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High-Resolution Mapping of Replication Fork Movement through the Amplified Dihydrofolate Reductase Domain in CHO Cells by In-Gel Renaturation Analysis

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Utilizing an in vivo labeling method on synchronized cultures, we have previously defined a 28-kilobase (kb) replication initiation locus in the amplified dihydrofolate reductase domain of a methotrexate-resistant Chinese hamster ovary cell line (CHOC 400) (N. H. Heintz and J. L. Hamlin, Proc. Natl. Acad. Sci. USA 79:4083–4087, 1982; N. H. Heintz and J. L. Hamlin, Biochemistry 22:3552–3557, 1983; N. H. Heintz, J. D. Milbrandt, K. S. Greisen, and J. L. Hamlin, Nature [London] 302:439–441, 1983). To locate the origin of replication in this 243-kb amplicon with more precision, we used an in-gel renaturation procedure (I. Roninson, Nucleic Acids Res. 11:5413–5431, 1983) to examine the labeling pattern of restriction fragments from the amplicon in the early S phase. This method eliminates background labeling from single-copy sequences and allows quantitation of the relative radioactivity in individual fragments. We used this procedure to follow the movement of replication forks through the amplicons, to roughly localize the initiation locus, and to estimate the rate of fork travel. We also used a slight modification of this method (termed hybridization enhancement) to illuminate the labeling pattern of smaller restriction fragments derived solely from the initiation locus itself, thereby increasing resolution. Our preliminary results suggest that there are actually two distinct initiation sites in the amplicon that are separated by ~22 kb.

In eucaryotic cells, the DNA in each chromosome is synthesized by thousands of tandemly arranged replication units (replicons) (17) whose synthesis must be tightly regulated to duplicate the chromosomes with fidelity. In recent years, several different experimental approaches have suggested the presence of a fixed origin within each replicon in eucaryotic chromosomes. One of the most powerful approaches has been the rescue of autonomously replicating sequence elements after shotgunning genomic libraries into the cell of origin by transfection. For example, many different autonomously replicating sequence elements have been isolated from the genome of the yeast *Saccharomyces cerevisiae* (6, 31) and more recently from mammalian cells (15). In addition, Johnson and Jelinek (20) have reported the autonomous replication of a human *Alu* sequence after transfection into monkey COS cells. Presumptive origins of replication have also been cloned from monkey cells by branch-migrating nascent DNA from small bubbles at the beginning of the S phase (32). At least one of these cloned fragments has been shown to replicate autonomously to a limited extent in short-term assays when reintroduced into monkey cells (10).

Most of the sequences isolated from eucaryotic cells by the approaches described above have not yet been shown to serve as initiation sites while resident in the chromosome. However, Hatton and colleagues (11a) have mapped fork movement through the c-*myc*, globin, and immunoglobulin loci in synchronous cell populations isolated by an elutriation technique. They found that forks move in one direction only through a particular locus in a given cell type at a given time during development, suggesting the presence of fixed origins. A similar conclusion was reached by James and Leffak (19), who used an in vitro runoff assay to show that forks move in one direction only through the alpha-globin locus in an avian cell line.

In our laboratory, we are interested in the nature of mammalian origins of replication, as well as the structural organization of replicons in chromosomes. As a model system, we have developed a methotrexate-resistant Chinese hamster ovary cell line (CHOC 400) that has amplified the dihydrofolate reductase gene (*DHFR*) and more than 200 kilobases (kb) of flanking sequence approximately 1,000 times (21, 22, 24). In previous gel electrophoretic studies, we took advantage of the high copy number of the amplified *DHFR* domain (amplicon) to show that only three *Eco*RI fragments derived from the amplicon are prominently labeled with radioactive thymidine at the beginning of the S phase (12). Because of background labeling from other early firing single-copy sequences, we were not able to determine which of these fragments had the highest specific radioactivity and therefore contained the origin. However, we showed that the early labeled fragments (ELFs) map together in the genome by isolating the initiation locus in the recombinant cosmid, cS21 (14), and in overlapping cosmids (21, 25). The ELFs defined a 28-kb initiation locus whose 5′ boundary lies ~10 kb downstream from the *DHFR* gene (Fig. 1A). In a recent publication, Burhans et al. (3) have presented evidence that DNA synthesis in the *DHFR* domain initiates within a single 4.3-kb *Xba*I fragment that is located near the 5′ end of the 28-kb initiation locus.

In the present study, we used an in-gel renaturation procedure developed by Roninson (30) to eliminate problems of background radioactivity from single-copy sequences that we experienced in our previous gel electrophoretic analysis

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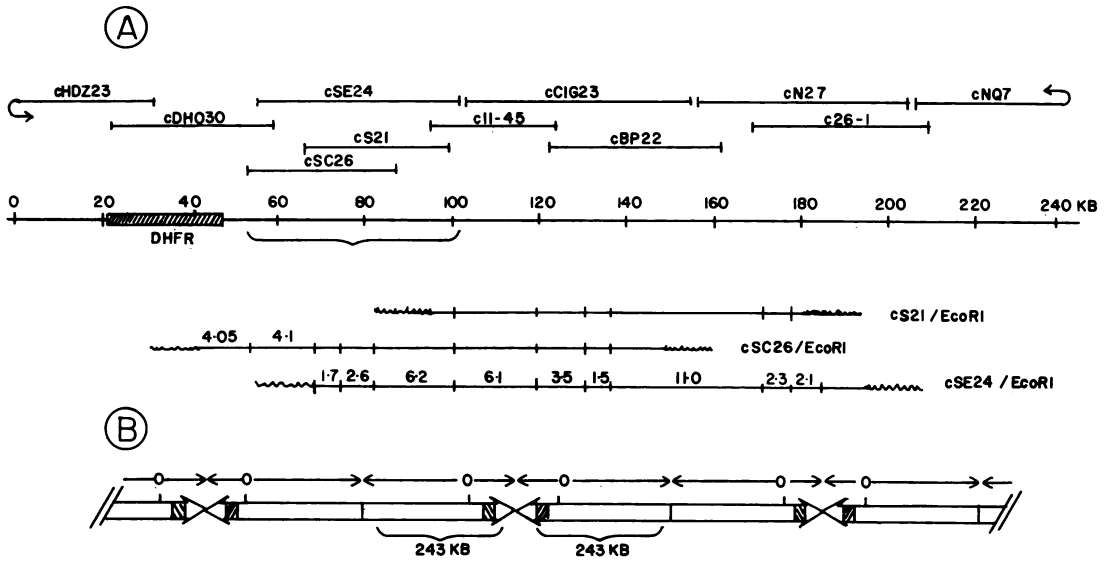

FIG. 1. Organization of the amplified *DHFR* locus in the CHOC 400 genome. (A) A representative series of overlapping recombinant cosmids that spans the major *DHFR* amplicon type in CHOC 400 cells is shown. The hairpin turns in the cosmids cHDZ23 and cNQ7 indicate the position of the palindromes that define the ends of the 243-kb amplicon. The *DHFR* gene itself is ~26 kb long and is indicated by the hatched box. The region containing the initiation locus is defined by the overlapping cosmids cS21, cSC26, and cSE24, which are bracketed, and *Eco*RI maps of these cosmids are shown below. (B) Schematic representation of the head-to-head and tail-to-tail arrangement of the major amplicon type in CHOC 400. The arrowhead in each repeating unit corresponds to the center of the palindrome in cHDZ23 (i.e., the head). The approximate center of the 28-kb initiation locus in each unit is indicated by an open circle (○).

of the labeling pattern of the amplicon in the early S phase. These studies enabled us to follow replication fork movement through this large domain and to localize the initiation sites in the *DHFR* amplicon with greater precision.

MATERIALS AND METHODS

Cell culture, synchrony, and labeling protocols. The methotrexate-resistant CHO cell line CHOC 400 was developed and maintained as previously described (24) and is resistant to 800 μM methotrexate. Cultures were arrested in the G_0 phase by isoleucine deprivation for 45 h, followed by release into complete medium containing 10 μg of aphidicolin per ml (12, 16). Twelve hours later (at the G1/S boundary), the cultures were released into the S phase by rapid washing with warm complete medium and were pulse-labeled with [^{3}H]thymidine (25 to 100 μCi/ml; 80 Ci/mmol) for various intervals in the early S phase. The plates were then either harvested immediately or were returned to fresh medium without label for the remainder of the S phase prior to harvest (see figure legends for details). Cells were washed twice with phosphate-buffered saline, followed by lysis in 10 mM Tris hydrochloride (pH 7.9)–10 mM EDTA–10 mM NaCl containing 1% sodium dodecyl sulfate and 100 μg of proteinase K (Merck & Co., Inc., Rahway, N.J.) per ml. Genomic DNA was purified as described in reference 11. All tissue culture supplies were purchased from GIBCO Laboratories (Grand Island, N.Y.), and aphidicolin and methotrexate were obtained from the National Cancer Institute.

Restriction digestion, labeling, and electrophoretic separation. Cosmids were isolated from two different CHOC 400 genomic DNA libraries (21, 23, 25). Genomic DNA samples and cosmids were digested with restriction enzymes under the conditions suggested by the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). A 1-kb ladder (Bethesda Research Laboratories) and digests of cosmids used as markers were end labeled with [^{32}P]dCTP by using T4 DNA polymerase (5). Genomic restriction digests and markers were separated on 1.0% agarose gels, and the gels were then either blotted directly onto GeneScreen Plus (Dupont, NEN Research Products, Boston, Mass.) by an alkaline transfer procedure (29) or were subjected to the in-gel renaturation or hybridization enhancement procedures prior to transfer (see below). In either case, the transfers were sprayed uniformly with En^{3}Hance (Dupont, NEN Research Products) and were placed next to preflashed X-Omat RP film (Eastman Kodak Co., Rochester, N.Y.) and an intensifying screen at −85°C. In the experiment depicted in Fig. 2B, after the tritium signal was obtained by autoradiography, the transfer was washed free of En^{3}Hance with toluene overnight at 24°C with gentle shaking and probed with a radioactive cosmid labeled with [^{32}P]dCTP by the random primer method (9). Prehybridization and hybridization procedures were as described in Dupont, NEN Research Products brochure no. NEF976, using the formulations containing 50% formamide.

In-gel renaturation and hybridization enhancement procedures. The in-gel renaturation experiments shown in Fig. 2 were performed on genomic restriction digests exactly as described by Roninson (30). In the hybridization enhancement experiments, 2 μg of genomic DNA digest was mixed with 5 μg of a nonradioactive digest of cosmid cSE24 prior to separation on 1.0% agarose. The gel was then subjected to two complete cycles of denaturation, renaturation, and S1 nuclease digestion as described by Roninson (30), except that the two renaturation steps were shortened from 2 and 12 h to 30 min each. Digests were then transferred to GeneScreen Plus, and the resulting X-ray films were scanned either with a Gilford spectrophotometer or by digitizing the autoradiographic images with a Dage video camera, an AT&T 6300 computer, and software supplied by the Computer Technology Center at the University of Virginia. The digitizer program has the advantage that it informs the

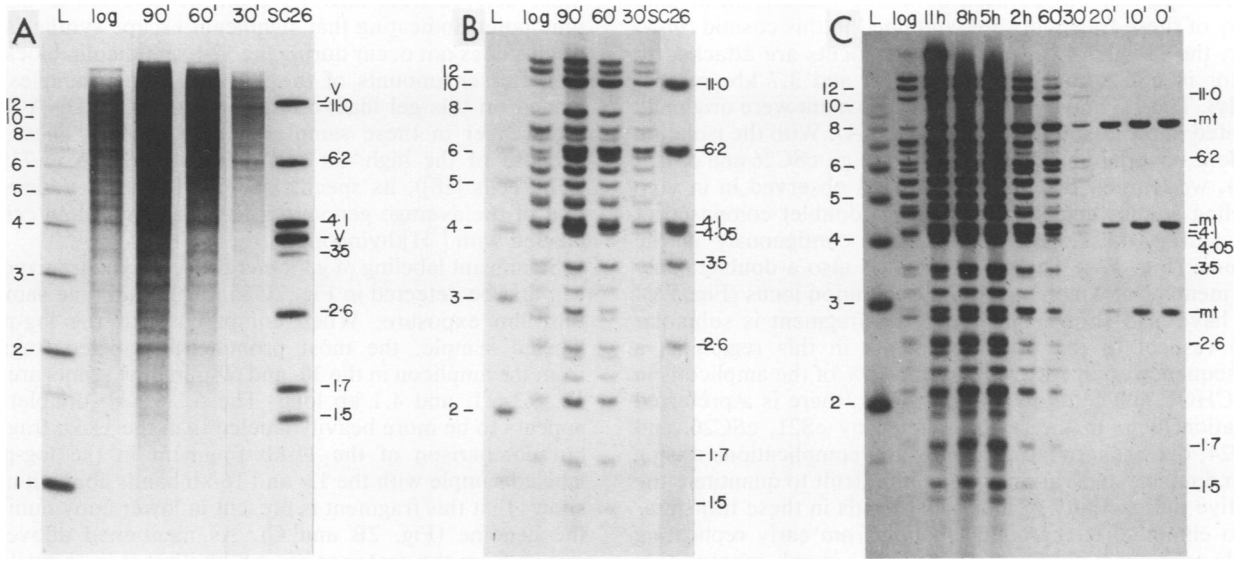

FIG. 2. Detection of ELFs in the *DHFR* amplicon. CHOC 400 cells were synchronized at the G1/S boundary as described in Materials and Methods. After removal of aphidicolin, cells were pulse-labeled for the indicated times with [^{3}H]thymidine. After each pulse, the label was washed out and chased with cold thymidine (see Materials and Methods for details). Cells were harvested 12 h after initial removal of aphidicolin. An exponential culture was also labeled for 12 h with 1 μCi of [^{3}H]thymidine per ml. Genomic DNA was then purified and digested with *Eco*RI, and 2 μg (A) or 6 μg (B) of each digest was separated on a 1% agarose gel along with an end-labeled 1-kb ladder. In panel A, an end-labeled sample of cosmid cSC26 was also included. (A) The agarose gel was blotted onto GeneScreen Plus, and the transfer was sprayed with En^{3}Hance and exposed to X-ray film for 1 day. The 11- and 4.05-kb genomic *Eco*RI fragments in cSC26 are both attached to vector and migrate at 11.5 and 3.7 kb, respectively (V in the figure). The sizes of the marker fragments in the 1-kb ladder and in cSC26 are indicated. (B) *Eco*RI digests (6 μg) of the same samples analyzed in panel A were separated on 1% agarose, the gel was subjected to two cycles of the in-gel renaturation procedure, and the gel was then blotted to GeneScreen Plus. The transfer was sprayed with En^{3}Hance and exposed to preflashed X-ray film for 6 days. The transfer was then washed overnight with toluene to remove the En^{3}Hance, and the blot was hybridized with ^{32}P-labeled cosmid cSC26 and exposed to X-ray film. The signals obtained from all the genomic digests after hybridization with the cSC26 probe were the same, but only the 90-min sample is shown (SC26 lane). (C) CHOC 400 cells were arrested in the G_0 phase by isoleucine deprivation and were released into medium containing 25 μCi of [^{3}H]thymidine per ml and 10 μg of aphidicolin per ml for 12 h. The cultures were then washed rapidly with warm medium to remove aphidicolin and were labeled for the indicated times with [^{3}H]thymidine. At the end of the pulse, the cells were harvested directly (no chase period), the purified DNA was digested with *Eco*RI, and digests (6 μg) were separated on an agarose gel. After two cycles of in-gel renaturation, the gel was transferred to GeneScreen Plus and the transfer was sprayed with En^{3}Hance and exposed to preflashed film for 21 days. The prominently labeled mtDNA bands are indicated (mt), as are the sizes of the *Eco*RI ELFs. The 20- and 30-min sample wells inadvertently received less DNA than the other wells.

operator when the film is optically saturated; this program was utilized to analyze the hybridization enhancement experiments depicted in Fig. 5. The reproducibility of the transfer to GeneScreen Plus within a given gel was determined by comparing the values of equivalent bands in the end-labeled 1-kb ladder separated in two different wells at opposite ends of a gel. The square root of the variance of a linear regression of one sample versus the other was found to be 1.64.

RESULTS

Map of the *DHFR* amplicon and the *DHFR* initiation locus in CHOC 400 cells. The ordered series of overlapping cosmids shown in Fig. 1A defines the predominant 243-kb amplicon type in CHOC 400 cells. The multiple copies of this amplicon are arranged in head-to-head and tail-to-tail configurations in homogeneously staining chromosome regions (Fig. 1B) (21, 22). The 28-kb initiation locus that we have previously defined by in vivo labeling experiments (12, 14) lies within the region contained in the overlapping cosmids cS21, cSC26, and cSE24 (indicated by the bracket in Fig. 1A). The left end of cSC26 lies ~10 kb downstream from the 3′ terminus of the *DHFR* gene. The initiation locus is not in the middle of the major CHOC 400 amplicon type, whose boundaries are defined by the center of the hairpins in cHDZ23 and cNQ7 (Fig. 1A and B) (14). The replication forks emanating from the initiation locus therefore appear to travel different distances to their respective termini, which presumably lie at the head-to-head and tail-to-tail interamplicon junctions (Fig. 1B).

Detection of ELFs in the *DHFR* domain by in-gel renaturation. To examine the replication pattern of the *DHFR* domain, we labeled synchronized CHOC 400 cells with [^{3}H]thymidine during the first 30, 60, or 90 min of the S phase. After the pulse, the cultures were returned to fresh medium and were harvested 12 h after removal of aphidicolin. A straightforward autoradiographic analysis of the labeling pattern of *Eco*RI digests of these samples after separation on a gel and transfer to GeneScreen Plus is shown in Fig. 2A. In the logarithmic (log)-phase-labeled sample, all the *Eco*RI bands derived from the amplicon are labeled (Fig. 2A; many of these bands are doublets and triplets). However, in the 30-min sample, a small subset of *Eco*RI fragments from the amplified *DHFR* domain was observed to be preferentially labeled against the background smear of radioactivity from single-copy sequences. The most prominent of these ELFs are 11, 6.2, and 4.1 kb long, as we have previously shown (12). Comparison with the end-labeled

*Eco*RI digest of cSC26 used as a size marker shows that many of these fragments are contained in this cosmid (however, the 11- and 4.05-kb *Eco*RI fragments are attached to vector in cSC26 and migrate at 11.5 and 3.7 kb, respectively). The 11- and a 6.2-kb *Eco*RI fragment were originally isolated in the cosmid cS21 (11) (Fig. 1A). With the isolation of clones overlapping cS21 to the left (e.g., cSC26 and cSE24 [21]), we learned that the 6.2-kb band observed in in vivo labeling studies actually represents a doublet composed of 6.2- and 6.1-kb fragments that map contiguously in the genome (Fig. 1A). The 4.1-kb band is also a doublet, only one member of which maps in the initiation locus (Fig. 1A). We have also shown that the 11-kb fragment is submolar with respect to the other fragments in this region as a consequence of an 8-kb variant in ~15% of the amplicons in the CHOC 400 cell line (14, 25). Thus, there is a preferred initiation locus in the region defined by cS21, cSC26, and cSE24, but background problems and complications arising from doublets and variants made it difficult to quantitate the relative radioactivity of individual bands in these transfers.

To eliminate background labeling from early replicating single-copy sequences, we used an in-gel renaturation method that was devised by Roninson (30) to analyze amplified sequences in mammalian DNA. Digests from the samples analyzed in Fig. 2A were separated on an agarose gel, and the digests in the gel were then subjected to two cycles of the following sequence: alkaline denaturation, renaturation, and S1 nuclease digestion. The renaturation steps were carried out long enough to allow amplified and other repeated (but not single-copy) restriction fragments to reanneal, and the single-copy fragments were eliminated from analysis by the S1 treatment. The digests were then transferred to a membrane and exposed to X-ray film after the transfer was sprayed with En^{3}Hance.

In the log-phase-labeled sample shown in Fig. 2B, approximately 30 amplified *Eco*RI bands (many of them doublets and triplets) can be discerned against a very clear background (compare with log-phase-labeled DNA from CHOC 400 cells in Fig. 2A). Thus, as shown by Roninson (30), the in-gel renaturation protocol effectively eliminates the interfering background of single-copy sequences. In the digests from cells labeled immediately upon release from aphidicolin for 30, 60, or 90 min, the most prominently labeled bands at all times are 11, 6.2-6.1, and 4.1 kb long, with the 6.2-6.1-kb doublet being slightly more labeled than the single *Eco*RI fragment at 11 kb (Fig. 2B). When this transfer was hybridized to the cosmid cSC26, all the prominent ELFs were seen to map within this cosmid (Fig. 2B and 1A).

The possibility existed that some initiation occurs at origins of replication in the presence of aphidicolin (7). Upon release of cells from the drug into [^{3}H]thymidine, labeling would then commence from positions flanking the actual origin, leaving the origin-containing fragment itself underlabeled. To test for this possibility, we performed a second experiment in which [^{3}H]thymidine was included in the medium during the aphidicolin block. One sample was harvested after 12 h in aphidicolin and [^{3}H]thymidine (the zero time point). The remaining cultures were released from the block into medium containing [^{3}H]thymidine of the same specific activity as the prelabel, and samples were harvested at the indicated times (Fig. 2C).

The most prominently labeled bands at all the early time points in this experiment arise from mitochondrial DNA (mtDNA), which is replicated throughout the cell cycle (2, 13) and is not significantly inhibited by aphidicolin (16, 18). However, no preferential labeling of fragments other than those derived from mtDNA could be detected at the zero time point, indicating that significant escape synthesis from origins does not occur during the 12-h aphidicolin block (Fig. 2C). Lesser amounts of the 20- and 30-min samples were loaded on this gel inadvertently; thus, the mtDNA signals are weaker in these samples than at earlier times. Also, because of the high G+C content of mtDNA (~71% for CHO cells [18]), its specific radioactivity will be less than that of the average genomic DNA fragment when cells are labeled with [^{3}H]thymidine.

Significant labeling of genomic DNA in synchronized cells can first be detected in Fig. 2C in the 30-min time sample at this film exposure. When compared with the log-phase-labeled sample, the most prominently labeled fragments from the amplicon in the 30- and 60-min time points are again 11, 6.2-6.1, and 4.1 kb long. The 6.2-6.1-kb doublet band appears to be more heavily labeled than the 11-kb fragment, but comparison of the 11-kb fragment in the log-phase-labeled sample with the 13- and 16-kb bands above it clearly shows that this fragment is present in lower copy number in the genome (Fig. 2B and C). As mentioned above, this results from the presence of an 8-kb variant in about 15% of the amplicons in CHOC 400 (14, 25). Since renaturation of amplified bands does not go to completion under the conditions of this experiment (T.-H. Leu, unpublished data) and since the rate of reannealing varies with the square of the concentration, the difference in copy number is magnified after two cycles of in-gel renaturation.

The samples isolated at later times show that all copies of the amplicon are not completely replicated until quite late in the S phase, in agreement with the labeling pattern of homogeneously staining regions in nonsynchronized cells (24). From lower film exposures, we have estimated that the 6.2- and 11-kb fragments do not attain maximum specific activity until 4 to 5 h after release from aphidicolin (data not shown).

To correct for differences in copy number of individual fragments as well as for differences in thymidine content, we compared the relative signal of a given ELF with its counterpart in log-phase-labeled DNA. The digests in the autoradiogram shown in Fig. 2B were scanned on a spectrophotometer, and representative scans from the log-phase-labeled and 30-min samples are compared in Fig. 3A (in this analysis, the signals from the largest 16-kb *Eco*RI fragment in the two samples were normalized to aid visualization). Preferential labeling is detected in the 30-min sample (open curves) in the previously identified bands at 11, 6.2-6.1, and 4.1 kb (ELFs C, F, and K), as well as in fragments migrating at 5.9, 4.05, 3.5, 2.3, 2.1, 1.7, 1.6, and about 1.5 kb (ELFs G, L, M, S, T, U, V, and W, respectively). All these fragments map in the previously defined initiation locus, with the exception of the 5.9-kb band, which maps just to the right of cosmid SE24 (see map, Fig. 1A). In Fig. 3B are plotted the ratios of densitometric signals from ELFs versus log-labeled fragments relative to map position. With the exception of ELFs F and F′, which map contiguously in the genome and are plotted as one point, this graph includes only those bands that are known to migrate as single amplified fragments on gels (determined from mapping studies [21]). Only six fragments (in addition to F and F′) fit this criterion, none of which maps in the region 3′ to fragment B (Fig. 3B).

In the 30-min sample, the highest labeling relative to log-labeled DNA was centered over the region defined by cosmid cSE24, with very little label detected outside of this region. At 60 min, the labeling curve was broader, and by 90 min, significant labeling was detected in regions that lie ~80

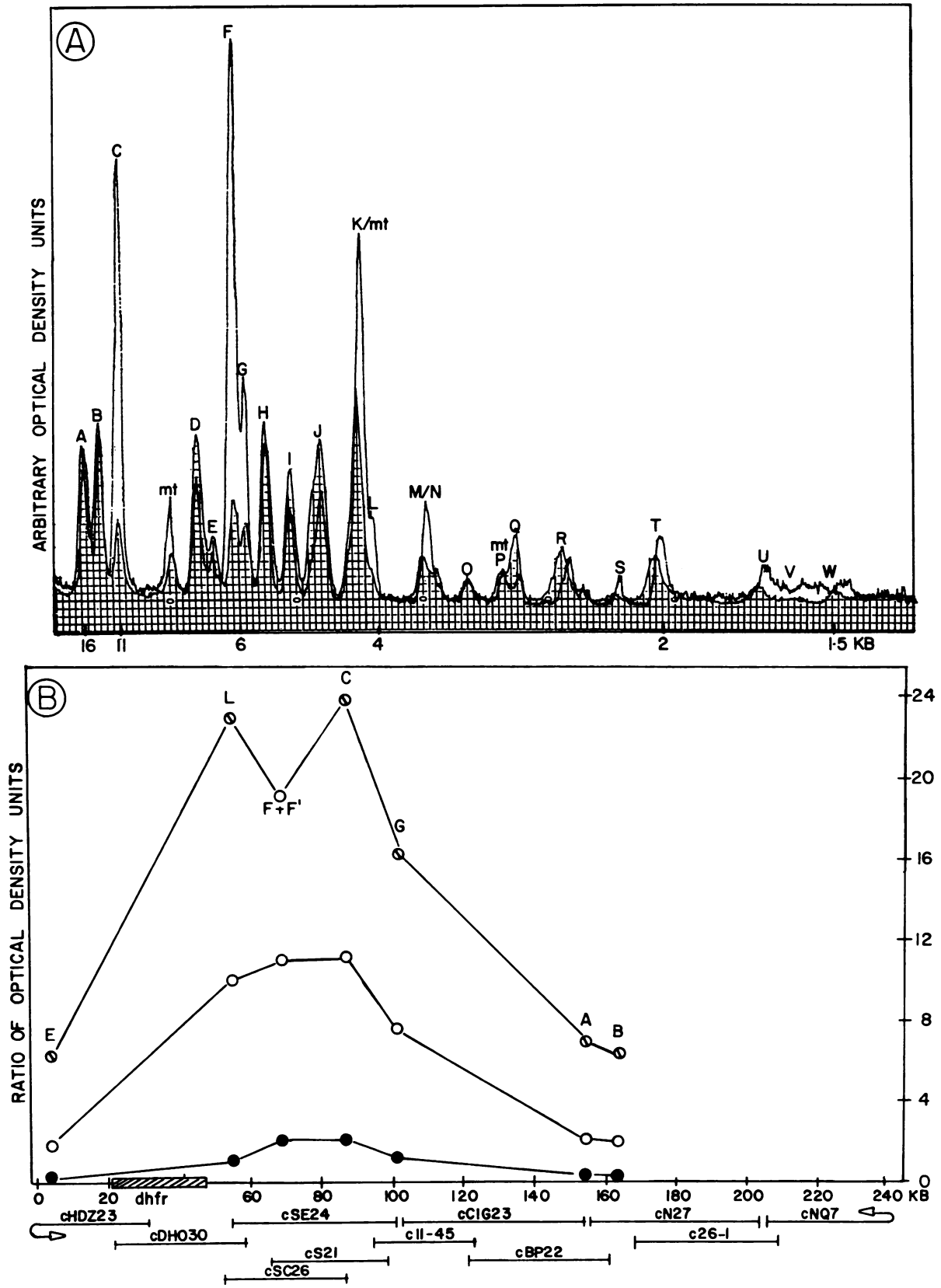

FIG. 3. Pattern of ELFs in the *DHFR* amplicon. (A) The log-phase-labeled and the 30-min samples from the experiment shown in Fig. 2B were scanned on a Gilford spectrophotometer. The peak heights of the largest *Eco*RI fragment (A) were normalized, and the two tracings were superimposed. The cross-hatched area represents the tracing from the log-phase-labeled sample, and the open curves are from the 30-min sample. The discernible peaks are labeled alphabetically, and the positions of mitochondrial fragments are indicated (mt). Note that the baseline goes down to zero absorbance in between most bands. (B) The log-phase-labeled, 30-, 60-, and 90-min samples from the experiment in Fig. 2B were each scanned as described above, except that the curves were not normalized to one another. The peak heights of individual bands in the early and log-phase-labeled samples were then expressed as a ratio, and this ratio is plotted as a function of map position in the amplicon. Only those bands that are known to contain single fragments were selected for analysis (with the exception of the two 6.2-kb *Eco*RI fragments [F+F'] that map together in the genome). Symbols: ●, 30-min/log phase ratios; ○, 60 min/log phase ratios; ○, 90 min/log phase ratios.

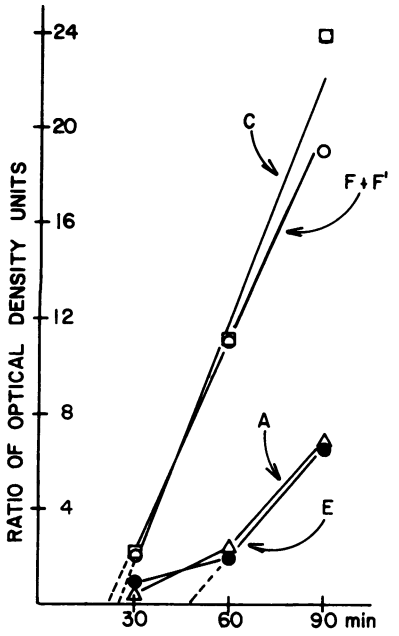

FIG. 4. Rate of labeling of selected amplicon fragments in the early S phase. The ratios determined for selected fragments in the experiment shown in Fig. 3B were plotted as a function of time in the early S phase. Fragments C and F+F' are ELFs discussed in the text, and fragments A and E are located ~80 kb to the left and right, respectively, of the center of the initiation locus in cSE24.

kb to the left and right of the center of cSE24 (fragments E and A). In addition, the relative labeling of fragments F-F' is almost identical to that of fragment C in the 30- and 60-min time points. The dip in fragments F-F' in the 90-min labeling curve is an artifact resulting from overexposure of the film in this strongly labeled band in the 90-min sample, with a consequent underestimate of its intensity (compare with the same band in the log-labeled sample in Fig. 2B).

In Fig. 4 are plotted the relative rates of labeling of the 11- and 6.2-6.1-kb ELFs (C and F-F'), as well as the distal 16- and 6.7-kb fragments (A and E) during the first 90 min of the S phase. The curves for ELFs C and F-F' are almost superimposable (except for the underestimated 90-min F-F' point) and appear to be linear for the 90-min period examined. Thus, initiation does not appear to occur simultaneously in all 1,000 copies of the amplified *DHFR* domain, but rather occurs over a considerable time. The two rate curves for C and F-F' intercept the abscissa at ~20 min, suggesting that significant initiation in this locus begins about 20 min after removal of aphidicolin. The curves for fragments E and A do not become linear for at least 60 min after removal of drug. A line between the last two points extrapolates to ~50 min on the abscissa, suggesting that the average fork moves, at most, 80 kb in 30 min, or ~2.5 kb/min.

Increased resolution of labeling patterns by hybridization enhancement. The resolution of the in-gel renaturation procedure is limited in two important ways. Because of the large size of the amplicon (~243 kb), very few amplified fragments are uniquely sized, regardless of the enzyme utilized to digest the DNA; as a consequence, the data from doublet or triplet bands are lost from analysis. The resolution of the method is also limited by the large size of the fragments analyzed; however, if we were to utilize restriction enzymes that digest the amplicon into smaller fragments, the problem of doublets and triplets would be even more pronounced.

We therefore devised a minor variation of the in-gel renaturation procedure that allows the analysis of smaller, uniquely sized fragments only within a circumscribed region of the amplicon. In this method, a great excess of an appropriate unlabeled cosmid digest is mixed with labeled genomic DNA digested with the same restriction enzyme, and the mixture is separated on agarose. The gel is then subjected to denaturation, renaturation, and S1 nuclease cycles. However, the renaturation step is considerably shortened relative to the standard procedure so that only those labeled sequences complementary to the cold driver cosmid fragments reanneal, while other amplified and single-copy sequences remain single stranded and are thus eliminated from analysis by the S1 treatment. The advantage of the method is that enzymes or enzyme combinations can be found that give smaller, uniquely sized fragments only in the region of interest, resulting in increased resolution in this region of the amplicon.

The autoradiogram in Fig. 5A shows the result of this analysis when the cosmid cSE24, which spans the initiation locus (Fig. 1A), was used to drive the in-gel renaturation of the same labeled genomic DNA samples that were analyzed in Fig. 2B and C. Both the cosmid and the genomic DNAs were digested with a combination of *Bam*HI and *Hin*dIII prior to separation on the gel. With the exception of the two mtDNA bands at 8.2 and 5.5 kb (Fig. 5B), a significant signal is recorded only from amplified fragments represented by the driver cosmid. Furthermore, no labeled bands are detected in log-labeled CHO DNA when mixed with cold cSE24 driver DNA, again except for faint mtDNA bands (Fig. 5A). The signals obtained from the labeled CHOC 400 DNA therefore must emanate from amplified genomic sequences (note that the 1.7-kb band is very light relative to other fragments, even in the log-phase-labeled control, owing to an extremely high G+C content [T.-H. Leu, unpublished observations]). There is a slight background distributed throughout each lane that is probably derived from incomplete washing after S1 digestion. The faint 8.2-kb mtDNA band in the 60-min sample shown in Fig. 5B probably results from the fact that, even though mtDNA seems to be present at approximately the same copy number per cell as fragments from the amplicon (see log-phase samples in Fig. 2A and B), mtDNA bands are extremely highly labeled relative to amplicon fragments in the samples that received [^{3}H]thymidine throughout the G1 phase (see Fig. 2C, and compare with Fig. 5A and 2B). However, the faint 5.5-kb band corresponds in size both to a mtDNA *Hin*dIII fragment (26) and to a truncated genomic fragment attached to vector. Hence, its signal is a complex mixture that cannot be resolved.

In the 30- and 60-min samples in Fig. 5A and B, the differences in radioactive signals among individual bands are not as apparent as in Fig. 2, since a much smaller region of the amplicon is being analyzed (~42 kb). However, subtle differences can be detected. For example, the relative intensities of the 6.3- and 5.3-kb bands in the 60-min samples are reversed compared with the log-phase samples in each experiment (also compare the 2.1- and 1.8-kb bands in the 30- and 60-min samples from the log-phase control). The intensities of bands in each of the samples in Fig. 5A and B were quantitated by densitometry, and the ratios of early labeled and log-phase-labeled signals are plotted in Fig. 6 as a function of map positions. Surprisingly, two peaks of radioactivity were observed in the 60-min samples (upper

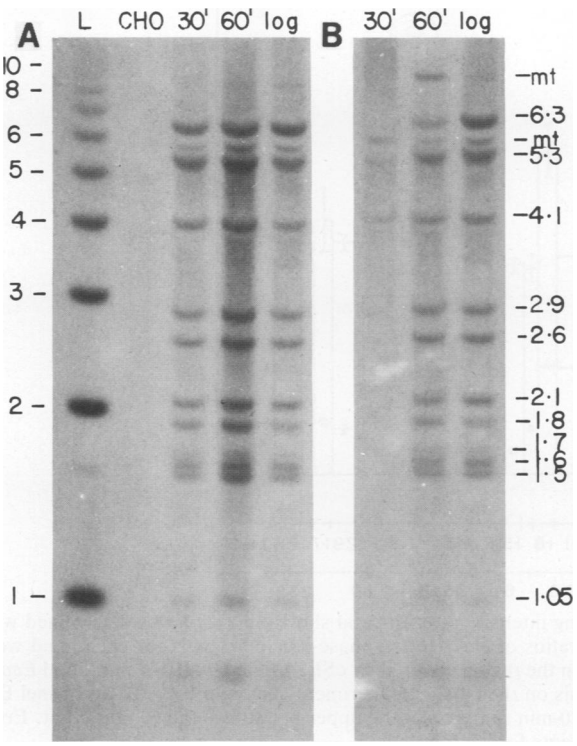

FIG. 5. Localization of initiation sites by hybridization enhancement. The labeled genomic DNA samples analyzed in Fig. 2B and C were digested with a combination of *Hin*dIII and *Bam*HI, and 5 μg of each was mixed with 2 μg of cSE24 digested with the same enzymes. The mixed digests were then separated on a 1% agarose gel, and the gel was subjected to two cycles of in-gel renaturation as described by Roninson (30), with the following modification. After each alkaline denaturation step, the gel was washed twice for 15 min with 50% formamide containing 3× SSPE (1× SSPE is 10 mM NaH_2PO_4, 0.18 M NaCl, and 1 mM EDTA, pH 7.0), but the subsequent 2-h (first cycle) and 12-h (second cycle) renaturation steps were each shortened to 30 min, followed by washing with S1 buffer, S1 nuclease treatment, and final washes as described by Roninson (30). The DNA was then transferred to GeneScreen Plus, and the transfer was sprayed with the fluorophor and exposed to preflashed X-ray film for 21 days. The sizes of individual fragments are indicated next to the gel, as are the positions of mtDNA fragments.

bar) in both of the previously analyzed experiments, flanking a dip over the 6.3-kb *Hin*dIII fragment. The same two peaks were also somewhat suggested in the 30-min samples in Fig. 6A and B (lower bars) but were not as dramatic, presumably because of the somewhat faint signals in these samples and consequent inaccuracies in measurement. Because of the relatively small size of most of the restriction fragments within the 42-kb region defined by cSE24, it is possible to locate the two peaks of radioactivity in the 60-min time points in or near the 1.8- and 1.7-kb *Hin*dIII-*Bam*HI fragments that map within ELF F′ and ELF C, respectively. The relative labeling intensities of these two fragments were shown to be approximately equal in the experiment in Fig. 3A and B. The loci corresponding to the 1.8- and 1.7-kb fragments are also extremely close to the two initiation sites identified by an independent method in the accompanying paper (1).

The replication of the 6.3-kb *Hin*dIII fragment between these two loci appears to be delayed beyond the time interval expected, since the center of this fragment is located only ~11 kb away from a local initiation site (e.g., in either the 1.8- or 1.7-kb fragment). However, a scan of shorter exposures of the film in Fig. 2C indicates that the fragments mapping in this region eventually do become labeled to the same relative extent as in log-phase-labeled DNA, but only after 5 to 6 h into the S phase (data not shown). Thus, suppressed labeling of this region in the early S phase is apparently not an artifact of the method used to analyze the autoradiograms and also is apparently not due to the relatively large size of the two fragments in the center of this locus.

DISCUSSION

By eliminating background labeling from single-copy sequences, the in-gel renaturation method enabled us to examine the replication pattern of the amplified *DHFR* domain in much greater detail than was formerly possible. We confirmed our previous suggestion that replication initiates preferentially in the region defined by the overlapping cosmids cS21, cSC26, and cSE24 (see map, Fig. 1A) (12–14). The rate of fork movement through the amplified *DHFR* domain appears to be slightly greater than 2.5 kb/min, which agrees well with estimates of fork rate in mammalian cells by DNA fiber autoradiography (17).

The time course of labeling of the initiation locus itself clearly shows that all the origins in the 1,000 copies of the *DHFR* domain do not fire at once. In fact, the specific activity of this locus continues to rise for approximately 5 h after release from aphidicolin (Fig. 2C). Although we cannot presently rule out the possibility that only some of the potential origins are utilized for initiation (i.e., that some amplicons are replicated by forks reading through from adjacent amplicons), this result suggests that activation of these early firing origins is a stochastic process that depends on interaction with some rate-limiting catalytic protein that is elaborated in the late G1 phase or in the early S phase or both. If replication complexes are attached to a nuclear matrix or scaffolding, as has been suggested previously (4, 8, 28; see reference 27 for a review), it is also conceivable that the concentration of these complexes in the zone of the matrix to which the amplicons are attached is not high enough to service all the *DHFR* amplicons simultaneously. Indeed, we have recently located a matrix attachment site in the left end of the 11-kb *Eco*RI fragment and have shown that fewer than ~20% of these fragments in the CHOC 400 cell line are attached at this site compared with more than 50% for parental CHO cells (7a).

The analysis of the replication pattern in the 28-kb initiation locus was considerably refined by our preliminary experiments with the hybridization enhancement method. When cSE24, which completely spans the initiation locus, was used as a driver, we found two distinct peaks of preferential labeling centered approximately over the 1.8- and 1.7-kb *Hin*dIII-*Bam*HI fragments, respectively. The same result was obtained from two different in vivo labeling protocols used in this study (and also in a different methotrexate-resistant Chinese hamster cell line with a larger *DHFR* amplicon that also initiates replication in this region [C. Ma, T. H. Leu, and J. L. Hamlin, manuscript in preparation; T.-H. Leu, unpublished observations]). In one of the experiments described in the present report, [^{3}H]thymidine was present in the G1 phase during the aphidicolin block, so that any replication forks moving slowly away from origins in the presence of the drug would be labeled. Thus, the dip in the labeling pattern between the two peaks cannot be

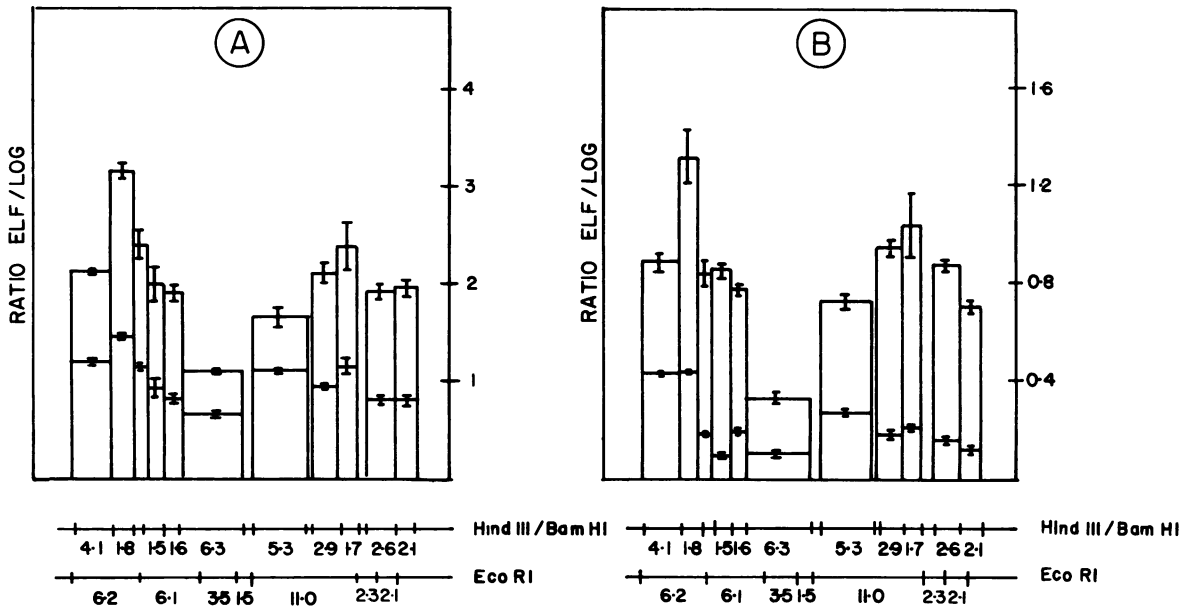

FIG. 6. Labeling pattern of the *DHFR* initiation locus. The relative labeling intensity of each band shown in Fig. 5 was determined with the Dage camera and computer program (Materials and Methods), and the ratios of early to log-phase-labeled signals for each band were calculated. These ratios are plotted in the figure as a function of map position in the region covered by cSE24. The *Hin*dIII-*Bam*HI and *Eco*RI maps are shown below each panel. Panel A displays the results of this analysis on the labeling experiment shown in Fig. 2B, and panel B is from the experiment in Fig. 2C. In each panel, the lower bar represents the 30-min sample and the upper bar shows the 60-min point. Error bars are shown for the standard error of the mean of at least four measurements for each point.

explained by escape synthesis in the absence of label during the aphidicolin block and agrees with our earlier studies (13). However, it is conceivable that some other form of aberrant replication induced by the presence of aphidicolin could result in the lower level of [^{3}H]thymidine uptake in the region centered over the 6.3-kb *Hin*dIII fragment (e.g., the synthesis of exceedingly long RNA primers). In the latter case, the origin would be expected to be located in or near the 6.3-kb fragment itself. Although we do not know of any precedent for the formation of such long RNA primers (~10 kb) during aphidicolin arrest (but see reference 7), we are attempting to investigate these possibilities by independent approaches.

Another shortcoming of the hybridization enhancement experiment presented in Fig. 5 and 6 is the fact that the low point between the two proposed initiation sites is located in the largest fragment in the digest (the 6.3-kb *Hin*dIII fragment), raising some uncertainty whether size could have an effect on our measurements. However, there is an unfortunate distribution of restriction sites in this region of the amplicon, since no double digests tested other than *Hin*dIII-*Bam*HI give a more suitable spectrum of fragments. This region has been mapped so far with *Eco*RI, *Kpn*I, *Pvu*II, *Xba*I, *Hin*dIII, and *Bam*HI and will require further characterization with other enzymes to clarify this uncertainty.

In the accompanying study (1), we used an independent method based on solution hybridization studies with replication intermediates to examine the labeling pattern of synchronized CHOC 400 cells in the early S phase. These studies also suggested the presence of two initiation sites in the *DHFR* domain. Thus, our results agree only in part with those of Burhans et al. (3), who identified only one initiation site mapping in *Eco*RI ELF F′ (Fig. 1A). The possible reasons for this discrepancy are addressed in the Discussion of the accompanying report (1). In any event, the in-gel renaturation methods described here helped us to identify and clone another initiation locus that we identified in the much larger *DHFR* amplicon of an independently isolated methotrexate-resistant Chinese hamster cell line (Ma et al., in preparation). An analysis of this additional locus should eventually help us to define the elements that are shared by chromosomal replication initiation sites.

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LITERATURE CITED

1. **Anachkova, B., and J. L. Hamlin.** 1988. 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. **Bogenhagen, D., and D. A. Clayton.** 1977. Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. Cell **11**:719–727.
3. **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.
4. **Carri, M. T., G. Micheli, E. Graziano, T. Pace, and M. Buongiorno-Nardelli.** 1986. The relationship between chromosomal origins and the nuclear matrix during the cell cycle. Exp. Cell Res. **164**:426–436.
5. **Challberg, M. D., and P. T. Englund.** 1980. Specific labeling of 3′ termini with T4 DNA polymerase. Methods Enzymol. **65**: 39–42.
6. **Chan, C. S. M., and B.-K. Tye.** 1980. Autonomously replicating sequences in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77**:6329–6333.
7. **Decker, R. S., M. Yamaguchi, R. Possenti, and M. L. DePamphilis.** 1986. Initiation of SV40 replication in vitro: aphidicolin

causes accumulation of early-replicating intermediates and allows determination of the initial direction of DNA synthesis. Mol. Cell. Biol. 6:3815–3825.

7a. **Dijkwel, P. A., and J. L. Hamlin.** 1988. Matrix attachment regions are positioned near replication initiation sites, genes, and an interamplicon junction in the amplified dihydrofolate reductase domain of Chinese hamster ovary cells. Mol. Cell. Biol. 8:5398–5409.

8. **Dijkwel, P. A., P. Wenink, and J. Poddighe.** 1986. Permanent attachment of replication origins to the nuclear matrix in BHK cells. Nucleic Acids Res. 14:3241–3249.

9. **Feinberg, A. P., and B. Vogelstein.** 1983. High specific activity labeling of DNA restriction endonuclease fragments. Anal. Biochem. 132:6–13.

10. **Frappier, L., and M. Zannis-Hadjopoulos.** 1987. Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences. Proc. Natl. Acad. Sci. USA 84:6668–6672.

11. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1978. Isolation of high molecular weight DNA from mammalian cells. Eur. J. Biochem. 36:32–38.

11a. **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.

12. **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.

13. **Heintz, N. H., and J. L. Hamlin.** 1983. *In vivo* effects of cytosine arabinoside on deoxyribonucleic acid replication in Chinese hamster ovary cells. I. Resolution of differential effects on mitochondrial and nuclear deoxyribonucleic acid synthesis. Biochemistry 22:3552–3557.

14. **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.

15. **Holst, A., F. Muller, G. Zastrow, H. Zentgraf, S. Schwender, E. Dinkl, and F. Grummt.** 1988. Murine genomic DNA sequences replicating autonomously in mouse L cells. Cell 52:355–365.

16. **Huberman, J. H.** 1981. New views of the biochemistry of eucaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase alpha. Cell 23:647–648.

17. **Huberman, J. H., and A. D. Riggs.** 1972. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. 32:327–341.

18. **Ikegami, S., T. Taguchi, M. Ohashi, M. Oguro, H. Naganu, and Y. Mano.** 1978. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-alpha. Nature (London) 275:458–460.

19. **James, D. C., and M. Leffak.** 1986. Polarity of DNA replication through the avian alpha-globin locus. Mol. Cell. Biol. 6:976–984.

20. **Johnson, E. M., and W. R. Jelinek.** 1986. Replication of a plasmid bearing a human Alu-family repeat in monkey COS-7 cells. Proc. Natl. Acad. Sci. USA 83:4660–4664.

21. **Looney, J. E., and J. L. Hamlin.** 1987. Isolation of the amplified dihydrofolate reductase domain from methotrexate-resistant Chinese hamster ovary cells. Mol. Cell. Biol. 7:569–577.

22. **Ma, C., J. E. Looney, T.-H. Leu, and J. L. Hamlin.** 1988. Organization and genesis of dihydrofolate reductase amplicons in the genome of a methotrexate-resistant Chinese hamster ovary cell line. Mol. Cell. Biol. 8:2316–2327.

23. **Milbrandt, J. D., J. C. Azizkhan, K. S. Greisen, and J. L. Hamlin.** 1983. Organization of a Chinese hamster ovary dihydrofolate reductase gene identified by phenotypic rescue. Mol. Cell. Biol. 3:1266–1273.

24. **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 78:6043–6047.

25. **Montoya-Zavala, M., and J. L. Hamlin.** 1985. Similar 150-kilobase DNA sequences are amplified in independently derived methotrexate-resistant Chinese hamster cells. Mol. Cell. Biol. 5:619–627.

26. **Nass, M. M. K.** 1983. Restriction map of Chinese hamster mitochondrial DNA containing replication coordinates: comparison with Syrian hamster mitochondrial genome. Gene 21: 249–255.

27. **Nelson, W. G., K. J. Pienta, E. R. Barrack, and D. S. Coffey.** 1986. The role of the nuclear matrix in the organization and function of DNA. Annu. Rev. Biophys. Biophys. Chem. 15: 457–475.

28. **Razin, S. V., M. G. Kekelidze, E. M. Lukanidin, K. Scherrer, and G. P. Georgiev.** 1986. Replication origins are attached to the nuclear matrix. Nucleic Acids Res. 14:8189–8207.

29. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. 13: 7207–7221.

30. **Roninson, I.** 1983. Detection and mapping of homologous, repeated and amplified DNA sequences by DNA renaturation in agarose gels. Nucleic Acids Res. 11:5413–5431.

31. **Stinchcomb, D. T., M. Thomas, J. Kelley, E. Selker, and R. W. Davis.** 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. Proc. Natl. Acad. Sci. USA 77:4559–4563.

32. **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 replication. Cell 27:155–163.