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THE REPLICATION TIMING OF THE AMPLIFIED DIHYDROFOLATE REDUCTASE GENES IN THE CHINESE HAMSTER OVARY CELL LINE CHOC 400

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We have examined the timing of replication of the amplified dihydrofolate reductase genes in the methotrexate-resistant Chinese hamster ovary cell line CHOC 400 using two synchronization procedures. DNA replicated in the presence of 5-bromodeoxyuridine was collected from cells of various times during the DNA synthesis phase and the extent of replication for defined sequences was determined by Southern blotting analysis of CsCl density gradient fractions. We report that under these conditions the DHFR gene replicates throughout the course of S phase in a mode similar to the bulk of the replicated genomic DNA. This contrasts with previous data that shows the non-amplified DHFR gene replicates during the first quarter of S phase. Therefore, we conclude that gene amplification alters the replication timing of the DHFR gene in CHOC 400 cells.

In eucaryotes DNA replication results from the activation of multiple initiation sites along the chromosomes (1). Examination of the replication timing of specific DNA sequences suggests that chromosomal segments are synthesized at defined times throughout the DNA synthesis (S) phase (reviewed in ref. 2). It has been demonstrated, by a number of groups, that the single copy Chinese hamster dihydrofolate reductase (DHFR) gene replicates during the first quarter of S phase (3,4, and refs. therein). We were interested in determining if the time of replication for the DHFR gene is altered in the methotrexate-resistant cell line CHOC 400, which contains about 1000 copies of the DHFR gene per cell (5). Several studies have defined a region located approximately 14 kilobases (kb) downstream from the DHFR gene as an origin of DNA replication (6, and refs. therein). In this study we used a probe from the origin region to define the replication timing of the DHFR gene in CHOC 400 cells; thus, establishing the activation profile of the DHFR origin region throughout S phase.

METHODS

Cell Culture, Synchronization, and BUdr Labelling - CHOC 400 cells were propagated in Eagle mimimum essential medium containing 5% donor bovine serum and 5% fetal bovine serum (GIBCO Laboratories). For synchrony, cells were first

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arrested in the G_1 period by incubation in isoleucine-deficient medium for 48 hrs, and were then incubated in complete medium containing 10 ug/ml of aphidicolin (Sigma) (7). After 12 hrs the cultures are poised at the G_1/S boundary. Cells were released into S phase by removing the inhibitor, washing with medium, and reincubation in complete medium and 50 um BUdr.

DNA Preparation, Restriction Endonuclease Digestion, and Isopycnic Centrifugation Total Genomic DNA was purified from CHOC 400 cells as previously described (7), and then digested with EcoRI under conditions suggested by the supplier (BRL). DNA concentrations were quantitated fluorometrically by the method of Labarca & Paigen (8). Between 25 and 35 ugs of genomic DNA was resolved on isopycnic CsCl gradients as described by Epner et al. (9). DNA was centrifuged in 15 mM NaCl-15 mM disodium EDTA (pH 7.5) 5.9 M CsCl (refractive index, 1.402) at 39,000 RPM for 60 to 72 hrs in a Beckman 50 Ti rotor at 20°C. Gradients were fractionated into 20 600 ul fractions. Fractions were diluted 1:3 with water and precipitated twice with NaCl and ethanol to remove the CsCl. Unsubstituted DNA (LL) sedimentated with a density of 1.68 to 1.70 g/ml; BUdr substituted DNA (HL) sedimentated with a density of 1.71 to 1.73 g/ml.

Gel Electrophoresis, Southern Blotting and Hybridization - Gradient fractions were resolved on 1% agarose gels in TAE buffer (40 mM Tris-acetate, pH 8.0; 1 mM EDTA), stained with ethidium bromide, and photographed. Southern transfer of DNA to nitrocellulose (Schleicher & Schuell) and hybridization procedures were as previously described (7). Plasmids pMCB, pSvMYC-1, and the cosmid C-5 were labeled with [^{32}P]dATP by random oligonucleotide synthesis (10) by the Klenow fragment of Polymerase I.

Densitometry - Densitometric analyses were performed with a Microscan 1000 scanning densitometer (TRI Inc.).

RESULTS

CHOC 400 cells were collected in G_1 with isoleucine-deficient medium, and synchronized at the G_1/S border with aphidicolin. At timepoints throughout S-phase DNA was isolated, digested with EcoRI, and replicated DNA (HL) containing BUdr was separated from non-replicated DNA (LL) by isopycnic centrifugation. Resolution of DNA from gradient fractions on agarose gels and Southern analysis permits the accurate assessment of the amount of any restriction fragment in unreplicated and replicated DNA.

Ethidium bromide stained gels of BUdr-substituted DNA from cells released into S phase from 0.5 to 8 hours is shown in figure 1A. Densitometry of photographic negatives of these gels reveals that less than 5% of the genomic DNA has replicated within the first 30 minutes of entry into S phase, by 10 hours 90% of the DNA has replicated (figure 3). S phase in CHOC 400 cells is approximately 8 to 11 hours long (7); thus, the remaining 10% of non-substituted DNA after 10 hours may represent replicons that have not yet been activated or is DNA from dead cells that did not survive the synchrony regime.

To compare the replication timing of the bulk chromosomal DNA to the replication timing of the DHFR gene, the gels in figure 1 and others from time points not shown were transferred to nitrocellulose and probed with a DHFR specific probe. The probe, plasmid pMCB, contains a 1.6 kb BamHI-HindIII fragment derived from the 6 kb early-labeled EcoRI fragment F' (ELF-F')(11). Figure 2A is a compilation of autoradiographs obtained by hybridization of pMCB

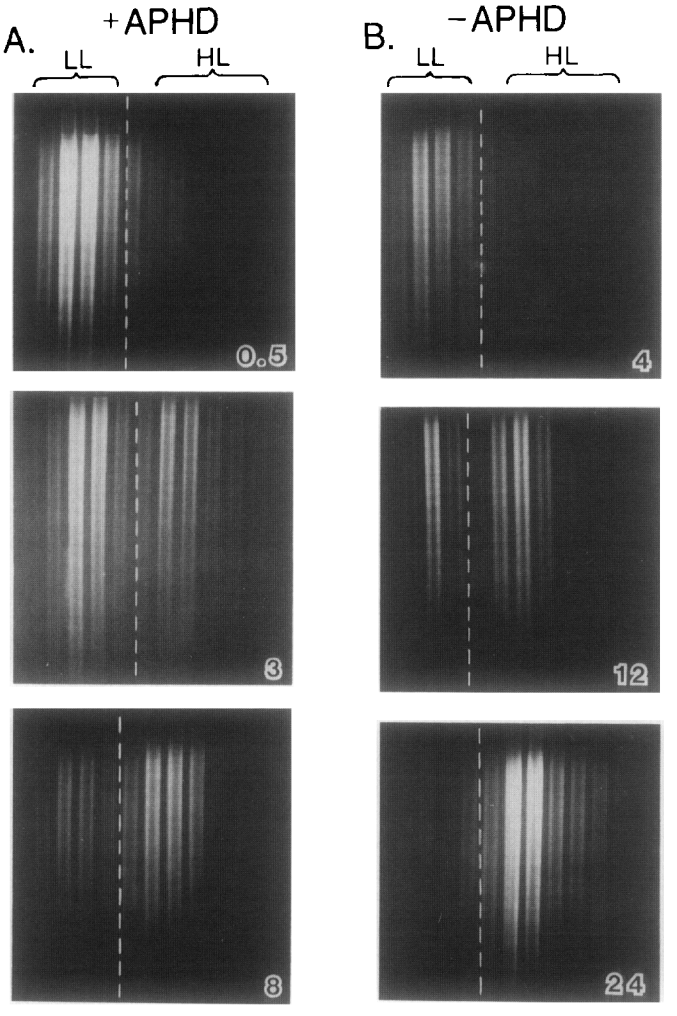


Fig. 1. Ethidium bromide stained gels of DNA from CHOC 400 cells synchronized with aphidicolin (APHD)(A) or (B) without aphidicolin. Cells were incubated in medium containing 50um BUdr after synchronization. DNA was isolated, digested with EcoRI, and resolved by isopycnic centrifugation in CsCl. Gels were processed as described in Materials and Methods. (LL); light-light unreplicated DNA. (HL); heavy-light replicated DNA.

to ELF-F' as a function of time in S; the probe does not hybridize to any other genomic fragment (data not shown). Densitometry of the autoradiographs reveals that about 5% of the DHFR origins have been activated after a 1 hour release from the aphidicolin block. After 4 hours only 50% of the DHFR origins have been replicated. Figure 3 illustrates the percentage of bulk DNA and DHFR genes replicated throughout S phase after release from the G_1/S block. From the graph, it can be inferred that replication of the DHFR genes follows the replication timing pattern of the bulk chromosomal DNA.

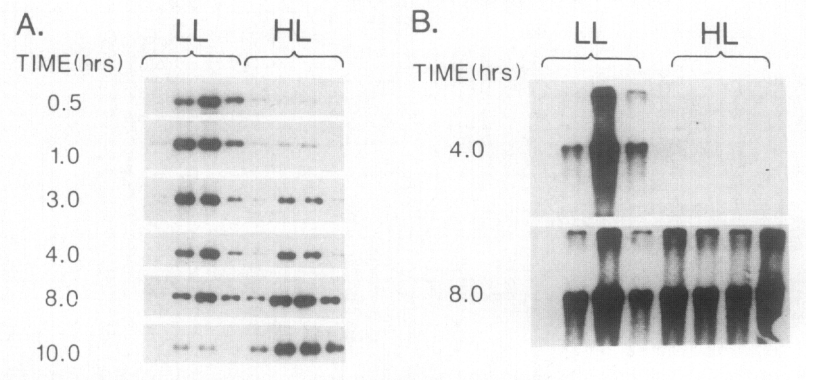


Fig. 2. The Gels from figure 1A, and also the gels for the 1, 4, and 10 hr timepoints (not shown) were transferred to nitrocellulose and probed with plasmid pMCB (A). Panel B shows the autoradiographs from reprobing the 4 and 8 hr blots with the centromeric probe C-5.

Next, we probed the blots from this experiment with sequences that replicate in either early or late S phase. Accordingly, as a late marker, we used a centromeric clone, C-5, which had been previously determined by cytological studies to be late replicating (N.H. Heintz, unpublished data). After 4 hours (figure 1B), in which nearly 50% of the cellular DNA and DHFR genes have replicated (figure 3), the centromeric fragments recognized by C-5 have scarcely begun to replicate. After 8 hours, when 75% of the genomic DNA and DHFR genes have replicated (figure 3), only 50% of the C-5 domain has replicated. Similarly, hybridization of these blots with a c-myc probe suggests that c-myc

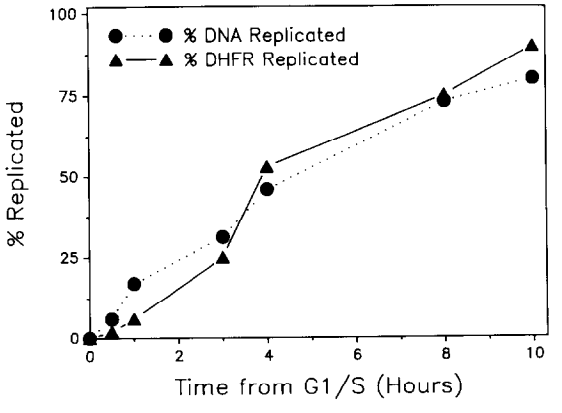
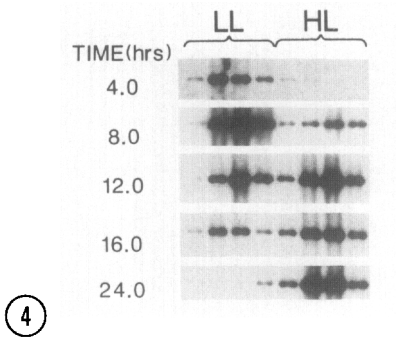


Fig. 3. Quantitation of the amount of bulk DNA and DHFR genes replicated throughout S phase after release from a G_1/S block induced by aphidicolin. The amount of bulk DNA that had replicated was calculated by densitometry of photographic negatives from figure 1A (1, 4, and 10 hr gels not shown). The portion of DHFR genes that had replicated was determined by densitometry of the autoradiographs shown in figure 2A.



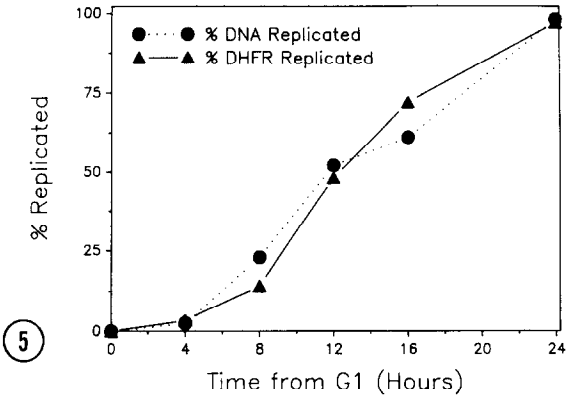


Fig. 4. The gels from figure 1B, and also gels for the 8 and 16 hr timepoints (not shown) were transferred to nitrocellulose and probed with plasmid pMCB as before. The early (c-myc) and late (C-5) S phase controls are not shown.

Fig. 5. The percentage of bulk DNA and DHFR genes replicated throughout S phase after release from a G_1 block. The amount of chromosomal DNA replicated was calculated by densitometry of photographic negatives from figure 1B (8 and 16 hr gels not shown). The portion of DHFR genes that had replicated was determined by densitometry of the autoradiographs shown in figure 4 as before.

replicates during the first half of S-phase (data not shown) as previously reported (12) . Hence, we can conclude that this technique is sufficiently sensitive to accurately delimit the replication timing of chromosomal domains into discrete intervals of S phase.

We were concerned that replication timing of the CHOC 400 cells was being altered by aphidicolin treatment during synchronization. This drug inhibits DNA synthesis by binding reversibly to DNA polymerase-α (13). Residual aphidicolin could delay DNA synthesis, or exposure to aphidicolin may damage the full activity of the polymerase after removal of the drug. Therefore, the previous experiment was repeated without the aphidicolin treatment. CHOC 400 cells were blocked in G_1 with isoleucine-free medium, and were then induced to re-enter the cell cycle by the addition of complete medium with 50 um BUdr. Cells treated in this manner are poised at different stages in early G_1; when they re-enter the cell cycle it takes the population 4 to 12 hours to begin S phase. Figure 1B shows the ethidium bromide stained gels of DNA collected from cells released from the G_1 block between 4 and 24 hours, digested with EcoRI, and resolved on cesium chloride gradients as before. At the fourth hour less than 3% of the DNA has shifted to heavy-light density; indicating that the majority of cells are still traversing the G_1 phase. By the sixteenth hour 75% of the DNA has shifted to heavy-light density (figure 5), and by hour 24 the cells have fully replicated their DNA. These gels were transferred to nitrocellulose and probed with pMCB (figure 4). The percentage of chromosomal DNA and DHFR genes replicated is plotted against time from G_1 in figure 5. As was observed in the previous

experiment using aphidicolin, the DHFR genes replicated with the same kinetics as bulk DNA. Both C-5 and c-myc show results identical to the previous experiment (not shown). Therefore, the cell synchrony regime with aphidicolin does not substantially alter the replication timing of CHOC 400 cells or the DHFR gene.

DISCUSSION

The DHFR gene in Chinese hamster cells replicates during the first quarter of S phase (3,4). We have found that in the Chinese hamster cell line, CHOC 400, the amplified DHFR genes replicate throughout S phase. There are several possible explanations for this alteration in timing. First, initiation factors required for the activation of the DHFR origin may be limiting. Second, the increased copy number of the DHFR gene may result in an altered chromatin structure that may limit the access of replication factors to DHFR initiation sites. In either instance, only a subset of replicons are able to be activated at any one time. The ability to quantitate the portion of DHFR origins activated in early S should permit us to search for growth factors or other mitogenic agents that increase the rate of origin activation, presumably by influencing the concentration of initiation factors available at the onset of S phase.

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