). Note that if this procedure is to be scaled up or down, the concentration of nuclei in LIS buffer should not exceed $\sim 1.5 \times 10^6$/ml. Extraction of histones and soluble nuclear proteins by LIS was allowed to proceed for 5 min at room temperature. The slightly turbid suspension was then divided among four 30-ml Corex tubes, and matrices were collected by centrifugation at 4,000 rpm for 15 min at 0°C in a Sorvall HB-4 rotor. The supernatants were discarded, and the pellets, which appear as gelatinous sheets adhering to the bottom of the tubes, were dislodged by forcible delivery of 3 ml of the appropriate 1× restriction buffer supplemented with 0.1% digitonin.

At this point, two pellets were combined ($\sim 0.5 \times 10^8$ to 1.0×10^8 nuclei), the volume was adjusted to 30 ml with restriction buffer containing 0.1% digitonin, and the matrices were centrifuged for 5 min at 4,000 rpm in the HB-4 rotor at 4°C. This washing procedure was repeated twice with restriction buffer only. Both pellets were then combined (i.e., all of the matrices from one 120-ml LIS extract) and were resuspended in 2 ml of the appropriate restriction buffer. When examined by fluorescence microscopy in the presence of 4 μg of ethidium bromide per ml, a brightly fluorescing matrix core surrounded by a fluorescent halo was observed. Restriction enzyme (2,000 U) was added, and the tube was placed at 37°C and swirled occasionally. After 5 min, the suspension was triturated through a wide-bore (2-mm) Gilson-1000 pipette tip until the large clumps were dispersed. After 15 min, the reaction was terminated by addition of EDTA to 20 mM. Then 13 ml of proteinase K buffer (PK buffer; 60 mM Tris-HCl [pH 7.9], 460 mM NaCl, 40 mM EDTA, 1% sarkosine) and 5 ml of a 10-mg/ml solution of proteinase K (in 10 mM Tris-HCl [pH 8], 0.1 mM EDTA) were added, and incubation was continued for 3 h at room temperature.

CsCl was then added to a refractive index of 1.4, and the sample was divided between two 25-ml tubes and centrifuged for 48 h at 30,000 rpm in a Beckman 50.2 Ti rotor at 20°C. Gradients were fractionated, and the tubes containing the majority of the DNA were pooled and dialyzed overnight at 4°C against 10 mM Tris-HCl (pH 7.9)–300 mM NaCl–1 mM EDTA–0.05 mM PMSF. After ethanol precipitation, the DNA was pelleted and dissolved in TEN (3 to 5 ml at 0°C for 7 min), 0.1 volume of 10× restriction buffer was added, and the DNA was digested to completion with 2,500 U of restriction enzyme for 45 min at 37°C.

Stabilization and purification of replication intermediates on the nuclear matrix (method E). Nuclei were isolated and extracted with LIS exactly as described above. The matrices from one 120-ml LIS extraction were brought up in 7.5 ml of the appropriate 1× restriction buffer. Restriction enzyme (1,000 U) was added, and the tube was placed at 37°C for 30 min. The matrices were pelleted (10 min at 4,000 rpm in the HB-4 rotor) and resuspended in 5 ml of fresh restriction buffer by trituration through the cut tip of a Gilson-1000 pipette. Another 1,000 U of restriction enzyme was added (meanwhile, the supernatant was transferred to a second tube and also placed at 37°C). After 15 min, RNase A (Sigma) was added to 100 μg/ml from a 10-mg/ml stock solution made up in 10 mM Tris–0.1 mM EDTA, pH 8.0. At 30 min, EDTA was added to 20 mM and the volume was adjusted to 15 ml with 1× restriction buffer.

Matrices were collected by centrifugation (10 min at 5,000 rpm in the HB-4 rotor at 0°C). The first and second supernatants were combined, and half of this sample was precipitated with 2 volumes of absolute ethanol to give the loop DNA fraction. The matrices and the loop DNA were each resuspended in 0.5 ml of dialysis buffer (10 mM Tris-HCl [pH 8.0], 300 mM NaCl, 1 mM EDTA) by trituration through the cut tip of a Gilson micropipette, after which 6 ml of PK buffer and 1.5 ml of proteinase K (10 mg of 10 mM Tris [pH 8.0]–0.1 mM EDTA per ml) were added. After 3 h at room temperature, the samples were dialyzed and ethanol precipitated as described above. The precipitates were dissolved in ice-cold TEN for 7 min, after which NaCl was added to 300 mM.

BND-cellulose chromatography. DNA samples prepared by methods A to E were applied to BND-cellulose columns (Sigma; 1.0-ml bed volume in a 0.8- by 4-cm Bio-Rad Polyprep column) equilibrated in 10 mM Tris (pH 8.0)–0.3 M NaCl–1 mM EDTA (19). Usually, no more than 100 μg of DNA digest was applied to each column to avoid overloading; however, for DNA prepared either by stabilization on the matrix or by organic extraction only (in which the percentage of replication intermediates is small), as much as 250 μg of DNA was sometimes applied to the column. Nonreplicating double-stranded DNA was eluted by gravity flow at room temperature with 3 ml 10 mM Tris-HCl (pH 7.9)–800 mM NaCl–1 mM EDTA (salt wash). The replication intermediates were then eluted with 3 ml of 1.8% caffeine in 10 mM Tris-HCl (pH 7.9)–1 mM EDTA–1 M NaCl (caffeine wash).

Both the salt and caffeine washes were centrifuged at room temperature for 10 min at setting 7 in an IEC bench-top centrifuge to remove any suspended BND-cellulose, after which the DNA was precipitated in 30-ml Corex tubes either with 2 volumes of absolute ethanol or, if the total volume was too large, with 1 volume of isopropanol. The precipitates were collected immediately by centrifugation (45 min at 10,000 rpm in the HB-4 rotor at 0°C) and were dissolved in 0.7 ml of TEN at 0°C. After 7 min, NaCl was added to 0.3 M and the samples were reprecipitated with 1.4 ml of ethanol. DNA was collected by centrifugation and redissolved in an appropriate volume of TEN, which was made 0.3 M in NaCl after 7 min at 0°C. Usually, the entire caffeine fraction was then loaded into a single well of a two-dimensional gel, and an equal amount of DNA from the salt wash was run as a control for the efficacy of the BND-cellulose fractionation.

More than 95% of replication intermediates routinely partition with the caffeine wash (29; data not shown).

Two-dimensional neutral/neutral gel electrophoretic analysis (3). Restriction digests were separated in the first dimension on a 0.4% agarose gel that was run for 40 to 60 h at 0.3 to 0.4 V/cm in TBE (89 mM Tris-HCl [pH 8.0], 89 mM boric acid, 2 mM EDTA) at room temperature (Bethesda Research Laboratories large horizontal gel apparatus). The gel was stained for 30 min in TBE containing 0.3 μg of ethidium bromide per ml, after which it was briefly illuminated with long-wavelength UV light to enable individual lanes to be excised with a scalpel. The excised lane, which was 1 by 10 cm and spanned DNA fragments 2 to 25 kb in length, was turned through a 90° angle and placed at the top of a 1% agarose gel made up in TBE containing 0.3 μg of ethidium bromide per ml. Separation in the second dimension was performed in the cold room for 12 h at 3 to 4 V/cm with buffer recirculation.

The gel was then soaked in 0.25 M HCl for 15 min and 0.5 M NaOH for 20 min. DNA was transferred to either GeneScreen or GeneScreen Plus (Dupont/New England Nuclear Corp.) by a modification of an alkaline blotting procedure (24). When GeneScreen Plus was used, 1.5 M NaCl was included in the NaOH solution. The GeneScreen transfers were then illuminated with UV light to cross-link DNA to the membrane (7), after which they were airdried. Prehybridizations and hybridizations were performed in the presence of 50% formamide and 10% dextran sulfate, as described in New England Nuclear bulletin NEF-976 for GeneScreen Plus, except that sonicated salmon sperm DNA was added to the probe before boiling and addition to the hybridization fluid (to a final concentration in the bag of 200 μg/ml). Specific hybridization probes were labeled with [^{32}P]dCTP by the random priming method (10). After 24 to 48 h of hybridization, the blots were washed sequentially with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min, with 2× SSC–1% sodium dodecyl sulfate (SDS) at 65°C for 20 min, with 0.5× SSC–1% SDS at 65°C for 30 min, and with 0.2× SSC at room temperature for 20 min (sometimes the intervening wash with 0.5× SSC–1% SDS was omitted). The blots were then exposed to X-ray film (Kodak X-Omat AR) at −70°C with the aid of an intensifying screen. If the transfers were to be reprobed, the previous probe was removed from GeneScreen by boiling twice for 10 min in 0.1× SSC–1% SDS or from GeneScreen Plus by washing for 30 min at 42°C, first with 0.4 M NaOH and then with 0.2× SSC–0.2 M Tris-HCl (pH 7.4)–0.1% SDS.

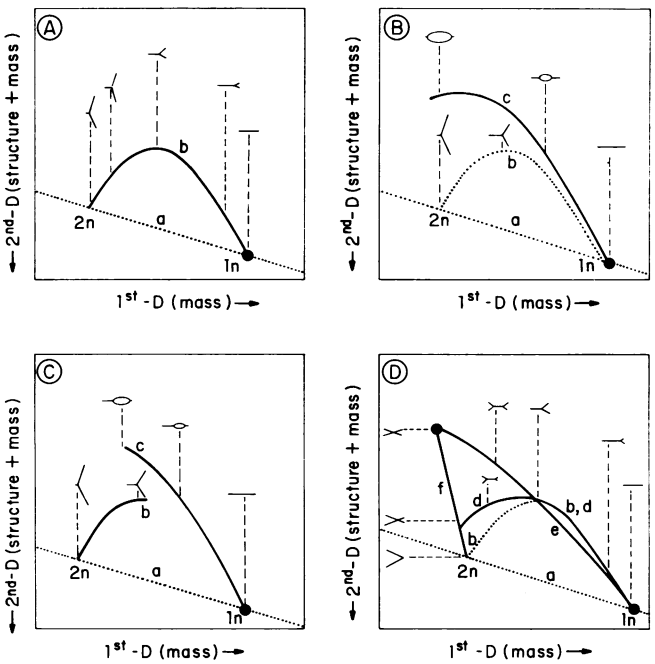

FIG. 1. Patterns of typical replication intermediates separated by two-dimensional neutral/neutral gel electrophoresis. Each panel shows an idealized autoradiographic image that would be obtained when a restriction digest of replicating DNA is hybridized with probes for fragments that contain different intermediates. (A) A complete simple Y or fork arc (b) resulting from a fragment that is replicated passively from an outside origin. Curve a represents the diagonal of nonreplicating fragments from the genome as a whole. (B) Pattern obtained when a fragment with a centered origin of replication is probed (curve c). Bubbles migrate more slowly at all extents of replication than do forks in a fragment of equal mass (b). (C) The presence of an off-centered origin in a fragment gives rise to an incomplete bubble arc (c), which then reverts to the fork arc when the bubble expands beyond the right-hand restriction site, resulting in a fork arc break. (D) When two forks approach each other in a fragment either symmetrically or asymmetrically, curve e or d, respectively, is obtained. If there is a fixed terminus in a fragment, the collected X-shaped structures would result in a concentrated spot somewhere on curve f. Recombination structures would also fall along curve f (3). The triangle formed between the simply Y arc and curves e and f contains a collection of double-forked structures differing in the extents of replication and the positions of the fork within the fragment.

RESULTS

Two-dimensional analysis of replication intermediates in DNA purified by organic extractions. In the neutral/neutral two-dimensional replicon mapping method (3), DNA is digested with a restriction enzyme and the fragments are separated on a neutral, low-percentage agarose gel according to molecular mass (Fig. 1). Nonreplicating fragments migrate as a single band, whereas replicating fragments migrate at a slower rate according to the extent to which the fragment has been replicated. In the second dimension, the fragments are separated on a higher-percentage gel under conditions in which shape contributes significantly to migration rate. Each class of replication intermediates traces a characteristic pattern, and fragments that contain initiation bubbles (Fig. 1B and C) can be distinguished from fragments replicated passively by forks entering from flanking regions (Fig. 1A and D).

In principle, this method has the potential to localize a fixed origin to within several hundred base pairs (3). In fact, we were readily able to map the initiation site in a cloned minimal polyomavirus origin with the neutral/neutral two-dimensional method after purifying the replicating DNA by a relatively standard method involving SDS-proteinase K treatment followed by organic extractions (method A, Materials and Methods) (28).

However, when genomic DNA was purified by the same method from synchronized CHOC 400 cells, the initial results of two-dimensional gel analysis were uninterpretable (Fig. 2; 28). When a transfer of an *Xba*I digest was probed specifically for a 4.3-kb *Xba*I fragment that is centered over the upstream zone of early labeling (Fig. 3E), we did not observe either of the patterns that indicate the presence of a

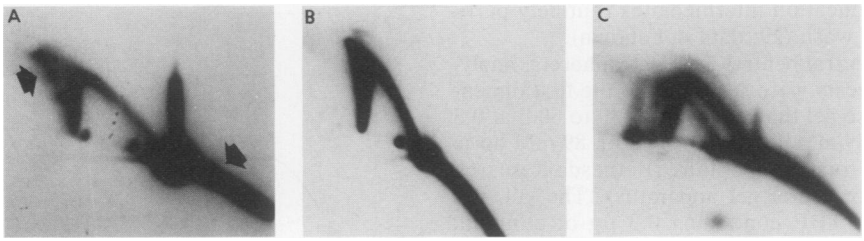

FIG. 2. Detection of bubble arcs under conditions that prevent branch migration. (A) Synchronized CHOC 400 cells were harvested 30 min after entry into S phase, and DNA was purified under low-ionic-strength conditions according to method A. After digestion with *Xba*I and fractionation on a BND-cellulose column, the DNA was separated on a two-dimensional gel and transferred to GeneScreen as described in Materials and Methods. (B) DNA was purified under conditions of high ionic strength during the organic extraction steps according to method B. (C) As in panel A except that DNA was cross-linked in vivo prior to entry into S phase and was purified 60 min after release from aphidicolin by method A. All of the DNA samples shown were analyzed by hybridization with a small probe from the center of the 4.3-kb *Xba*I fragment (see Fig. 3E).

fixed origin. Instead, an intense fork arc was observed that extended all the way from the 1*n* to the 2*n* positions in the mass dimension (Fig. 2A; 28). After very long film exposures, we did not observe even a faint bubble arc that would suggest infrequent initiations in this fragment in some of the copies of the amplicon. At face value, this result suggested that the 4.3-kb *Xba*I fragment is usually replicated passively by forks emanating from an initiation site or sites outside of the fragment. However, we were also not able to detect bubble arcs in any of several other overlapping and flanking fragments in the previously defined 28-kb initiation locus; instead, all fragments analyzed displayed only complete fork arcs (data not shown).

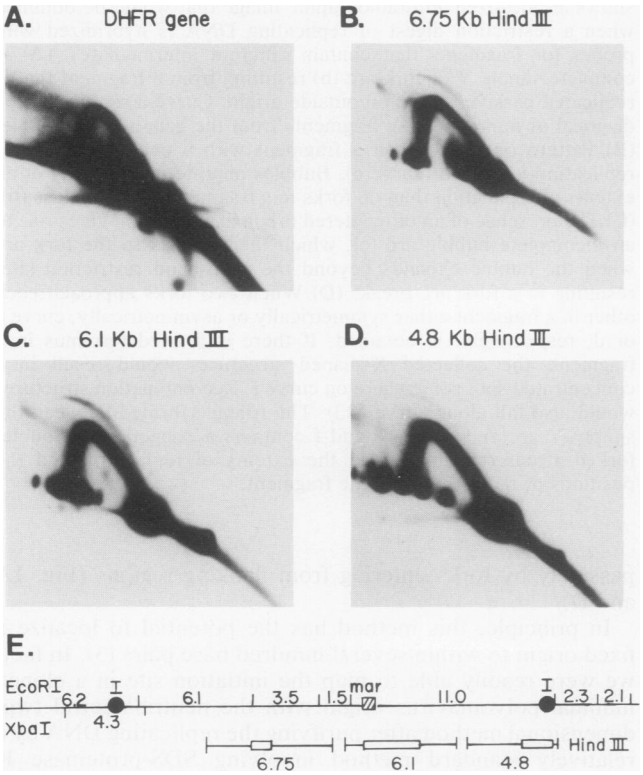

FIG. 3. Detection of replication initiation in several adjacent fragments in DNA prepared on the nuclear matrix from synchronized CHOC 400 cells. Replicating DNA was purified by method D, using *Hin*dIII to release loop DNA from the matrices. After separation on a two-dimensional gel and transfer to GeneScreen Plus, the membrane was hybridized sequentially with a probe from the body of the DHFR gene (A) and with three probes specific for the indicated fragments from the initiation locus (B through D). (E) Map of fragments used as probes.

Specific probing for the 4.3-kb *Xba*I fragment, as well as for several other neighboring fragments, displayed prominent signals at the position expected of either recombination intermediates or fragments containing converging replication forks (triangular smear indicated by the upper arrow in Fig. 2A). This signal did not appear to result from simple recombination in the amplified domain, since it was absent from fragments that were not actively replicating at the time of sampling (data not shown). Furthermore, the triangular smear was unlikely to have resulted from replication forks converging on a terminus in this fragment (Fig. 1D), since every fragment analyzed, whether from within or outside of the initiation locus, displayed a similar pattern (data not shown).

In addition to the triangular smear, there was a large but variable amount of hybridization on the diagonal below the 1*n* spot (Fig. 2A, lower arrow). Since the amount of both this material and the triangular smear relative to the fork arc was extremely variable from experiment to experiment, it seemed likely that both signals resulted from destabilization that occurred during DNA isolation, possibly by branch migration or shear. The triangular smear and the $<1n$ material were not unique to phenol-chloroform extraction, since both signals were also observed when the same DNA sample was purified by a guanidine chloride method that avoids organic extractions completely (27a). These two novel signals were also not observed in a previous analysis of the cloned polyomavirus origin (28), probably because the plasmid is relatively small (~7 kb) and is therefore not particularly subject to shear forces. Second, the plasmid probably remains supercoiled during isolation, which should suppress branch migration (31).

Reduction of branch migration and shear preserves the integrity of replication bubbles. Several different protocols were tested for their ability to stabilize replication intermediates in high-molecular-weight genomic DNA. In the first approach (method B), the ionic strength was increased to 0.5 M with EDTA in the standard purification scheme involving organic solvent extractions, and DNA was digested without a prior ethanol precipitation step. When the 4.3-kb *Xba*I fragment from the upstream initiation zone was analyzed in this DNA preparation, the triangular smear was, in fact, not

greatly reduced in intensity relative to the fork arc. However, a faint bubble arc was now visible (although the smaller bubbles in this particular preparation were lost; Fig. 2B).

To attempt to eliminate loss of replication bubbles completely, the DNA of CHOC 400 cells was cross-linked in vivo with trioxsalen and UV light at ~1-kb intervals 30 min after entry into the S period (method C). The DNA was then isolated by the standard low-ionic-strength phenol-chloroform extraction procedure (method A) and was digested with *Xba*I. When a transfer of this digest was probed for the 4.3-kb *Xba*I fragment (Fig. 2C; 28), the bubble arc, though faint, now seemed complete (i.e., extended all the way from the 1*n* to the 2*n* positions). Moreover, the triangular smear was greatly reduced in intensity relative to the fork arc when compared with the pattern observed in Fig. 2A. Again, however, the most prominent signal emanating from this fragment was the simple Y arc, suggesting that although initiation sometimes occurs in the 4.3-kb *Xba*I fragment, it is primarily replicated passively by forks from flanking regions.

We were concerned that cross-linking per se might induce artifactual bubblelike structures in genomic DNA. In addition, cross-linking often results in incomplete digestion by restriction enzymes (notice the shadow arcs in Fig. 2C). Alternative stabilization methods were therefore investigated. In one approach, synchronized CHOC 400 cells were harvested 30 min after entry into the S period by encapsulation in agarose beads according to the method of Cook (8). When a transfer of this DNA preparation was probed specifically for a 6.2-kb *Eco*RI fragment that straddles the upstream peak of labeling (Fig. 3E), the triangular smear virtually disappeared and the signal on the diagonal at the >1*n* position was reduced (27a). This result again suggested that most, if not all, of the material migrating at this position is an artifact of destabilization processes that occur during standard DNA purification protocols.

An independent purification scheme was developed in which nuclear matrices were isolated and the associated DNA was digested with restriction enzymes in loco (method D). This method and an important variation (method E) proved to be the most reliable for the retention of bubble structures and the elimination of artifactual signals resulting from branch migration or shear. Nuclei were isolated from synchronized CHOC 400 cells 30 min after entry into the S period, and histones and other chromosomal proteins were extracted with LIS detergent to yield matrix-halo structures (22, 29). The genomic DNA, which remains supercoiled by virtue of periodic attachment to the matrix, was then briefly digested with *Hin*dIII, which reduces the size of the DNA and eliminates most of the effects of shear during subsequent manipulations. After proteinase K-SDS treatment, the DNA (matrix-associated and loop fractions combined) was purified further on a CsCl density gradient. After a second digestion to completion with *Hin*dIII, replication intermediates were purified by BND-cellulose chromatography and were separated on a two-dimensional gel. The transfer was then hybridized consecutively with probes specific for three *Hin*dIII fragments in the initiation locus and, finally, with a probe for a fragment in the DHFR gene. The latter probe lies ~5 kb upstream from the 3′ end of the gene, which, in turn, lies ~12 kb upstream from the 5′ end of the map shown in Fig. 3E.

The triangular smear was virtually eliminated by the use of this novel isolation method. In addition, in the examples shown in Fig. 3B to D, the <1*n* material was considerably reduced (the transfer in Fig. 3A was purposely overexposed and is therefore not representative). In each of the four fragments analyzed, prominent and complete fork arcs were observed, suggesting that all four are replicated primarily by forks emanating from initiations in flanking fragments. However, less prominent but complete bubble arcs were also detected in the three fragments from the initiation locus (Fig. 3B to D). In contrast, no trace of a bubble arc was seen in the fragment from the DHFR gene, even at extremely long film exposures.

We conclude from this experiment that the bubble arcs observed in previous experiments were not artifacts of organic extractions or of cross-linking, but rather represent legitimate replication intermediates that result from initiations occurring in this region of the DHFR amplicon.

The matrix isolation procedure facilitates enrichment for stable replication intermediates. A complete bubble arc results from the presence of a centered origin in a fragment (Fig. 1B). However, combining the data in Fig. 3 with the results of previous studies (28), we have now observed faint but complete bubble arcs in several contiguous and overlapping fragments in a 30- to 35-kb region of the DHFR amplicon that completely encompasses the two zones of early labeling defined in previous studies (1, 18). The chance that each of these fragments contains a centered origin is extremely unlikely. A more likely explanation is that replication initiates at many sites scattered over a broad zone surrounding each of the two early-labeled zones. In this model, a composite pattern containing both a complete bubble arc and a complete fork arc results when a given restriction fragment is replicated passively in one amplicon or cell but contains an active initiation site at different positions in different amplicons or cells.

This unexpected result raised the possibility that an unusual form of delocalized initiation might have been induced by the amplification process per se. To address this possibility, it was necessary to analyze the pattern of replication of the single-copy DHFR locus in parental CHO cells. However, this would be impossible on theoretical grounds without some form of additional enrichment of replication intermediates, particularly if initiation is delocalized in CHO cells as well.

We took advantage of the observation that replication forks appear to partition preferentially with the nuclear matrix when matrix-halo structures are digested with either DNase I or restriction enzymes (9, 29). The method that we devised (method E) is as follows. Nuclei are isolated and extracted with LIS detergent, after which the matrix-halo structures are subjected to two sequential digestions with an appropriate restriction enzyme. The DNA that remains attached to the nuclear matrix (4 to 5% of total with a restriction endonuclease that has a 6-bp recognition sequence) is separated from the loop fraction by centrifugation. This matrix-attached DNA fraction is then further enriched in replication intermediates by fractionation on a BND-cellulose column (19).

Figure 4 shows the results of this enrichment scheme with DNA from an exponentially growing culture of CHOC 400 cells. Ten micrograms of an *Eco*RI digest of total, nonenriched DNA was separated on a two-dimensional gel, transferred to GeneScreen Plus, and hybridized with a probe specific for the 6.2-kb *Eco*RI fragment (Fig. 3E). In Fig. 4A, the 1*n* spot is clearly visible at this film exposure, but no replication intermediates can be detected.

A second aliquot of the total DNA fraction was subjected to BND-cellulose chromatography, and a 10-μg sample of the caffeine wash was analyzed on a two-dimensional gel. As seen in Fig. 4B, a fork arc is now detectable in this

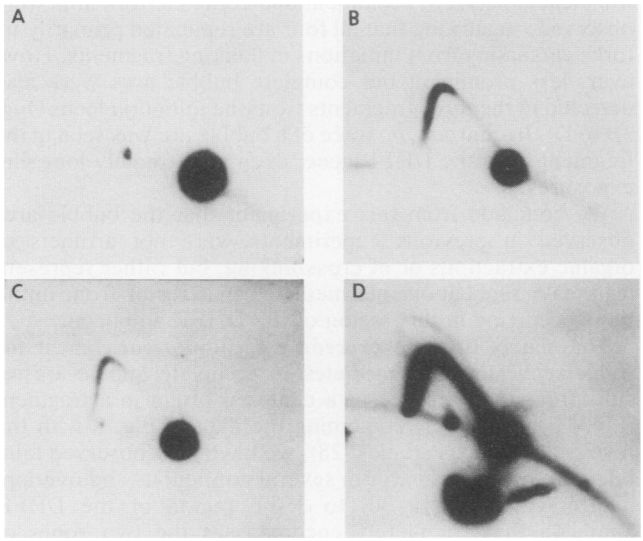

FIG. 4. Evidence that replication intermediates can be highly purified by a combination of isolation on the nuclear matrix and fractionation on BND-cellulose. Matrices were prepared from exponentially growing CHOC 400 cells, the DNA loops were digested in situ, and the digest was divided into four parts. (A) DNA was purified as in method E, and a 10-μg sample (total DNA) was separated on a two-dimensional gel. (B) After purification as described above, the DNA was subjected to fractionation on BND-cellulose prior to separation on a gel. (C) Matrix-attached DNA was purified as in method E and was then separated on a gel. (D) Matrix-attached DNA purified as in method E was fractionated on BND-cellulose and separated on a two-dimensional gel. After transfer to GeneScreen Plus, all DNA samples were hybridized with a probe located in the middle of the 6.2-kb *Eco*RI fragment that straddles the upstream initiation zone (see Fig. 3E).

BND-cellulose-enriched sample. A similar pattern was observed when 10 μg of the matrix-attached DNA fraction was analyzed (Fig. 4C). Therefore, replication intermediates do indeed partition with the nuclear matrix, and enrichment by the latter method is comparable to that achieved with BND-cellulose chromatography.

Finally, an aliquot of matrix-attached DNA was passed over BND-cellulose, and 10 μg of the DNA eluted with caffeine was subjected to two-dimensional gel analysis. A clear bubble arc is now detectable when the 6.2-kb *Eco*RI fragment is probed, and the intensity of the fork arc relative to the 1*n* spot is considerably greater than with either of the two enrichment procedures alone. The same enrichment factor is not attained by simply running the same DNA digest through the BND-cellulose column a second time (14a). Thus, the mechanism by which replicating DNA binds to BND-cellulose (presumably at single-stranded gaps between Okazaki fragments and at the replication fork; 19) is distinct from the mode by which replicating DNA binds to the nuclear matrix. In the best preparations, the matrix isolation and BND-cellulose procedures can be used sequentially to obtain a DNA fraction that is enriched in replication intermediates 200 to 250 times.

To address the possibility that any replication intermediate not partitioning with the nuclear matrix (e.g., a termination structure) might be lost from analysis, the experiment described above was repeated in a slightly different way. Instead of loading an equal number of micrograms of each DNA fraction for comparison, DNA samples from equivalent cell numbers were compared. When the transfers of these gels were hybridized with a probe specific for the 6.2-kb *Eco*RI fragment, all detectable replication intermediates again partitioned with the matrix and virtually none were detected in the detached DNA fraction (data not shown). It is therefore unlikely that selective loss of particular replication intermediates during the enrichment procedures significantly affects the outcome of two-dimensional gel electrophoretic analysis.

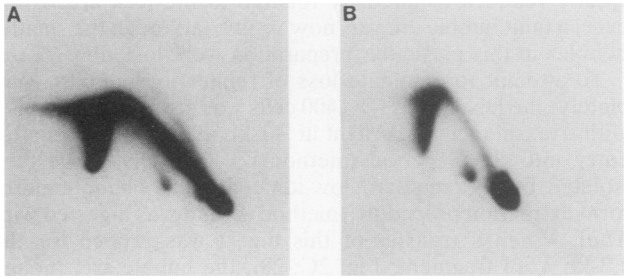

FIG. 5. Detection of initiation in the single-copy DHFR locus in CHO cells. Matrices were isolated from synchronized CHO cells 20 min after entry into the S period (A) or from exponentially growing cells (B). The DNA was released from the matrices with *Xba*I, and DNA was isolated according to method E. After separation on a gel and transfer to GeneScreen Plus, the transfers were hybridized with a probe from the middle of the 4.3-kb *Xba*I fragment that straddles the upstream initiation zone (see Fig. 3E).

Analysis of the single-copy DHFR locus in CHO cells. The ability to purify replication intermediates more than 100-fold from total genomic DNA suggested that it would be possible to detect replication intermediates in the single-copy locus of parental CHO cells if sufficient numbers of cells were used as starting material. Matrices were therefore prepared from 5×10^8 synchronized CHO cells 20 min after entry into the S period, and the matrix-attached DNA was separated from loop DNA by *Xba*I digestion. After passage through BND-cellulose, the digest was subjected to two-dimensional gel analysis and transferred to GeneScreen Plus. Figure 5A shows the autoradiogram obtained when the transfer was hybridized with a probe specific for the 4.3-kb *Xba*I fragment that straddles the upstream early-labeled zone. Just as in CHOC 400 cells, a faint and complete bubble arc is observed in addition to an intense, complete fork arc.

To control for any artifacts caused by the cell-synchronizing regimen, the same double-enrichment procedure was applied to CHO cells in exponential growth (10^9 cell equivalents per well). As shown in Fig. 5B, a complete fork arc was again observed, indicating that this fragment is probably usually replicated passively and does not contain a fixed initiation site by the criterion of two-dimensional gel analysis. Note, however, that the left half of the fork arc is more intense than the right half, because a small centered hybridization probe was used that will detect two probe equivalents in any fragment replicated by more than 50%. Note also that a bubble arc could not be detected in this experiment, even after prolonged film exposures, because not enough DNA was loaded onto the gel.

DISCUSSION

In this report, we have described methods for stabilizing branched replication intermediates in mammalian genomic DNA. This study was initiated because replication interme-

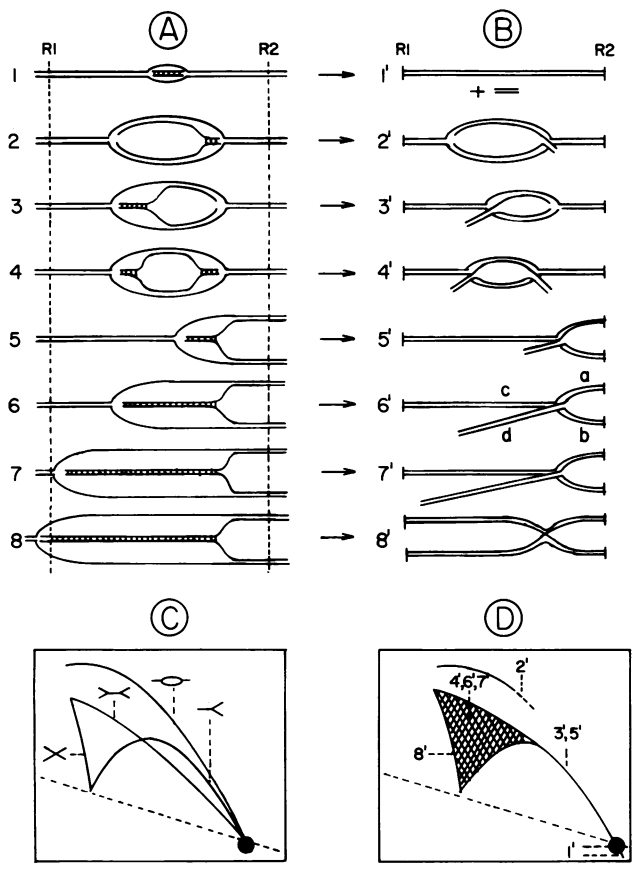

FIG. 6. Predicted patterns of branch-migrated intermediates on two-dimensional gels. The structures that would be expected as a result of branch migration of small (case 1), medium (cases 2 to 4), and large (cases 5 to 8) replication bubbles are shown. (A) Beginning stages of branch migration; (B) expected resulting structures; (C and D) predicted patterns on two-dimensional gels of stable and unstable replication intermediates, respectively.

diates proved to be unstable in DNA purified by standard isolation protocols. No bubble arcs could be detected in any fragments from the previously defined initiation locus in the DHFR domain of CHOC 400 cells, and two novel signals appeared in autoradiograms that did not correspond to the patterns expected for known replication intermediates: (i) material traveling on the diagonal below the 1*n* spot and (ii) a prominent triangular smear situated above the usual position of termination structures (Fig. 2). The former material must be due to shear forces generated during the manipulations involved in purifying long, relaxed DNA strands. The triangular smear most likely results from branch migration of replication intermediates, since it was not observed in DNA prepared by a variety of methods designed to stabilize DNA (e.g., cross-linking and isolation on the nuclear matrix).

Figure 6A and B show some of the variant structures that could be generated by branch migration; Fig. 6C and D show the expected two-dimensional gel patterns of normal and branch-migrated intermediates, respectively. Replication intermediates will generate different variant structures depending on the position of the fork(s) relative to the restriction sites defining the fragment ends.

Branch-migrated nascent strands from very small bubbles would be completely lost from analysis (case 1), while large bubbles completely contained within the fragment would probably be stable. Intermediate-size bubbles could travel on the bubble arc if the bubble is still partly intact and branch migration is limited (case 2). If branch migration is more extensive, rendering the bubble small and the new fork relatively large (case 3), the resulting intermediate could behave as a single-forked structure; intermediate levels of branch migration would result in a series of curves between the bubble and Y arc (not shown). If both ends of the nascent strands branch migrate (case 4), the intermediate could travel as a double-forked structure.

Branch migration in single-forked fragments will generate aberrant double-forked structures in which only the arms of the original fork (designated a and b in case 6) will be equal in length. If branch migration occurs when the replication forks are small (case 5), the resulting variant forms will probably lie along (or close to) the single-forked arc. However, as the fragment replicates more extensively (cases 6 and 7), the branch-migrated double-forked structures will trace a unique triangular smear in the gel that results from different extents of replication and branch migration in the same fragment. The aberrant 2*n* spike diagrammed in Fig. 6D results from nascent strands annealing with each other in a region that crosses a restriction site, with the result that both the parental and the nascent double strands are cut by the enzyme (Fig. 6B, case 8). Digestion therefore generates a 2*n* X-shaped molecule similar to true double-forked termination or recombination structures.

It should be noted that we have observed patterns that probably represent legitimate termination structures (e.g., Fig. 3C and D and 4D). A triangular pattern is also generated by forks colliding at random positions within a restriction fragment (3). In this case, however, the upper side of the triangle actually emerges from the single fork arc below its highest point (compare Fig. 6C and D). We assume that in synchronized cells, these double-forked structures result from the collision of two replication forks emanating from adjacent initiation sites located in the same DHFR amplicon. In exponentially growing cells, double-forked fragments could additionally result from the collision of forks from two different amplicons.

When DNA was stabilized (e.g., by cross-linking or by isolation on the nuclear matrix), bubble arcs could be detected in the DHFR amplicons of CHOC 400 cells after only a single enrichment step on BND-cellulose (Fig. 3; 27b). This experiment is possible because the copy number of the DHFR locus (1,000) per cell is approximately equal to a single-copy fragment in the yeast genome.

This introduces the important point that two-dimensional gel mapping methods are limited by the amount of DNA in micrograms that can be fractionated in an agarose gel without trailing (as is observed in Fig. 3A and 5) or distortion of the patterns on subsequent autoradiograms (~50 μg per well in our experience). The more complex the genome, the more DNA has to be loaded in order to detect replication intermediates in a particular fragment. Thus, without a further enrichment step, it would not be possible to analyze replication intermediates in a single-copy locus of a mammalian genome. A necessary consequence of the complexity problem is that even after the double-enrichment scheme, replication intermediates must be prepared from large numbers of mammalian cells in order to be detected by standard hybridization procedures (0.5×10^9 to 1.0×10^9 cell equivalents per well).

Although all three stabilization methods that we have attempted (cross-linking, encapsulation in agarose beads,

and isolation on the nuclear matrix) yield comparable patterns of replication intermediates on gels, the latter method is preferable for several reasons. In the cross-linking procedure (method C), cells are maintained in a nonphysiological condition for up to 1 h during treatment with trioxsalen and UV light, which could activate nucleases and affect the stability of replication intermediates. In addition, the preferential introduction of cross-links into DNA at thymidines (1, 25) interferes with the action of several common restriction enzymes, usually resulting in partial digests (e.g., Fig. 2C). Finally, only limited numbers of cells can be conveniently processed in each experiment because of the many manipulations involved, which effectively precludes the use of this method for the analysis of single-copy loci in mammalian genomes. The encapsulation method (8) is also practically limited for this reason.

The matrix stabilization procedure has the advantage that large numbers of cells can be conveniently processed, and the procedure can be modified to allow for enrichment of replication intermediates (method E). Theoretically, when a restriction endonuclease that has a 6-bp recognition sequence is used to remove loop DNA from matrix-halo structures, 4 to 5% of total DNA should remain attached to the matrix, assuming an average fragment size of 4 kb and an average loop size of ~100 kb (30). If all replicating DNA partitions with the nuclear matrix, as our previous studies show (9, 29), then matrix-attached DNA should be enriched in replication intermediates by a factor of 20 to 25. However, some nascent DNA is invariably lost because of detachment from the matrix during the washing procedures or because a fraction of the matrices may disintegrate. Consequently, the average enrichment factor is usually only 10 to 20. After the matrix-attached DNA is fractionated on BND-cellulose, which can enrich for replicating DNA from log-phase cells by 20- to 30-fold (19), a total enrichment of 200- to 600-fold should still be possible. This assumes that the mechanisms by which the matrix and BND-cellulose bind replication intermediates are distinct. The data in Fig. 5 argue that they are. Generally, however, enrichment factors of 150 to 200 are obtained (as in the experiment shown in Fig. 5B).

The matrix enrichment procedure has an additional advantage. In a recent modification of the neutral/neutral two-dimensional gel electrophoretic technique in which it is possible to determine the direction of replication fork travel through a region of interest (4a), DNA has to be digested to completion with a second restriction enzyme in the agarose gel after separation in the first dimension. This may prove to be difficult to accomplish when there is a large amount of DNA in the gel. By analyzing matrix-enriched DNA, the amount of material loaded can be reduced by a factor of ~200-fold (27b).

When replicating DNA from synchronized CHOC 400 cells was purified by the matrix stabilization scheme and analyzed on two-dimensional gels, several contiguous and overlapping fragments were found to display faint but reproducible bubble arcs (Fig. 2 and 3), in agreement with earlier studies (28). We also showed previously that forks move in both directions in fragments analyzed in the initiation locus of CHOC 400 cells by the neutral/alkaline two-dimensional gel mapping method (28). The simplest interpretation of these data is that in different amplicons or cells, replication initiates at different (possibly random) sites scattered throughout a broad region encompassing both zones of early labeling (28).

To exclude the possibility that this unusual delocalized form of initiation might be induced by the amplification process per se, it was necessary to analyze initiation reactions in the parental single-copy DHFR locus. Using the double-enrichment procedure, we were able to detect bubble arcs in the 4.3-kb *Xba*I fragment from the initiation locus in synchronized CHO cells (Fig. 5A). Again, this fragment appeared to be replicated mainly by forks entering from flanking regions, since the fork arc is so intense relative to the faint bubble arc. Unfortunately, the signals were not strong enough to detect bubble arcs in the DNA from exponential-phase CHO cultures, in which only a very low percentage of fragments is being replicated at the time of sampling (Fig. 5B). However, the presence of a complete fork arc in the 4.3-kb *Xba*I fragment also argues that replication may initiate at many sites in this locus in parental CHO cells.

On the surface, the results reported here and previously (28) seem to contradict a recent report in which most Okazaki fragments were observed to switch from one template strand to another within a single 400-bp fragment in the upstream peak of early labeling in the DHFR amplicon, implying the existence of a well-defined initiation site (6). Although it is difficult to quantitate the actual number of initiations occurring per unit length of DNA in the neutral/neutral two-dimensional technique used in the study reported here, qualitatively our data do not suggest a strongly preferred initiation site in this region of the previously defined initiation locus. However, recent experiments on CHO cells seem to suggest a slight preference for initiation in a 4.3-kb *Xba*I fragment that includes the proposed strand-switching site (8a).

These seemingly disparate results can be accommodated by a replication model for higher eukaryotic cells in which melting of large stretches of the helix occurs prior to the actual initiation of nascent strands (2). Since the strand-switching study (6) examined replication intermediates synthesized in vitro under circumstances that would not be expected to maintain nuclear architecture, initiation may actually occur in that system within a small zone encompassing a true *cis*-regulatory origin of replication. However, in the in vivo situation (as in our study), the subtleties of chromatin architecture may result in the melting of large regions of DNA surrounding a *cis*-regulatory origin as a consequence of the binding of an initiation protein. Initiation of nascent chains could then begin at any site within the melted region, resulting in the pattern of delocalized random initiation that we have observed in the mammalian DHFR initiation locus. Of course, as soon as the cells are lysed for preparation of the DNA, those parts of the melted DNA that have not yet replicated will reanneal to form double-stranded intermediates again and thus would not appear as single-stranded intermediates anywhere in the neutral/neutral gel system.

ACKNOWLEDGMENTS

We are very grateful to both Joel Huberman and Bonny Brewer, as well as the other members of our laboratory group, for many stimulating and helpful discussions. Carlton White and Kevin Cox deserve our deep gratitude for the hundreds of plates of cells and many DNA probes that they have provided for this study.

This work was supported by grants to J.L.H. from the NIH (GM26108) and the American Cancer Society (CD298). J.P.V. was supported on an NIH predoctoral training grant (T32 GM08136).

REFERENCES

1. **Anachkova, B., and J. L. Hamlin.** 1989. Replication in the amplified dihydrofolate reductase domain in CHO cells may

initiate at two distinct sites, one of which is a repetitive sequence element. Mol. Cell. Biol. **9**:532–540.
2. **Benbow, R. M., M. F. Gaudette, P. J. Hines, and M. Shioda.** 1985. Initiation of DNA replication in eukaryotes. Control Anim. Cell Prolif. **1**:449–483.
3. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins in ARS plasmids in S. cerevisiae. Cell **51**: 463–471.
4. **Brewer, B. J., and W. L. Fangman.** 1988. A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. Cell **55**:637–643.

4a. **Brewer, B. J., and W. L. Fangman.** Unpublished data.

5. **Burhans, W. C., J. E. Selegue, and N. H. Heintz.** 1986. Isolation of the origin of replication associated with the amplified Chinese hamster dihydrofolate reductase domain. Proc. Natl. Acad. Sci. USA **83**:7790–7794.
6. **Burhans, W. C., L. T. Vassilev, M. S. Caddle, N. H. Heintz, and M. L. DePamphilis.** 1990. Identification of an origin of bidirectional replication in mammalian chromosomes. Cell **62**:955–965.
7. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
8. **Cook, P. R.** 1984. A general method for preparing intact nuclear DNA. EMBO J. **3**:1837–1842.

8a. **Dijkwel, P. A.** Unpublished data.

9. **Dijkwel, P. A., L. H. F. Mullenders, and F. Wanka.** 1979. Analysis of the attachment of replicating DNA to a nuclear matrix in mammalian interphase nuclei. Nucleic Acids Res. **6**:219–230.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. High specific activity labeling of DNA restriction endonuclease fragments. Anal. Biochem. **132**:6–13.
11. **Handeli, S., A. Klar, M. Meuth, and H. Cedar.** 1989. Mapping replication units in animal cells. Cell **57**:909–918.
12. **Hatton, K. S., V. Dhar, E. H. Brown, M. A. Iqbal, S. Stuart, V. T. Didamo, and C. L. Schildkraut.** 1988. Replication program of active and inactive multigene families in mammalian cells. Mol. Cell. Biol. **8**:2149–2158.
13. **Heck, M. M. S., and A. C. Spradling.** 1990. Multiple replication origins are used during Drosophila chorion gene amplification. J. Cell Biol. **110**:903–914.
14. **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. USA **79**:4083–4087.

14a. **Huberman, J. A.** Personal communication.

15. **Huberman, J. A., and A. D. Riggs.** 1968. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. **32**:327–337.
16. **Huberman, J. A., J. Zhu, L. R. Davis, and C. S. Newlon.** 1988. Close association of a DNA replication origin and an ARS element on chromosome III of the yeast S. cerevisiae. Nucleic Acids Res. **16**:6363–6384.
17. **James, D. C., and M. Leffak.** 1986. Polarity of DNA replication through the avian alpha-globin locus. Mol. Cell. Biol. **6**:976–984.
18. **Leu, T.-H., and J. L. Hamlin.** 1989. High resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation. Mol. Cell. Biol. **9**:523–531.
19. **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**:549–565.
20. **Linskens, M. H. K., and J. A. Huberman.** 1988. Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4927–4935.
21. **Milbrandt, J. D., N. H. Heintz, W. C. White, S. M. Rothman, and J. L. Hamlin.** 1981. Methotrexate-resistant Chinese hamster ovary cells have amplified a 135 kilobase pair region that includes the gene for dihydrofolate reductase. Proc. Natl. Acad. Sci. USA **78**:6042–6047.
22. **Mirkovitch, J., M.-E. Mireault, and U. K. Laemmli.** 1984. Organization of the higher order chromatin loop: specific DNA attachment sites on the nuclear scaffold. Cell **39**:223–232.
23. **Nawotka, K. A., and J. A. Huberman.** 1988. Two-dimensional gel electrophoretic method for mapping DNA replicons. Mol. Cell. Biol. **8**:1408–1413.
24. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**:7207–7221.
25. **Russev, G., and L. Vassilev.** 1982. Isolation of a DNA fraction from Ehrlich ascites tumor cells containing the putative origin of replication. J. Mol. Biol. **161**:77–87.
26. **Struhl, K., D. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76**:1035–1039.
27. **Umek, R. M., M. H. K. Linskens, D. Kowalski, and J. A. Huberman.** 1989. New beginnings in studies of eukaryotic DNA replication origins. Biochim. Biophys. Acta **1007**:1–14.

27a. **Vaughn, J. P.** Unpublished data.

27b. **Vaughn, J. P., and P. A. Dijkwel.** Unpublished data.

28. **Vaughn, J. P., P. A. Dijkwel, and J. L. Hamlin.** 1990. Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell **61**:1075–1087.
29. **Vaughn, J. P., P. A. Dijkwel, L. H. Mullenders, and J. L. Hamlin.** 1990. Replication forks are associated with the nuclear matrix. Nucleic Acids Res. **18**:1965–1969.
30. **Vogelstein, B., D. M. Pardoll, and D. S. Coffey.** 1981. Supercoiled loops and eukaryotic DNA replication. Cell **22**:79–85.
31. **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.