Isolation of the origin of replication associated with the amplified Chinese hamster dihydrofolate reductase domain

(DNA synthesis/replication initiation/cell synchrony)

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ABSTRACT Autoradiography of restriction digests of DNA labeled in early S phase indicates that replication of the amplified dihydrofolate reductase (DHFR) domain of methotrexate-resistant CHOC 400 cells initiates within a 6.1-kilobase pair (kb) EcoRI-doubtlet located on the 3' side of the DHFR gene. To localize the DHFR origin fragment, synchronized CHOC 400 cells were either pulse labeled with [3H]thymidine in vivo or permeabilized and incubated with [32P]dATP under conditions that support limited chromosomal DNA replication. The temporal order of replication of amplified fragments was determined by hybridization of the in vivo or in vitro replication products to cloned fragments spanning the earliest-replicating portion of the DHFR domain. At the G1/S boundary, the labeled products derived from the replication of amplified sequences, either in whole or permeabilized cells, are distributed about an amplified 4.3-kb Xba I fragment that maps 14 kb downstream from the DHFR gene. As cells progress through the S phase, bidirectional replication away from this site is observed. These studies indicate that the 4.3-kb Xba I fragment contains the origin of replication associated with the amplified DHFR domain.

To isolate DNA sequences that serve as initiation sites in mammalian chromosomes, we have studied the replication timing of restriction endonuclease fragments derived from the amplified dihydrofolate reductase (DHFR) domain of a methotrexate-resistant Chinese hamster ovary (CHO) cell line, CHOC 400 (1). The high copy number and homogeneity of the amplified DHFR domains (2) reduce the relative complexity of cellular replication studies several hundred times, thereby permitting functional delineation of the DHFR origin region in intact chromosomes prior to physical and biological characterization of isolated origin sequences.

Pulse-labeling of synchronized cells has shown that replication of the amplified DHFR domains is initiated within a specific group of restriction fragments (3). These early-labeled fragments (ELFs) have been isolated by molecular cloning (4) and located by restriction mapping to a single contiguous region downstream from the DHFR gene (5). We demonstrated that replication intermediates formed during initiation of DNA synthesis in CHOC 400 cells are enriched for sequences derived from one amplified restriction fragment (6). These studies suggest that replication of each repeated DHFR domain is initiated from a single origin of replication located 10–30 kilobase pairs (kb) distal to the DHFR gene. Here we describe pulse-labeling experiments in whole and permeabilized cells that locate the origin of replication associated with the amplified DHFR domain to a 4.3-kb Xba I restriction fragment that maps 14 kb downstream from the DHFR gene.

MATERIALS AND METHODS

Cell Culture and Synchrony. CHOC 400 cells were propagated in Eagle’s minimum essential medium (3). For synchrony, cells were first arrested in the G1 period by incubation in isoleucine-deficient medium (7) and were then collected at the G1/S boundary by incubation for 12 hr in complete medium containing either aphidicolin at 5 μg/ml (National Cancer Institute) or 10 μM fluorodeoxyuridine (FdUrd) (Sigma). The efficacy of synchronization regimens was determined by fluorescent-activated cell sorting of propidium iodide-stained nuclei (8).

Pulse Labeling, DNA Preparation, and Fluorography. Cultures collected at the G1/S boundary with aphidicolin or FdUrd were released into the S period by removing the inhibitor, washing the cultures with fresh medium, and reincubation at 37°C. At selected intervals, cells were labeled with medium containing [3H]thymidine at 10–20 μCi/ml (ICN; 62 Ci/mmol; 1 Ci = 37 GBq) or [methyl-3H]thymidine at 1 μCi/ml (50–60 mCi/mmol, Amersham). DNA was prepared from lysed cells, digested with restriction enzymes, and blotted as described (4). DNA concentrations were determined fluorometrically (9). For fluorography, gels were impregnated with 5% (wt/vol) 2,5-diphenyloxazole (Sigma) in ethanol, rinsed in H2O, dried, and exposed to preflashed x-ray film (XAR-5, Kodak) with an intensifying screen at –70°C for 3 wk. Digests labeled with [32P]thymidine were separated by agarose gel electrophoresis, transferred to nitrocellulose, and exposed to x-ray film for 20–30 days.

Restriction Mapping and Subcloning. Recombinant cosmids S13, S14, and S21 were mapped by agarose gel analysis of restriction digests performed separately or in combination. For subcloning, Xba I fragments from cosmids S13, S14, and S21 were purified from agarose gels by the method of Benson (10), and subcloned into pUC12 or pAN3 by standard ligation and transformation techniques (11). Plasmids were propagated in Escherichia coli HB101.

Large-scale preparations of plasmid DNA were obtained by alkaline lysis and equilibrium density sedimentation in CsCl2 (11).

Preparation of Dot Blots. Plasmid DNA to be bound to dot blots was incubated in 0.1 M NaOH at 37°C for 12 hr, diluted 1:2 (vol/vol) in 20× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0), and 2-μg aliquots of each plasmid were filtered through nitrocellulose sheets that had been preequilibrated with 10× SSC. The filters were air dried, rinsed in distilled water, and baked in vacuo at 80°C for 2 hr. Triplicate samples of each plasmid were applied. Dot blots were prehybridized for at least 6 hr at 42°C in hybridization buffer [5× SSC/5× Denhardt’s/20 mM NaH2PO4, pH 6.5/50% (vol/vol) deionized formamide, sonicated herring

Abbreviations: DHFR, dihydrofolate reductase; CHO, Chinese hamster ovary; ELFs, early-labeled fragments; kb, kilobase pair(s); FdUrd, fluorodeoxyuridine; Ara-Cyt, cytosine arabinonucleoside.

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sperm DNA at 100 μg/ml, 10% (wt/vol) dextran sulfate] as described (12).

Hybridization of Labeled CHOC 400 DNA to Dot Blots. In most experiments, labeled DNA was enriched 50-fold for replication intermediates by chromatography on benzoylated, naphthoylated DEAE-cellulose as described (6). Labeled DNA (0.8–2.0 x 10^6 cpm per blot) was reduced to a modal size of 0.5 kb by sonication, heated to 100°C for 5 min, and mixed with 2 ml of hybridization buffer; hybridization proceeded at 42°C for 36 hr. The filters were rinsed at room temperature in 0.2 x SSC/0.1% NaDodSO4, washed in several changes of the same buffer at 60°C for 2 hr, rinsed 5 x in fresh 0.2 x SSC/0.1% NaDodSO4, and dried. Each dot was then excised, and the amount of bound radioactivity was quantitated by liquid scintillation counting. As a control for repetitive DNA, plasmids containing AluI family repetitive sequences were included on each blot. The average amount of radioactivity hybridized to immobilized vector sequences was used as a measure of nonspecific hybridization and was subtracted as background from each experimental value. The amount of radioactivity specifically hybridized to each fragment was then normalized for insert length.

Replication Assays in Permeabilized Cells. For in vitro replication assays, synchronized cells were washed in ice-cold medium without serum, scraped from the plates, and collected by centrifugation. The cell pellet was resuspended in 1.1 vol of ice-cold 2 x replication buffer [1 x replication buffer = 50 mM Hepes, pH 7.8/100 μg of heat-inactivated, nuclease-free bovine serum albumin/1 mM dithiothreitol/0.1 mM each dGTP, dCTP, and dTTP/0.2 mM each CTP, GTP, and UTP/4 mM ATP/15% (vol/vol) glycerol; 10 mM MgCl2]. A quantity of [3H]dATP (3000 Ci/mm, 10 mCi/ml, ICN) equal to 0.1 vol of the cell pellet was added, and the reaction mix was made 0.4% Nonidet P-40 (Sigma). After incubation at 37°C for 15–30 min, the reaction was chased by the addition of 0.01 vol of 10 mM unlabeled dATP and reincubation for 30 min.

The replication reaction was terminated by the addition of an equal volume of lysis buffer [1.2% (wt/vol) NaDodSO4/200 mM NaCl/40 mM EDTA/100 mM Tris-HCl, pH 7.9]. and DNA was prepared and quantitated as before. For hybridization, the reaction products were sonicated, denatured, and hybridized to Southern blots for 16–24 hr at 42°C as described (12). Blots were rinsed in 2 x SSC/0.1% NaDodSO4 at room temperature, washed in several changes of 0.1 x SSC/0.1% NaDodSO4 for 2 hr at 60°C, and air dried. Blots were exposed to XAR-5 film (Kodak) for 24–48 hr at -70°C with intensifying screens.

Densitometry. Autoradiographic signal intensities were quantitated with a computer-based densitometer as described (13). Estimations of peak areas within the linear response range of both the film and the densitometer were determined using the program GELSCAN 5.0.

RESULTS

Organization of the Amplified DHFR Domain. Overlapping recombinant cosmids representing the entire 150- to 175-kb amplified DHFR domain have been isolated (2). Depicted in Fig. 1A are several recombinant cosmids clones that span 70 kb of the earliest-replicating portion of the amplified domain, including the entire 25-kb DHFR gene (14, 15). In earlier pulse-labeling studies, the synthesis of 5 or 6 amplified EcoRI ELFs was detected within the first 20 min of the CHOC 400 S phase (3). Two of the more prominent ELFs, denoted EcoRI ELF-C and ELF-F, were located by cloning and mapping experiments to a single region on the 3' side of the DHFR gene (4, 5). Using standard methods for "chromosomal walking" and restriction mapping, we have verified that ELF-C represents a single amplified restriction fragment. EcoRI ELF-F, however, represents two 6.1- to 6.3-kb fragments (herein denoted as ELF-F and ELF-F') located side by side in the Chinese hamster genome (Fig. 1A). To distinguish between ELF-F and ELF-F' and to facilitate

![Fig. 1.](image-url)
Fig. 2. Replication of the EcoRI ELF-F doublet precedes replication of ELF-C. (A) Fluorographic analysis of DNA synthesis during the onset of the S period. CHOC 400 cells synchronized with aphidicolin were pulse labeled with [3H]thymidine for the indicated period (in min) after release into the S phase. EcoRI digests of the labeled DNA were separated by agarose gel electrophoresis. The gel was processed for fluorography, dried, and exposed to x-ray film for 20 days. The size of bands derived from the synthesis of mitochondrial DNA (mt) is indicated in kb. The positions of EcoRI ELF-F and ELF-C are denoted by F and C, respectively. (B) Autoradiographic analysis of replication in cells entering the S period in the presence of a chain terminator. CHOC 400 cells were synchronized with aphidicolin prior to release into the S phase for the indicated period (in min) in [3H]thymidine with or without Ara-Cyt (5 μg/ml). EcoRI digests of labeled DNA were separated by agarose gel electrophoresis prior to blotting to nitrocellulose paper. The blot was exposed to x-ray film for 30 days.

Detailed replication studies, we have subcloned Xba I fragments representing 31 kb of the earliest replicating portion of the amplified domain into several plasmid vectors (Fig. 1B).

Replication Timing of Restriction Fragments Derived from the Amplified DHFR Domain. To determine the order in which sequences from the amplified region are synthesized, we have examined the labeling pattern of amplified restriction fragments in early S phase. Using fluorography and brief pulse-labeling intervals, we have detected those amplified fragments synthesized within the first 5–10 min of the S period (Fig. 2). In cells synchronized with aphidicolin, very low levels of replication are detected within the first 5 min of release into the S phase, and synthesis of specific amplified fragments is not observed (Fig. 2A, lane 1). By 10 min after aphidicolin release, the replication of several bands is readily apparent (Fig. 2A, lane 2), the most prominent of which are derived from mitochondrial DNA (16). During this interval, synthesis of amplified fragments is largely limited to the 6.1-kb EcoRI bands ELF-F and ELF-F'. With longer labeling intervals, or by labeling at different times during the S period, the synthesis of additional amplified fragments, including ELF-C, is detected (Fig. 2A, lanes 3 and 4). Densitometric analysis of experiments of this type, corrected for fragment copy number and size, indicates that synthesis of the ELF-F doublet precedes the synthesis of ELF-C (data not shown).

Replication of the ELF-F doublet prior to ELF-C is also observed when cells are released into the S period in high concentrations of the chain terminator, cytosine arabinonucleoside (Ara-Cyt) (Fig. 2B). Since Ara-Cyt reduces the rate of chain elongation in mammalian cells without interfering with either RNA or protein synthesis (17, 18), chromosomal DNA synthesis in cells entering the S period in the presence of the drug is limited to those regions including initiation sites (6). Synthesis of the ELF-F doublet prior to ELF-C in the presence of Ara-Cyt also suggests that the origin of replication associated with amplified domains is located within or near the EcoRI ELF-F doublet.

Hybridization of Replication Intermediates to Dot Blots. To determine which fragment from the ELF-F region is replicated first, synchronized CHOC 400 cells were pulse labeled with [3H]thymidine at various intervals during the S phase, and the labeled replication products were hybridized to replicate dot blots of recombinant plasmids representing 31 kb of DNA from the 3' early-replicating region (Fig. 1B). To achieve maximal rates of incorporation of labeled thymidine during the pulse, cells were collected at the G1/S boundary withFdUrd rather than aphidicolin (19). In most experiments, the unlabeled genomic template sequences that compete against the fixed plasmid sequences for binding of replication intermediates during hybridization were removed by chromatography over benzoylated, naphthoylated DEAE-cellulose. When bulk labeled DNA not purified by chromatography is used in plasmid hybridization experiments, the binding efficiency is less by a factor of 3, but the qualitative nature of the binding histogram is unchanged (data not shown).

Fig. 3 depicts the histograms obtained by hybridizing DNA from cultures pulse labeled with [3H]thymidine during various intervals in the S phase to dot blots of immobilized plasmids. Within the first 5 min following release from the FdUrd blockade, synthesis of sequences homologous to amplified fragments is observed (Fig. 3A). This binding profile shows a somewhat symmetrical distribution of replication intermediates about a 4.3-kb Xba I fragment (S13X-24) that maps 14 kb distal to the 3' end of the DHFR gene. Note that S13X-24 overlaps the 6.3-kb EcoRI fragment ELF-F' that maps immediately to the 5' side of the fragment referred to in earlier reports as ELF-F. The binding of labeled sequences to the 4.3-kb Xba I fragment is nearly twice that observed for Chinese hamster Alu family sequences, a highly repetitive, interspersed DNA sequence in the hamster genome (20).
By 30 min into the S period (Fig. 3B), binding to S13X-24 is reduced, and synthesis of sequences homologous to those fragments surrounding the 4.3-kb Xba I fragment continues to be detected. The hybridization pattern obtained with total DNA isolated from synchronized CHO cells labeled for the first 60 min of the S phase indicates that uniform synthesis of the entire early-replicating region has occurred by this time (Fig. 3C). The binding data suggest that replication of the amplified DHFR domain is initiated at a site within or near S13X-24 and that synthesis proceeds in a bidirectional fashion away from this site as cells progress through the S phase.

**Temporal Specificity of DNA Replication in Permeabilized Cells.** In an effort to confirm our in vivo findings, we examined the order of synthesis of amplified restriction fragments in a modified permeabilized cell replication assay (21) using labeled precursors of high specific activity. The replication timing of amplified sequences was examined by hybridizing DNA synthesized in permeabilized cells, synchronized at various points in the S phase, to Southern blots of the recombinant cosmid clones that span the 70 kb of the amplified domain depicted in Fig. 1. To distinguish products synthesized by DNA polymerase α from those synthesized by other polymerases, hybridization experiments were conducted with replication products synthesized in both the presence and absence of aphidicolin. As shown in Fig. 4B, little product homologous to the amplified DHFR sequences is synthesized in vitro in the presence of aphidicolin (20 μg/ml), a result consistent with earlier studies that demonstrated that replication of the amplified domain in intact cells is sensitive to this drug. In the absence of aphidicolin, labeled products synthesized in permeabilized G1/S cells hybridize to several fragments derived from the amplified domain (Fig. 4C). Quantitative densitometric analysis of this hybridization pattern and display of the relative densities as a function of the restriction map (Fig. 5) show that the G1/S autoradiographic signal is symmetrically distributed about the same 4.3-kb Xba I fragment identified as the earliest-replicating fragment in the plasmid hybridization studies. In cells released into the S period for 30 min prior to permeabilization, labeled products homologous to fragments extending 30 kb in either direction from the 4.3-kb Xba I fragment are synthesized (Fig. 4D), resulting in a nearly uniform distribution of signal (Fig. 4B). At 3 hr into the S phase, replication of these sequences is still observed (Fig. 4E), producing an autoradiographic pattern similar to, but fainter than, that obtained at 30 min after release into the S phase. Thus, these in vitro experiments confirm our in vivo studies and indicate that subclone S13X-24, which maps 14 kb downstream from the...
DHFR gene, contains the origin of replication associated with CHO 400 amplified domain.

**DISCUSSION**

Using pulse-labeling and hybridization techniques, we have examined the replication timing of restriction fragments derived from the amplified DHFR domain of CHO 400 cells. Analysis of replication products from either intact or permeabilized cells indicates that replication is initiated within (or very near) a 4.3-kb Xba I fragment (S13X-24) that maps 14 kb downstream from the last exon of the DHFR gene. Replication proceeds in a bidirectional fashion away from this site as cells progress through the S phase. Although exact localization of the origin sequence within S13X-24 will require Okazaki fragment mapping experiments similar to those reported for simian virus 40 (22) and λ phage DNA (23), our present evidence strongly supports the notion that synthesis of this chromosomal replication is initiated at a precise site and/or nucleotide sequence.

The synthesis of this chromosomal region is sensitive to aphidicolin, both in intact and permeabilized cells, suggesting that replication of the amplified domain is accomplished by the major chromosomal replicative polymerase, DNA polymerase α (24). The replicative capacity of permeabilized cells, at any point in the S period, appears quite limited, with synthesis likely limited to extending preexisting replication forks no more than several thousand nucleotides (N.H.H., unpublished data). The hybridization pattern obtained from G1/S incubations indicates that replication forks may be distributed in a nearly symmetrical fashion about the origin in G1/S cells prior to permeabilization (see Fig. 5). Thus, initiation of DNA synthesis likely occurs in the presence of aphidicolin, which greatly impedes, but does not abolish, movement away from the origin. Interestingly, a small amount of product synthesized in vitro in G1/S cells in the presence of aphidicolin is homologous to the 4.3-kb Xba I fragment (Fig. 4B). Inasmuch as simian virus 40 DNA synthesis is limited to the immediate origin region by aphidicolin (R. S. Decker and M. DePamphilis, personal communication), these minor products may represent an aphidicolin-insensitive step in the initiation process, perhaps due to primase activity not suppressed by the drug (25). Thus, it may be possible to dissect the initiation process in permeabilized cells.

The amplified DHFR domains of CHO 400 cells are stable chromosomal domains that likely respond to the same trans-acting cellular signals as do other replicons that are coordinately activated immediately upon entry into the S phase. As we have discussed in earlier reports, continued replication of amplified sequences throughout the first few hours of the S phase is likely due to both decay of synchrony within the cell population and failure of all the DHFR origins to be activated in concert (3, 6). Although the S13X-24 origin fragment does not hybrdize AluI family repeats (unpublished observations), this fragment does contain repetitive elements that are interspersed throughout the Chinese hamster genome. Preliminary experiments in CHO cells suggest that these repetitive elements are located in additional early-replicating replicons distinct from that containing the DHFR gene (N.H.H., unpublished observations). Thus, the DHFR origin may represent a prototypical, dispersed Chinese hamster chromosomal origin of replication.

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