# Replication Intermediates Formed during Initiation of DNA Synthesis in Methotrexate-Resistant CHOC 400 Cells Are Enriched for Sequences Derived from a Specific, Amplified Restriction Fragment†

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ABSTRACT: 1-β-D-Arabinofuranosylcytosine (ara-C) inhibits nuclear DNA replication in Chinese hamster ovary cells by an efficient chain termination mechanism without affecting the rate at which cells traverse G1 and enter S [Heintz, N. H., & Hamlin, J. L. (1983) *Biochemistry 22*, 3557–3562]. Here we have employed ara-C to enrich for replication intermediates formed during initiation of DNA synthesis in synchronized CHOC 400 cells, a methotrexate-resistant derivative of Chinese hamster ovary cells that contains approximately 1000 copies of an early replicating 150-kb chromosomal domain. This highly amplified domain includes the gene for dihydrofolate reductase (DHFR). CHOC 400 cells were collected at the G1/S boundary of the cell cycle with aphidicolin prior to release into S in the presence of both [*methyl*-$^3$H]thymidine and various concentrations of ara-C. Chromatographic fractionation of restriction endonuclease digests over benzoylated naphthoylated DEAE-cellulose (BND-cellulose) showed that high concentrations of ara-C inhibited the maturation of chromosomal replication intermediates containing ssDNA (replication forks) into dsDNA for up to 60 min. The effect of ara-C on the sequence complexity of replication intermediates formed during early S phase was determined by hybridizing purified intermediates labeled with $^{32}$P in vitro to Southern blots of genomic DNA derived from both methotrexate-sensitive and methotrexate-resistant Chinese hamster ovary cells. In the absence of ara-C, $^{32}$P-labeled ssDNA BND-cellulose fractions from cultures released into S for 30–60 min hybridized to a spectrum of restriction fragments encompassing 40–50 kb of the amplified DHFR domain. In contrast, high concentrations of ara-C limited hybridization within the amplified domains to a single 4.3-kb *Xba*I fragment. These data indicate that progression of replication forks away from chromosomal initiation sites can be prevented in a dose-dependent fashion by ara-C and suggest that replication of each amplified DHFR domain is initiated from sequences contained within a single amplified restriction fragment.

Our laboratory is interested in the control of initiation of chromosomal DNA synthesis in mammalian cells. As a model system, we utilize methotrexate-resistant CHOC 400 cells that contain approximately 1000 copies of a 150-kb early-replicating domain that includes the gene for dihydrofolate reductase (DHFR)[1] (Milbrandt et al., 1981). Autoradiographic analysis of restriction endonuclease digests of DNA labeled with [*methyl*-$^{14}$C]thymidine during the onset of S in synchronized CHOC 400 cells has demonstrated that replication of the amplified domains initiates within a defined subset of amplified restriction fragments (Heintz & Hamlin, 1982). Molecular cloning and restriction mapping have localized these fragments to a single locus spanning approximately 30 kb of DNA (Heintz et al., 1983) distal to the 3′-end of the DHFR gene (Hamlin et al., 1983). These studies strongly support the notion that each amplified DHFR domain contains a single origin of replication located downstream from the 3′-end of the DHFR gene.

In an effort to precisely localize the origin of replication associated with the amplified DHFR domains, we have attempted to isolate nascent DNA synthesized during the onset of S in synchronized CHOC 400 cells and to map the position of these sequences by hybridization to DNA derived from the amplified DHFR domains. This approach requires that a large proportion of the synchronized cell population enter S during a very short period of time. Autoradiographic analysis of nuclei from CHOC 400 cultures released into S phase from an aphidicolin-induced G1/S block has shown that only a portion of the population (15–20%) enters S immediately upon removal on the inhibitor. Nearly 30–40 min is required for the remainder of the population to initiate DNA synthesis (Heintz & Hamlin, 1982). Although cultures synchronized in this manner allowed the identification of a group of amplified restriction fragments that replicate in early S, the variable rates with which cells enter S after release from the aphidicolin block limit their usefulness as a source of nascent DNA for fine mapping of initiation sites. We therefore have sought alternative methods for limiting DNA synthesis during the onset of S to only those regions including and surrounding origins of replication.

In bacteria, the chain terminating properties of 1-β-D-arabinofuranosylcytosine (ara-C) have been used to prolong the half-life of replication intermediates formed during initiation of DNA synthesis, permitting Okazaki and colleagues to map the exact initiation site within the *Escherichia coli* chromosomal origin of replication (Hirose et al., 1983).

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[1] Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; DHFR, dihydrofolate reductase; BND-cellulose, benzoylated naphthoylated DEAE-cellulose; CHO, Chinese hamster ovary; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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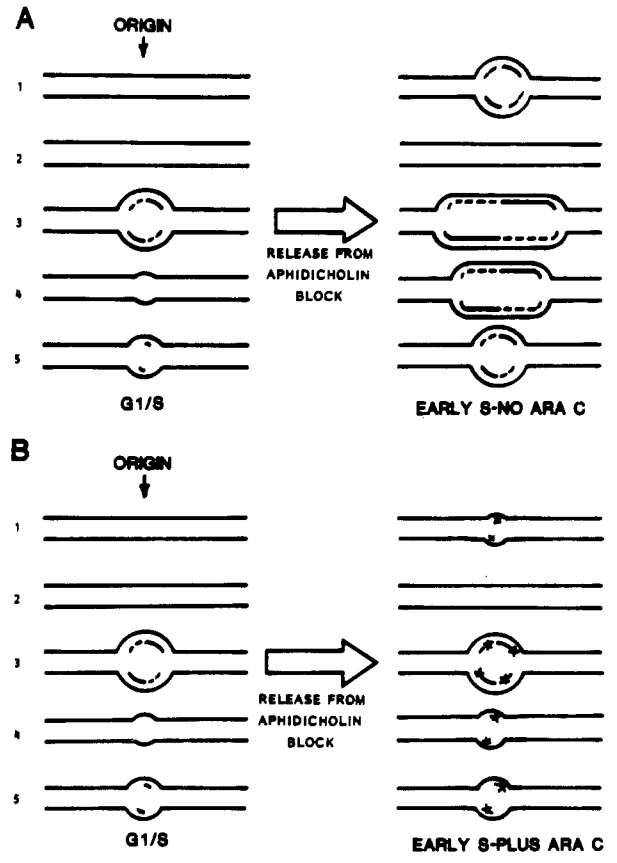


FIGURE 1: Experimental approach for limiting DNA synthesis to initiation regions. Depicted are the regions surrounding the origin of replication in five replicons after collection at the G1/S boundary with aphidicolin. Some replicons have not initiated DNA synthesis (1 and 2) whereas others may have synthesized varying but small amounts of DNA (3–5). After release from the aphidicolin block (panel A), cells enter S parasynchronously over a 45–60-min period, resulting in the replication of variable amounts of DNA in individual replicons at any time after release from aphidicolin block. When cells are released from the aphidicolin block in the presence of high concentrations of ara-C (panel B), the chain terminating action of the drug stalls elongation, permitting the collection of initiation events as cells enter S. Ara-C residues incorporated into DNA are depicted with (×).

Studies in mammalian cells have shown that ara-C reduces the rate of thymidine incorporation into replicating nuclear DNA in a dose-dependent fashion (Graham & Whitmore, 1970; Zahn et al., 1972; Momparler, 1969, 1972; Kufe & Major, 1982) without affecting the proportion of cycling cells that traverse G1 and enter S (Yataganas et al., 1974; Heintz & Hamlin, 1983a). Since the drug appears to act as an efficient but not absolute DNA chain terminator [reviewed by Cozzarelli (1977)], we have investigated the use of ara-C for enriching replication intermediates formed during initiation of DNA synthesis.

Diagrammed in Figure 1 is the rationale for utilizing ara-C to terminate nascent DNA synthesis at sites close to replication origins in mammalian chromosomes. We have previously demonstrated that the drug alters the rate but not the order in which restriction fragments derived from the amplified DHFR domains are replicated (Heintz & Hamlin, 1983b), indicating that the sites normally utilized for initiation of replication are used in the presence of the drug. Here we report that high doses of ara-C inhibit the progression of replication forks away from chromosomal initiation sites and present evidence that replication of the amplified DHFR domains of CHOC 400 cells is initiated within a single amplified restriction fragment.

## Materials and Methods

*Cell Culture, Cell Synchronization, and Pulse Labeling.* CHOC 400 cells were propagated in Eagle's minimum essential medium (EMEM) supplemented with 10% donor calf serum and 50 μg/mL gentamicin sulfate in a water-saturated 95% air/5% $CO_2$ environment. Synchronized cultures were obtained by incubation in EMEM lacking isoleucine supplemented with 10% dialyzed bovine serum for 50 h followed by incubation in complete medium containing 5–30 μg/mL aphidicolin as previously described (Heintz & Hamlin, 1982). Cell culture supplies were obtained from GIBCO. Cell synchrony was evaluated by fluorescent-activated cell sorting of propidium iodide stained cells prepared by the method of Vindelov et al. (1983) on an Ortho Cytofluorograph Model 50 HH cell sorter equipped with a 2150 computer system. Cells synchronized with aphidicolin alone were pulse-labeled during the onset of S with 1 μCi/mL [*methyl*-$^{14}$C]thymidine as previously described (Heintz & Hamlin, 1982).

*DNA Preparation, Restriction Endonuclease Digestion, Gel Electrophoresis, and Southern Blotting.* DNA was prepared from CHO or CHOC 400 cells by rinsing cultures with ice-cold phosphate-buffered saline, pH 7.4, followed by lysis in 0.6% SDS, 100 mM NaCl, 50 mM Tris (pH 7.8), and 20 mM EDTA. After overnight digestion with proteinase K (Boehringer Mannheim), lysates were extracted twice with $H_2O$-saturated phenol, followed by extraction with chloroform–isoamyl alcohol (24:1 v/v) prior to precipitation of the aqueous phase with ethanol. The DNA precipitate was collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 10 mM Tris–1 mM EDTA, pH 8.0 (TE buffer). Aliquots of DNA samples were digested with restriction endonucleases under the conditions suggested by the supplier. Agarose electrophoresis and Southern transfer of restriction endonuclease digests to nitrocellulose paper (Schleicher & Schuell) were as previously described (Milbrandt et al., 1983). DNA concentrations were determined fluorometrically with the Hoechst 33258 staining method of Labarca & Paigen (1980). The amount of [*methyl*-$^{3}$H]thymidine incorporated into DNA was determined by scintillation counting in ready-Solv EP (Beckman).

*Denaturing Gel Electrophoresis and Fluorography.* Agarose–formaldehyde gels were prepared as described by Maniatis et al. (1982) in 20 mM 3-(*N*-morpholine)propanesulfonic acid (MOPS), pH 7, 5 mM sodium acetate, and 1 mM EDTA (gel running buffer). Twenty-microliter DNA samples in TE were heated to 95 °C for 5 min, quick chilled on ice, mixed with 18 μL of formaldehyde (37% solution), 10 μL of 10× gel running buffer, and 50 μL of deionized formamide (BRL), and reheated to 65 °C for 5 min prior to addition of 10 μL of 50% glycerol containing 0.05% bromophenol blue. After electrophoresis, gels were dehydrated by agitation in 3 volumes of 100% ethanol for 1 h; three to four changes of ethanol were required for complete dehydration. Gels were then agitated for 1 h in ethanol containing 3% 2,5-diphenyloxazole (PPO) followed by precipitation of the PPO by soaking in distilled $H_2O$. Impregnated gels were then dried and exposed for 2–3 weeks to Kodak AR X-OMAT film with a Du Pont Cronex Lightning-Plus intensifying screen. DNA markers were labeled with $^{32}$P by filling in 5′ overhanging ends generated by restriction endonucleases with the large fragment of DNA polymerase I (New England Biolabs) as described by Maniatis et al. (1982).

*Benzoylated Naphthoylated DEAE-cellulose Chromatography.* Benzoylated naphthoylated DEAE-cellulose (BND-cellulose) (Sigma) was washed twice in 50 mM Tris–1 mM

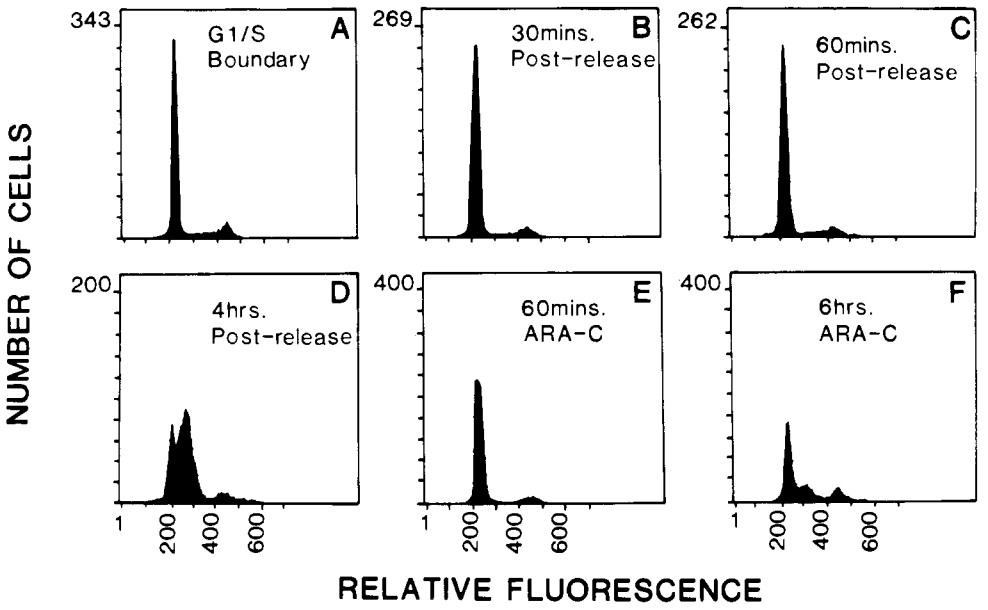


FIGURE 2: Effect of ara-C on the progression of synchronized CHOC 400 cells through S phase. Cell synchrony under various experimental conditions was evaluated by fluorescence-activated cell sorting (FACS) of nuclei stained with propidium iodide. CHOC 400 cells were collected in G1 by incubation in medium lacking isoleucine for 48 h and were then collected at the G1/S boundary by incubation with 5 μg of aphidicolin/mL for 12 (panel A). Cells were released from the G1/S boundary by incubation in complete medium lacking inhibitors, and progress through S was monitored by increased DNA content (panels B–D). Cells released from the G1/S boundary in 5 μg of ara-C/mL traverse S very slowly (panels E and F). Cells in G1 display a median fluorescence of 215 ± 5 relative fluorescence units.

EDTA, pH 8, and 0.3-mL columns were packed by gravity in 1-mL disposable plastic syringes. Approximately 30-μg DNA samples were loaded on the column in either TE or restriction endonuclease buffer, and the applied material was washed 2 mL of column buffer (50 mM Tris, 1 mM EDTA, pH 8) containing 0.3 M NaCl. Up to 50 μg of DNA sample is quantitatively retained under such conditions. Predominantly double-stranded DNA (dsDNA) was eluted in 4-drop fractions (approximately 130–150 μL/fraction) with 2 mL of column buffer containing 1 M NaCl. DNA containing substantial single-stranded regions (ssDNA) was then eluted in 4-drop fractions with 2 mL of column buffer containing 2 M NaCl and 2% caffeine as described by Strauss (1981). DNA fractions were desalted by microspin column chromatography in 1 mL of Sephadex G-50 in TE buffer as described (Maniatis et al., 1982). DNA concentrations of desalted fractions were determined by the method of Labarca & Paigen (1980); incorporated [$^3$H]thymidine was measured by scintillation counting as before. The areas under various column peaks were determined with an Apple II-Plus microcomputer equipped with Optomax image analysis software.

*Labeling of DNA Fractions with $^{32}P$ in Vitro.* Desalted BND-cellulose column fractions were labeled with [α-$^{32}$P]-dCTP (3000 Ci/mM, ICN) in vitro by three different methods. Samples were labeled by nick translation with DNase I (Boehringer Mannheim) and *E. coli* DNA polymerase I (New England Biolabs) as described by Rigby et al. (1977). Samples were also labeled by incubation with the large fragment of *E. coli* DNA polymerase I (New England Biolabs) in 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10 μM β-mercaptoethanol, 50 μg/mL bovine serum albumin, and 25 μM unlabeled dNTPs for 1–3 h at 12 °C. Alternatively, samples were labeled with T4 DNA polymerase (New England Biolabs) by incubation in 33 mM Tris–acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 100 μg/mL bovine serum albumin, and 25 μM unlabeled dNTPs for 2–4 h at 15 °C. Samples were incubated with T4 DNA polymerase for 3–15 min prior to the addition of labeled dCTP and/or the unlabeled dNTPs. Unincorporated radioactivity was removed from the reaction mixture by microcolumn spin chromatography as before. Labeled probes were denatured at 95 °C for 5 min prior to use at (1–5) × $10^6$ dpm/mL in subsequent hybridization experiments. Conditions for hybridizing column fractions labeled with $^{32}$P to Southern blots were as described by Wahl et al. (1979).

*Reagents and Chemicals.* Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs; [*methyl*-$^3$H]thymidine (72 Ci/mM) and [α-$^{32}$P]dCTP (3000 Ci/mM) were from New England Nuclear or ICN. Other reagents were from Sigma.

## RESULTS

*Effect of Ara-C on Entry into and Progression through S.* Synchronization of CHOC 400 cells has been achieved with either hydroxyurea or aphidicolin as the blocking agent (Heintz & Hamlin, 1982; Hamlin et al., 1983). Synchronization with aphidicolin, as evaluated by percent labeled nuclei measurements, resulted in a more uniform traversal of cell populations through S (Heintz & Hamlin, 1982). Thus, in this study, CHOC 400 cells were collected at the G1/S boundary with a combination of isoleucine deprivation and incubation in aphidicolin as before. The position of cell populations in the cell cycle was determined by autoradiography of labeled nuclei and/or by fluorescent-activated cell sorting of nuclei stained with propidium iodide. Both methods gave similar results. Cell sorting profiles indicate that greater than 95% of the population is poised at the G1/S boundary after synchronization with aphidicolin (Figure 2A). After being washed and refed with medium lacking aphidicolin, nearly 90% of the cells enter and progress through S in a parasynchronous wave (Figure 2B–D). Progression of the population away from the G1/S boundary is not detectable by cell sorting until at least 60 min postrelease. If cultures are preequilibrated with 5 μg/mL ara-C for 60 min prior to release and are subsequently permitted to enter S in the same concentration of drug, progression away from the G1/S boundary is severely inhibited (Figure 2E–F).

This progression is a dose-dependent phenomenon that correlates with the rate of thymidine incorporation into nascent

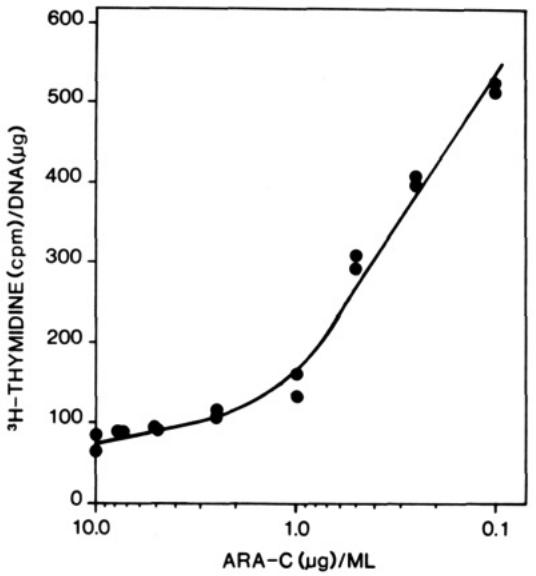


FIGURE 3: Effect of ara-C on [*methyl*-$^3$H]thymidine incorporation during the onset of S. Replica cultures on CHOC 400 cells were collected at the G1/S boundary with aphidicolin and were pre-equilibrated with the indicated concentration of ara-C for 60 min prior to release into S in the presence of the same concentration of drug and 1 μCi/mL [*methyl*-$^3$H]thymidine. After 30 min, the cultures were lysed, DNA was purified, and the amount of labeled thymidine incorporated per microgram of DNA was determined by scintillation counting. DNA labeled for 30 min in the absence of ara-C had an average specific activity of 1950 cpm/μg.

DNA (Figure 3). Incorporation of [*methyl*-$^3$H]thymidine into nascent DNA during the onset of S is inhibited in a dose-dependent fashion by ara-C until concentrations exceeding 2 μg/mL are attained (Figure 3). At higher concentrations, continued low levels of incorporation (3–5% of controls) are minimally inhibited. The cellular component whose synthesis is refractory to the action of the drug at high concentrations (greater than 2.5–5 μg/mL) has been identified as mitochondrial DNA (Heintz & Hamlin, 1983a). Thus, thymidine incorporation curves generated under these conditions represent composite effects on nuclear and mitochondrial DNA synthesis.

*Effect of Ara-C on Synthesis of Replication Intermediates.* To assess the effects of ara-C on the synthesis of replication intermediates formed during the onset of S, DNA labeled with [*methyl*-$^3$H]thymidine in the presence of varying concentrations of ara-C after release from an aphidicolin-induced G1/S block was electrophoresed under denaturing conditions on formaldehyde–agarose gels. Label incorporated into nascent DNA strands was detected by fluorography (Figure 4). In the absence of drug, thymidine incorporation during a 10-min pulse occurs in a uniform spectrum of DNA strands that migrate throughout the entire gel (Figure 4, lane 1). In contrast, in the presence of ara-C incorporation of [*methyl*-$^3$H]thymidine into high molecular weight DNA is almost completely inhibited (Figure 4, lanes 2–4), and labeling is limited to two species less than 600 bases in length. One species (indicated by the bracket, Figure 4) migrates as a faint smear at 200–400 bases; extraction of this DNA followed by labeling with $^{32}$P by oligonucleotide-primed extension in vitro and hybridization to Southern blots of CHO cellular DNAs revealed that this DNA is enriched for sequences derived from the mitochondrial heavy strand origin of replication (N. H. Heintz, unpublished observations). Thus, we assume this short species represents mitochondrial D-loop DNA.

The second species migrates as a compact band of approximately 140 bases (arrow, Figure 4). When DNA of this

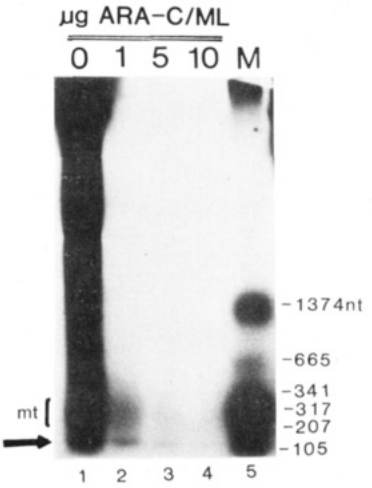


FIGURE 4: Labeling of nascent DNA in the presence of ara-C. DNA labeled during the first 10 min of S phase with 10 μCi/mL [*methyl*-$^3$H]thymidine in the presence of the indicated concentration of ara-C was electrophoresed on a formaldehyde–agarose gel under denaturing conditions, and the gel was processed for fluorography. Incorporated label was detected by autoradiography with X-ray film for 3 weeks. "mt" indicates DNA derived from the mitochondrial D loop; the arrow indicates the position of presumptive Okazaki fragments. The positions of denatured pBR322 marker restriction fragments generated with *Sau*3A and labeled with $^{32}$P (M) are indicated.

mobility is isolated from log phase cultures, labeled with $^{32}$P in vitro, and hybridized to Southern blots of genomic DNA, hybridization to every amplified fragment is observed (not shown). On the basis of the insensitivity of this species to RNase A, its mobility on denaturing gels, and its hybridization characteristics, this DNA likely represents Okazaki fragments synthesized on lagging strands of replication forks during semidiscontinuous replication.

Labeling of both species appears to be sensitive to increasing concentrations of ara-C (Figure 4, lanes 2–4). At doses exceeding 5 μg of ara-C/mL, synthesis of the mitochondrial species is greatly reduced, and synthesis of presumed Okazaki fragments is nearly undetectable. These observation suggest that ara-C inhibits the rate of nuclear DNA synthesis during the onset of S by suppressing production of the normal intermediates of semidiscontinuous replication. Thus, the terminator may indeed impede the movement of replication forks away from initiation sites. Curiously, synthesis of mitochondrial D-loop DNA appears sensitive to ara-C, even though total mitochondrial DNA replication is highly resistant to the drug (Heintz & Hamlin, 1983a).

*Fractionation of Replication Intermediates by Benzoylated Naphthoylated DEAE-cellulose Chromatography.* Although the basis for separation is not well understood, benzoylated naphthoylated DEAE-cellulose differentially retains completely dsDNA fragments and molecules, such as replication forks, that contain substantial ssDNA regions (Sedat et al., 1967). This property has been used to separate dsDNA bacteriophages and animal cell viruses from their replicating forms (Kiger & Sinsheimer, 1967). In order to investigate the effect of ara-C on the maturation of replication forks into completely dsDNA, restriction digests of DNA samples from cultures labeled in the presence and absence of the drug were chromatographed on BND-cellulose. dsDNA was eluted with 1 M NaCl; DNA containing ss regions (henceforth referred to as ssDNA) was eluted with 2 M NaCl–2% caffeine as described (Strauss, 1981). Recovery of DNA from the column approached 90%.

The efficacy of BND-cellulose for separating replication intermediates from bulk cellular DNA was tested by com-

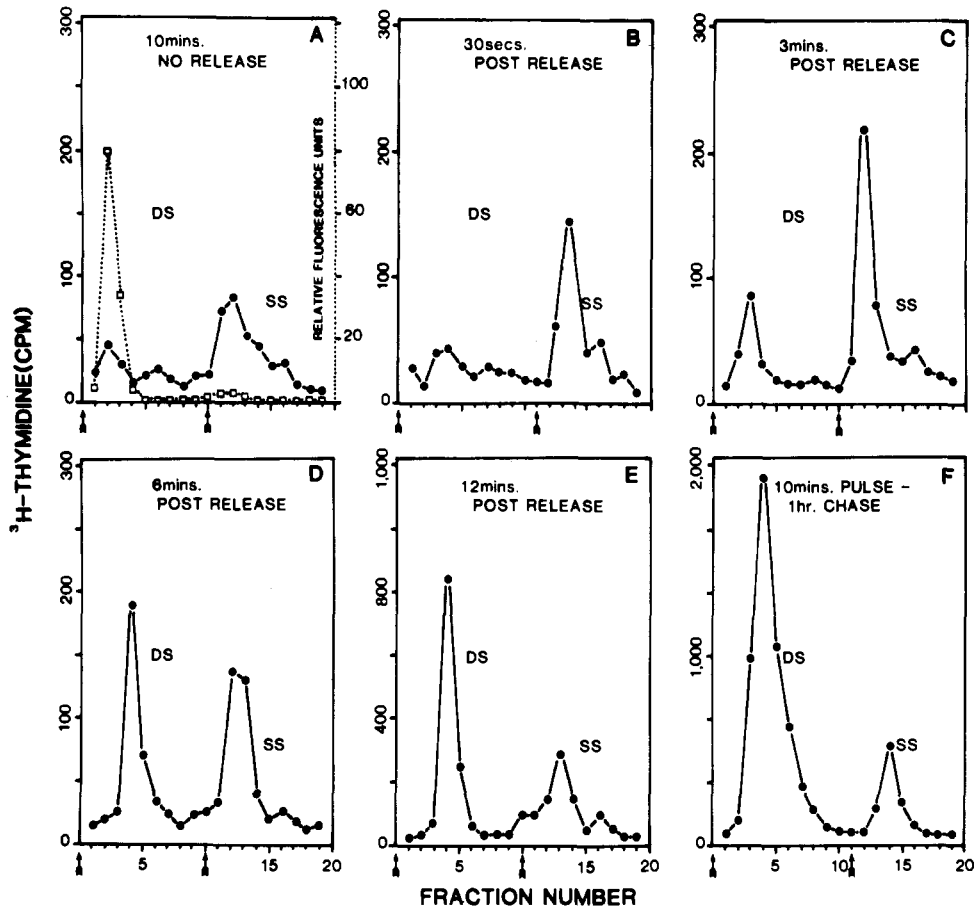


FIGURE 5: Fractionation of replication intermediates by BND-cellulose chromatography. CHOC 400 cells were collected at the G1/S boundary with aphidicolin and were then released into S phase for the indicated period of time by removal of the aphidicolin and incubation in complete medium containing 5 μCi/mL [*methyl*-$^3$H]thymidine. DNA was prepared and digested with *Bam*HI, and 30 μg of each sample was fractionated on BND-cellulose columns. Predominantly double-stranded DNA (ds) was eluted by the application of column buffer containing 1 M NaCl (left-hand arrow); DNA containing substantial single-stranded regions (ss) was eluted by the application of column buffer containing 2 M NaCl–2% caffeine (right-hand arrow). The amount of [*methyl*-$^3$H]thymidine (●) in each fraction was determined by scintillation counting. The amount of DNA (□) eluting in each fraction was determined by the Hoescht 33258 fluorescence staining assay of Labarca & Paigen (1980). The DNA elution profiles for panels B–F were identical with that presented in (A); approximately 1–2% of the applied DNA eluted in the ssDNA fractions regardless of the experimental conditions. As a control for mitochondrial DNA synthesis, one culture was labeled for 10 min in the presence of aphidicolin (panel A). Maturation of replication intermediates containing ss regions into dsDNA was demonstrated by chasing a culture pulse-labeled for 10 min with complete medium lacking exogenous labeled or unlabeled thymidine for 1 h (panel F).

paring the thymidine incorporation profiles of DNA digested with restriction endonucleases and fractionated by BND-cellulose column chromatography from cultures pulse-labeled for 2–10 min with [*methyl*-$^3$H]thymidine (72 mCi/mmol; 10 μCi/mL) to that obtained from fractionating DNA labeled for the same period of time followed by a 1-h chase (Figure 5). At short labeling times (less than 2–4 min) in either synchronized or unsynchronized CHOC 400 cells (data for unsynchronized cells not shown), greater than 85% of the incorporated radioactivity is retained in the ssDNA fractions. After a 1-h chase in the absence of unlabeled thymidine, greater than 85% of the incorporated radioactivity is recovered in the dsDNA fracitons (Figure 5, panel F). Electron microscopy (not shown) and hybridization experiments (see below) indicate that a portion of the label remaining in the ssDNA fractions after the chase may be due to the retention of replicating mitochondrial DNA molecules. The amount of DNA recovered in the ssDNA fractions remained constant throughout these experiments and averaged 1–2% of the applied material (for example, see profiles presented in Figures 5 and 6).

Time course labeling experiments in CHOC 400 cells released from an aphidicolin-induced G1/S block showed that the ratio of radioactivity in the dsDNA containing fractions to that recovered in the ssDNA fractions reaches 1 by 6 min postrelease (Figure 5, panels B–E), even though the peak rate of DNA synthesis is not reached until at least 20 min later. By 12-15 min post release, greater than 75% of the incorporated precursor is recovered in the dsDNA fractions (Figure 5, panel E). Since it requires at least 40 min for the entire population to enter S after release from the aphidicolin block (Heintz & Hamlin, 1982), these column profiles indicate that many restriction fragments have been completely synthesized in some cells before other cells have even entered S.

*Effect of Ara-C on Maturation of Replication Intermediates.* The effect of ara-C on the maturation of replication intermediates containing ssDNA regions into dsDNA during the onset of S was determined by releasing cultures from an aphidicolin-induced G1/S block into S in the presence of 5 μg of ara-C/mL and then determining the amount of incorporated [*methyl*-$^3$H]thymidine retained in the ssDNA fractions (Figure 6). In an effort to avoid possible effects related to slower transport and/or phosphorylation of ara-C (Chu & Fischer, 1968), cultures were prequilibrated for 30–60 min with the same concentration of drug used during the subsequent pulse.

After 30 min of labeling in the absence of ara-C, greater than 60% of the incorporated radioactivity is recovered in the

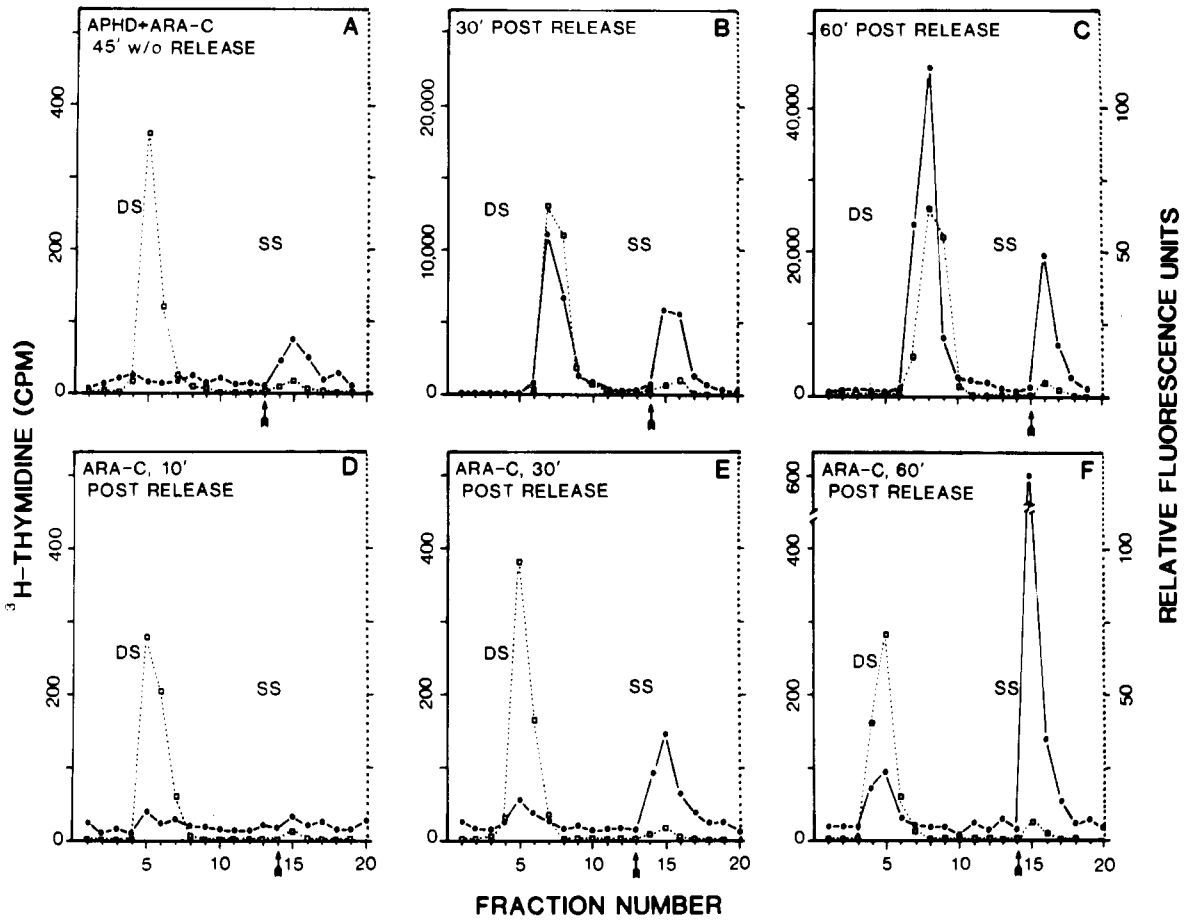


FIGURE 6: Maturation of replication intermediates formed during the onset of S phase is delayed by high concentrations of ara-C. Cell cultures were collected at the G1/S boundary with aphidicolin and were released into S phase in complete medium containing 5 μCi/mL [*methyl*-$^3$H]thymidine either without ara-C (panels B and C) or in the presence of 5 μg/mL ara-C (panels D–F). After the indicated period of time, DNA was prepared, digested with *Bam*HI, and fractionated by BND-cellulose chromatography as before (see legend to Figure 5). The amount of labeled thymidine (●) and mass of DNA (□) present in each fraction are presented. One culture was labeled for 45 min in the presence of both 5 μg/mL aphidicolin and 5 μg/mL ara-C(panel A) as a control for mitochondrial DNA synthesis.

dsDNA fractions, indicating complete synthesis of many restriction fragments. Note that, due to ongoing DNA replication, approximately 40% of the radioactivity is retained in the ssDNA fractions, suggesting the presence of active replication forks. By 60 min, thymidine incorporation has reached its maximum rate, and the specific activity of the ssDNA fractions begins to plateau.

In the presence of 5 μg/mL ara-C, a dose that inhibits the rate of thymidine incorporation by 95% (Figure 3), the overall rate of incorporation into both dsDNA and ssDNA fractions is drastically reduced (Figure 6D–F). However, by 30 min postrelease, all the incorporated radioactivity is recovered in the ssDNA BND-cellulose fractions, even though the total amount of incorporated precursor is greater than that observed in the absence of the drug at 6 min postrelease (Figure 5D). Incorporation of [*methyl*-$^3$H]thymidine exclusively into the ssDNA peak continues until approximately 60 min, when maturation into dsDNA is first observed (Figure 6F).

Autoradiography of restriction digests separated by neutral gel electrophoresis shows that [*methyl*-$^3$H]thymidine is incorporated into both mitochondrial and nuclear DNA under these conditions (Heintz & Hamlin, 1983a). Labeling for the same period of time in the presence of aphidicolin (no release, Figure 6A) indicates that the effect of ara-C on the maturation of newly replicated DNA from intermediates containing ss regions into dsDNA is not due solely to mitochondrial DNA replication, which is resistant to the action of both ara-C and aphidicolin (Spadari et al., 1984; Heintz & Hamlin, 1983a). Thus, at a fixed dose of drug that inhibits the rate of chain elongation by greater than 95%, the maturation of replicating restriction fragments containing ssDNA regions into mature dsDNA is almost completely inhibited for up to 60 min. If cells do indeed enter S and initiate DNA synthesis in the presence of ara-C, then the ssBND fractions from replication-arrested cells must be enriched for intermediates formed during initiation of DNA synthesis.

*Hybridization of BND-cellulose Column Fractions to Southern Blots of CHO and CHOC 400 Genomic DNA.* The thymidine incorporation profiles obtained by BND-cellulose chromatography suggested that the ssBND fractions from ara-C-treated cultures contained, albeit in unknown amounts, replication intermediates formed during the onset of S. The sequence complexity of the replicating DNA present in the ssBND fractions was investigated by hybridizing BND-cellulose column fractions labeled with $^{32}$P in vitro to Southern blots of restriction endonuclease digests of DNA derived from CHOC 400 cells.

Labeling of the BND fractions in vitro was achieved in several different ways, each of which gave similar results. First, BND-cellulose fractions were labeled by nick translation with DNase I and *E. coli* DNA polymerase I by a standard protocol (Rigby et al., 1977). Second, fractions were labeled with the large fragment of *E. coli* DNA polymerase I in the absence of DNase I in an effort to preferentially label replication intermediates by extension of those primed templates present in the fractions. And finally, BND-cellulose fractions

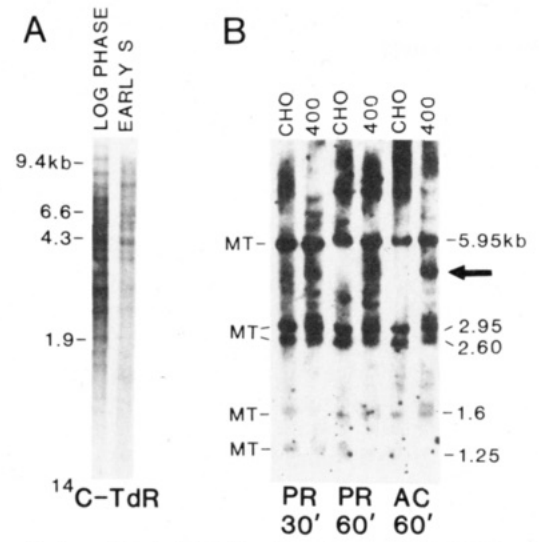


FIGURE 7: Ara-C limits hybridization of the ssDNA BND-cellulose fractions to a single restriction fragment derived from the amplified DHFR domain. (Panel A) Autoradiography of CHOC 400 restriction fragments labeled with [*methyl*-$^{14}$C]thymidine during log phase or during early S. CHOC 400 cultures were labeled with 1 $\mu$Ci/mL [*methyl*-$^{14}$C]thymidine for 30 min during exponential growth (left lane) or for the first 30 min of S in cells synchronized with aphidicolin (right lane). DNA from each culture was purified and digested with *Xba*I, and 2.5 $\mu$g of each sample was electrophoresed on a 0.8% neutral agarose gel. The DNA was blotted onto nitrocellulose paper, and labeled fragments were visualized by autoradiography with X-ray film for 20 days. The size in kilobases and positions of marker restriction fragments generated by digestion of $\lambda$ phage DNA with *Hin*dIII are indicated. (Panel B) Hybridization of ssDNA BND-cellulose fractions to Southern blots of CHO and CHOC 400 cellular DNA. DNA from CHO and CHOC 400 cells was purified, digested with *Xba*I, and electrophoresed on 0.8% neutral agarose gels prior to transfer to nitrocellulose paper. Replica blots were then hybridized with ssDNA BND-cellulose fractions from synchronized CHOC 400 cultures released into S for 30 (PR 30′), 60 min (PR 60′), or 60 min in the presence of 5 $\mu$g of ara-C/mL (AC 60′). In preparation for hybridization, the ssDNA BND-cellulose fractions were pooled and labeled by incubation with [$\alpha$-$^{32}$P]dCTP and the large fragment of *E. coli* DNA polymerase I as described under Materials and Methods. Each blot was hybridized with (1–5) × 10$^6$ dpm/mL of hybridization buffer for 16–20 h before washing and exposure to X-ray film for 3–5 days. "MT" indicates the position of *Xba*I restriction fragments derived from mitochondrial DNA, the size of these fragments in kilobases is indicated on the right. The arrow indicates the amplified 4.3-kb *Xba*I restriction fragment discussed in the text.

were incubated with T4 polymerase in the absence of dCTP for 2–10 min prior to the addition of labeled dCTP in an effort to remove terminal ara-C residues that might inhibit subsequent elongation by the exogenously added enzyme (hybridization data not shown). Depicted in Figure 7B are the autoradiographic patterns obtained by hybridizing ssDNA BND-cellulose fractions labeled with $^{32}$P by incubation with the large fragments of *E. coli* DNA polymerase I to Southern blots of genomic DNAs digested with the restriction endonuclease *Xba*I. In order to identify hybridization derived specifically from the amplified DHFR domain, DNA digests from methotrexate-sensitive CHO cells were electrophoresed and blotted in companion lanes.

For comparison, the autoradiographic patterns obtained from CHOC 400 DNA labeled with [*methyl*-$^{14}$C]thymidine during log phase or during the first 30 min of S in cells synchronized with aphidicolin are also presented (Figure 7). Autoradiography of *Xba*I restriction digests of CHOC 400 DNA labeled during log phase growth and fractionated on neutral agarose gels reveales 35–40 bands derived from the amplified DHFR domains (Figure 7A). In contrast, labeling of the amplified DHFR domains during the first 30 min of S is limited to approximately six to eight fragments, representing at least 40 kb of DNA, with several prominent bands visible between 3.8 and 6.4 kb.

The hybridization patterns obtained from the ssDNA BND-cellulose fractions on genomic blots (Figure 7B) permit several interesting observations. First, the presence of mitochondrial DNA in all of the ssBND column fractions is confirmed by equivalent hybridization to restriction fragments from both CHO and CHOC 400 DNA that migrate at the positions of mitochondrial DNA restriction fragments (Nass, 1983). Since mitochondrial DNA exists at the same copy number in both cell lines (Heintz & Hamlin, 1983a), it provides an appropriate internal control for determining if signals are obtained from the amplified sequences derived from the DHFR domain. Second, hybridization of ssDNA BND fractions from cells released into S for 30 min generates a spectrum of signals encompassing many amplified fragments. These fragments encompass at least 40–50 kb of amplified DNA and appear to include many fragments of the same size as those identified by whole cell pulse-labeling experiments such as that depicted in Figure 7A. The pattern obtained with ssDNA BND fractions from cells released into S for 60 min is even more complex, with the intensity and number of signals considerably different from those obtained from the 30-min fractions. These results indicate that retention of various fragments in the ssDNA BND fractions is a function of the position of cells within S phase and that the wide variety of fragments retained reflects the highly variable position of replication forks within individual replicons after release from the G1/S boundary. Clearly, synchronous synthesis of the amplified DHFR domains has not been achieved at a molecular level under these conditions.

In contrast, the hybridization pattern obtained with ssDNA BND-cellulose fractions from cells labeled for 60 min in the presence of 5 $\mu$g of ara-C/mL is considerably less complex, with the most intense hybridization occurring at a *Xba*I band (or bands) migrating at 4.3 kb (arrow, Figure 7B). Although mapping studies have identified at least four *Xba*I fragments between 4.2 and 4.8 kb within the early-replicating region of the amplified DHFR domain, at this level of resolution it is not possible to conclude that this hybridization signal is derived only from the earliest replicating portion of the amplified domain. With the exception of those fragments derived from mitochondrial DNA, hybridization to CHO genomic DNA occurred in a uniform smear encompassing fragments throughout the length of the blot.

In control experiments not shown, hybridization of dsDNA BND-cellulose fractions to genomic DNA was shown to generate signals from every amplified fragment, in addition to repetitive fragments common to both genomes, and ssBND fractions obtained from cells labeled late in S revealed a complex but different hybridization pattern.

## Discussion

The mechanism by which ara-C interferes with DNA synthesis has been extensively studied. Ara-C, which has little effect on protein or RNA synthesis, appears to inhibit DNA synthesis directly at the level of elongation by a chain termination mechanism [for review, see Cozzarelli (1977)]. At very high concentrations of drug, approximately 5% of the analogue is found at the termini of nascent DNA chains, while the remainder is found in internucleotide linkages (Zahn et al., 1972; Kufe & Major; 1982). Analysis of the effect of ara-C on the progression of cells through the cell cycle by flow cytometry has shown that high concentrations of the drug block cells at the G1/S boundary (Yataganas et al., 1974).

In previous experiments, we have demonstrated that CHOC 400 cells arrested in G1 by isoleucine deprivation will traverse the remainder of G1 and enter S in the presence of ara-C (Heintz & Hamlin, 1983a) and that the pattern of restriction fragments labeled during this interval is identical with that observed when other inhibitors are used as the synchronizing agent (Heintz & Hamlin, 1983b).

In this study, denaturing gel electrophoresis of DNA labeled in the presence of ara-C during the onset of S (Figure 4) indicated that the drug inhibits the production of presumed Okazaki fragments in a dose-dependent fashion. Since ara-C does not alter the fidelity with which polymerases incorporate other nucleotides into DNA (Furlong & Gresham, 1971; Kufe & Major; 1983), it appears that the drug affects only the rate and not the mechanism by which DNA is normally synthesized. This contention is supported by the studies of Leffak (1983), which showed that DNA replicated in the presence of ara-C is condensed into chromatin that resembles normal nascent chromatin with regard to nucleosome structure (Leffak, 1983). Thus, the chain terminating properties of the drug appear to be ideally suited for limiting the rate of replication fork travel without perturbing the ability of cells to initiate DNA synthesis. Here we have investigated the use of ara-C for arresting chain elongation as cells enter S in order to enrich for nascent DNA synthesized during initiation of DNA replication.

In these experiments, the ability of ara-C to inhibit the conversion of replication intermediates containing ssDNA regions (i.e., replication forks) into mature dsDNA was monitored by chromatography of restriction digests over BND-cellulose columns. Retention of sequences on the column in the presence of 1 M NaCl requires substantial regions of single strandedness, and a variety of studies with replicating bacteriophages (Kiger & Sinsheimer, 1969) or animal cell viruses (Hay & DePhamphilis, 1982) have shown that the single-stranded regions of replication forks are selectively retained by the resin. Comparison of the profiles obtained for thymidine incorporation with the quantity of DNA eluting in each column peak shows that restriction fragments containing replication intermediates can be effectively purified greater than 50-fold from bulk dsDNA by chromatography on BND-cellulose. Pulse–chase experiments such as those depicted in Figure 5 show that the replication intermediates detected in ssDNA BND-cellulose can be chased into mature dsDNA. Thus, fractionation by BND-cellulose chromatography appears to be an effective method for isolating replication intermediates as well as for monitoring the maturation of replication intermediates containing single-stranded regions into dsDNA.

The BND-cellulose column profiles for thymidine incorporation show that mature, replicated ds restriction fragments are detectable by 3–6 min after release from an aphidicolin-induced G1/S blockade. Percent labeled nuclei studies indicate that less than 15–20% of the cell population has entered S by this time (Heintz & Hamlin, 1982), and the maximum rate of DNA synthesis is not reached until 30–40 min later. Therefore, the amount of DNA derived specifically from initiation events under these conditions must be very small. By 30 min postrelease, greater than 70% of the cells have entered S, and labeling of a subset of the restriction fragments derived from the amplified DHFR domains is observed (Figure 7A). Replication forks are positioned within many restriction fragments by this time, as evidenced by the hybridization pattern obtained with $^{32}$P-labeled ssDNA BND-cellulose fractions on Southern blots of genomic DNA (Figure 7B). By 60 min postrelease, the positions of the replication forks have changed, resulting in a different hybridization pattern. These disperse hybridization signals indicate that isolation of replication forks poised at various positions within the amplified DHFR domains is a function of the position of cells in the cell cycle and are indicative of the relatively variable progression of cells through S phase.

In contrast, ara-C appears to limit fork travel, and hence the variability of the position of replication forks within the amplified domains, in a dose-dependent fashion. At high doses of drug (greater than 2.5 $\mu$g/mL), thymidine incorporation is highly reduced, and progression of cells away from the G1/S boundary (measured by cell sorting profiles) is greatly inhibited. Thus, the extent that cells are able to progress through S shows a dependence on time in a fixed dose of drug and on dose within a fixed labeling period. After 60 min in the presence of 5 $\mu$g/mL ara-C, the ssDNA BND-cellulose fractions are considerably enriched for sequences homologous to a 4.3-kb *Xba*I fragment (or fragments) derived from the amplified domain, while signals obtained from other sequences observed in the 30- and 60-min control samples are nearly absent. These data suggest that initiation of replication of the amplified units occurs at a site within or near an amplified 4.3-kb *Xba*I fragment and that movement of replication forks away from this site can be impeded by high concentrations of ara-C.

These studies strongly support the notion that replication of the amplified DHFR replicon is initiated at a fixed origin of replication. Hybridization of initiation intermediates obtained by arresting replication with ara-C to recombinant clones spanning the entire 150-kb amplified DHFR domain should permit localization of the origin of replication associated with this region.

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# Preparation and Characterization of a Viral DNA Molecule Containing a Site-Specific 2-Aminofluorene Adduct: A New Probe for Mutagenesis by Carcinogens†

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ABSTRACT: The synthetic oligonucleotide heptamer 5′-ATCCGTC-3′ was reacted in vitro with *N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene and the resulting product isolated by reverse-phase high-performance liquid chromatography (HPLC). This purified oligonucleotide, which was shown by chemical and enzymatic analysis to be a heptamer containing a single *N*-(deoxyguanin-8-yl)-2-aminofluorene adduct, was then used to situate the putatively mutagenic aminofluorene lesion within the genome of M13 mp9 by ligating it into a complementary single-stranded region located at a specific site in the negative strand of the duplex M13 mp9 DNA molecule. The presence of the adduct at the anticipated location was confirmed by taking advantage of the facts that AF adducts inhibit many restriction enzymes when located in or near their restriction sites and that the AF moiety should be contained within the *Hin*cII recognition sequence on M13 mp9 DNA. Upon attempted cleavage of the M13 DNA containing the site-specific AF adduct with *Hin*cII, we find that the large majority of the DNA remained circular, demonstrating the incorporation of the AF adduct in high yield into the DNA molecule at this location. This system should prove useful in vivo for the study of mutagenesis by chemical carcinogens and in vitro to study the interaction of purified DNA metabolizing proteins with a template containing a site-specific lesion.

The formation of covalent adducts between reactive electrophilic metabolites of chemical carcinogens and the nucleophilic sites of DNA is considered to be a critical step in chemical carcinogenesis (Miller, 1978). Chemical modification of cellular DNA presents a serious challenge to cells since mutation or cell death may result upon exposure to an agent with such potential. There is substantial evidence suggesting that modification of cellular DNA is the premier event of the multistep carcinogenic process (Weinstein, 1981; King, 1985), and it is generally accepted that the majority of ultimate carcinogens are mutagens (Ames & McCann, 1979), which mediate their effect through covalent binding to DNA (Singer & Grunberger, 1984).

Aromatic amines have been shown conclusively to induce urinary bladder cancer in humans and tumors in a wide variety of organs in experimental animals [for reviews, see King (1982, 1985) and Garner et al. (1984)]. The carcinogenic activity of this class of compounds, as with agents having other structures, appears to depend on their conversion to reactive metabolites that alter the macromolecules of the target tissues (King, 1985). 2-(Acetylamino)fluorene (AAF),[1] one of the most studied aromatic amines, is believed to form DNA adducts following N-oxidation and a subsequent metabolic activation involving conjugation of the hydroxamic acid with sulfate in rat liver or O-acetylation of the hydroxylamine in

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[1] Abbreviations: AAF, 2-(acetylamino)fluorene; AF, 2-aminofluorene; RF, replicative form; TE buffer, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA; GHD, gapped heteroduplex DNA (M13 mp9 DNA containing a seven-nucleotide gap in the negative strand from position 6251 to position 6257); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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