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Nuclear DNA Synthesis In Vitro Is Mediated via Stable Replication Forks Assembled in a Temporally Specific Fashion In Vivo

NICHOLAS H. HEINTZ[1]* AND BRUCE W. STILLMAN[2]

Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont 05405,[1] and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[2]

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A cell-free nuclear replication system that is S-phase specific, that requires the activity of DNA polymerase alpha, and that is stimulated three- to eightfold by cytoplasmic factors from S-phase cells was used to examine the temporal specificity of chromosomal DNA synthesis in vitro. Temporal specificity of DNA synthesis in isolated nuclei was assessed directly by examining the replication of restriction fragments derived from the amplified 200-kilobase dihydrofolate reductase domain of methotrexate-resistant CHOC 400 cells as a function of the cell cycle. In nuclei prepared from cells collected at the G1/S boundary of the cell cycle, synthesis of amplified sequences commenced within the immediate dihydrofolate reductase origin region and elongation continued for 60 to 80 min. The order of synthesis of amplified restriction fragments in nuclei from early S-phase cells in vitro appeared to be indistinguishable from that in vivo. Nuclei prepared from CHOC 400 cells poised at later times in the S phase synthesized characteristic subsets of other amplified fragments. The specificity of fragment labeling patterns was stable to short-term storage at 4°C. The occurrence of stimulatory factors in cytosol extracts was cell cycle dependent in that minimal stimulation was observed with early G1-phase extracts, whereas maximal stimulation was observed with cytosol extracts from S-phase cells. Chromosomal synthesis was not observed in nuclei from G1 cells, nor did cytosol extracts from S-phase cells induce chromosomal replication in G1 nuclei. In contrast to chromosomal DNA synthesis, mitochondrial DNA replication in vitro was not stimulated by cytoplasmic factors and occurred at equivalent rates throughout the G1 and S phases. These studies show that chromosomal DNA replication in isolated nuclei is mediated by stable replication forks that are assembled in a temporally specific fashion in vivo and indicate that the synthetic mechanisms observed in vitro accurately reflect those operative in vivo.

Chromosomal DNA synthesis is a highly regulated event limited to the S phase of the eucaryotic cell cycle. Although little is known about the molecular mechanisms that regulate entry of eucaryotic cells into the S phase, substantial genetic and biochemical evidence (reviewed in references 7, 10, and 14) suggests that replication is initiated at specific sites (or origins of replication) and proceeds bidirectionally (10) in a semidiscontinuous fashion (19). Selection of those replicons slated to be synthesized immediately upon entry into the S phase may be regulated at the level of either initiation or elongation, perhaps by the interaction of *trans*-acting initiation factors with specific origin sequences or by differential elongation of performed initiation complexes. The elongation phase of DNA synthesis is known to require multiple protein factors with a myriad of enzymatic activities (7). To date, dissection of the processes leading to site-specific initiation of DNA replication, as well as identification of factors required for efficient elongation, has been best approached by studying the replication of animal cell virus DNA. These studies have provided a foundation for investigating chromosomal DNA replication in isolated nuclei.

Owing to the size and complexity of most vertebrate genomes, study of the replication of any specific, single-copy chromosomal sequence can be achieved only via indirect methods (3, 4, 18). In an effort to overcome the difficulties of studying the replication of single-copy sequences, drug-resistant cell lines that contain multiply repeated copies of large genetic domains have been developed. To this end, a Chinese hamster cell variant, CHOC 400, which is resistant to very high concentrations of the folate analog methotrexate, has been isolated and characterized. These cells are resistant to high levels of methotrexate (400 μg/ml of culture medium) by virtue of a 500-fold amplification of a ca. 200-kilobase (kb) domain that includes the gene for dihydrofolate reductase (*dhfr*) (25). The amplified *dhfr* domains are stable chromosomal entities that are arranged in tandem arrays in several marker chromosomes (23, 25). The entire amplified *dhfr* domain of CHOC 400 cells has been cloned in a series of overlapping recombinant cosmids (13, 23, 26), and a restriction map encompassing the entire unit repeat has been constructed (23). Retroactive labeling studies of metaphase chromosomes have shown that the chromosomal regions containing the amplified *dhfr* domains initiate replication immediately upon entry of cells into the S phase and that synthesis of this region is completed in the first half of the S phase (12, 15, 25). Pulse-labeling studies with synchronized cells have shown that replication of the amplified domains is initiated within a specific subset of restriction fragments derived from the amplified region (15). These early-labeled fragments (ELFs) were isolated by molecular cloning (16) and were localized by restriction mapping to a single contiguous region downstream from the *dhfr* gene (13). Hybridization of early replication intermediates synthesized in synchronized CHOC 400 cells to cloned ELFs indicates that the origin of replication lies within, or very close to, a 4.3-kb *Xba*I fragment that maps 14 kb downstream from the last exon of the *dhfr* gene (5, 6).

Although pulse-labeling studies of whole or permeabilized

* Corresponding author.

cells have led to the identification of a fragment that contains, or is very close to, the origin of replication associated with the amplified *dhfr* domain, definitive delineation of the *dhfr* origin sequences, as well as functional characterization of origin activity, requires an in vitro system amenable to experimental manipulations. Ideally, such a system should require components necessary for regulated DNA replication in vivo and should respond to those factors elaborated at various times during the cell cycle that control site-specific initiation of DNA synthesis. As a step toward establishing such a system, we have adapted conditions developed for the identification of cellular factors required for simian virus 40 DNA synthesis (22, 29) to examine DNA replication in nuclei purified from synchronized CHOC 400 cells. In this report, we show that chromosomal DNA replication in purified nuclei is highly stimulated by cytoplasmic factors, requires the activity of DNA polymerase alpha, is temporally exact, and is mediated by stable replication forks. The cytoplasmic factors that stimulate DNA synthesis are cell cycle regulated and probably act at the level of elongation rather than initiation. Furthermore, the order of synthesis of amplified restriction fragments in vitro appears identical to that previously observed in vivo. Thus, the mechanism by which nuclear DNA is replicated in vitro appears to accurately reflect that used in whole cells.

MATERIALS AND METHODS

Cell culture and synchronization. CHOC 400 cells were propagated in Eagle minimum essential medium containing 10% donor bovine serum (GIBCO Laboratories). For synchrony, cells were first arrested in the G1 period by incubation in isoleucine-deficient medium (15, 32) and were then collected at the G1/S boundary by incubation in complete medium containing 10 μg of aphidicolin (Natural Products Division, National Cancer Institute) per ml. These cultures were allowed to proceed into the S period by having the inhibitor removed, being washed with fresh complete medium, and being reincubated at 37°C. The efficacy of the synchrony regimen has been documented previously (5, 6, 12). HeLa cells were propagated in monolayer cultures in Dulbecco minimal essential medium containing 10% fetal bovine serum (Armour Pharmaceutical Co.).

Preparation of nuclei and cytosol. Cells were propagated in either 100- or 150-cm^2 tissue culture plates before both nuclear and cytoplasmic extracts were isolated essentially by the method of Li and Kelly (22). Each plate was washed once with 10 ml of phosphate-buffered saline (8 g of NaCl per liter, 0.2 g of KCl per liter, 1.5 g of sodium phosphate [dibasic] per liter, 1.5 sodium phosphate [monobasic] per liter) and then rinsed twice with ice-cold hypotonic buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]–KOH [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol) (22). Plates were drained extensively to remove as much hypotonic buffer as possible before the cells were harvested with a rubber policeman. Under these conditions, each 150-cm^2 plate yielded approximately 200 μl of cell lysate. The cells were incubated on ice for 10 min and then disrupted by Dounce homogenization until 95% of the cells were broken (20 strokes of a B pestle). The cell lysate was left on ice for 30 min, and the suspension was then centrifuged at 10,000 rpm in a Sorvall HB4 rotor for 10 min at 4°C. The supernatant, or cytosol fraction, was removed and either used immediately or frozen at −70°C. Concentrations of protein in the cytoplasmic extracts were determined with a protein assay kit (Bio-Rad Laboratories) (2) and ranged from 1.9 to 3.4 mg/ml.

The nuclear pellet was suspended either in 75 μl of hypotonic buffer per plate (1×10^5 to 5×10^5 nuclei per μl) with or without 10% sucrose (nuclear fraction) or directly in the cytosolic extract. Phase-contrast microscopy indicated that sucrose improves the morphology of nuclei incubated in vitro, but its presence did not appreciably improve the synthetic capacity of the nuclear fractions.

DNA synthesis. Reaction mixtures contained (final concentration) 30 mM HEPES-KOH (pH 7.5), 7 mM $MgCl_2$, 0.8 mM dithiothreitol, 100 μM each dTTP, dGTP, and dCTP, 25 μM [α-^{32}P]dATP, 200 μM each CTP, GTP, and UTP, 4 mM ATP, 40 mM creatine phosphate, and 20 μg of creatine phosphokinase (rabbit muscle type I; Sigma Chemical Co.) per ml. Standard reaction mixtures for titration experiments were prepared by adding 10 μl of a 5× buffer mix containing the above components along with various amounts of nuclei and cytosol to a final volume of 50 μl. Reaction mixtures were prepared on ice and incubated at 37°C for the indicated time.

For synchrony experiments, lysate equivalent to cells from one 150-cm^2 plate (5×10^6 to 10×10^6 nuclei per plate) was used for each time point. Standard reaction mixtures contained 50 μl of 5× reaction mix and 5×10^6 to 10×10^6 nuclei suspended with 200 μl of cytosolic extract. Reactions were terminated by the addition of an equal volume of lysis buffer (40 mM EDTA, 1.2% sodium dodecyl sulfate, 100 mM NaCl, 50 mM Tris hydrochloride [pH 7.8]). The lysate (500 μl) was incubated with proteinase K at 37°C overnight, extracted twice with water-saturated phenol, extracted once with chloroform-isoamyl alcohol (24:1, vol/vol), and precipitated twice with ethanol. Restriction enzyme digestion was performed under the conditions suggested by the vendor. DNA concentrations were determined by the fluorometric method of Labarca and Paigen (20).

Analysis of replication products. For determination of the amount of dAMP incorporated during the in vitro incubations, EDTA (pH 8.0) was added to a final concentration of 10 mM, and 50 μg of denatured, sheared calf thymus DNA was added as carrier. Samples were then mixed with 1 ml of trichloroacetic acid–1% sodium PP_i, incubated for 15 min on ice, and filtered onto Enzo EGF glass filters. (Enzo Biochemical, Inc.). The filters were washed three times with 10 ml of ice-cold trichloroacetic acid-sodium PP_i solution and twice with ice-cold 95% ethanol and dried, and radioactivity was determined by scintillation counting. Agarose gel electrophoresis was performed with TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA) as described previously (24). Gels were rinsed, stained with ethidium bromide, photographed, and dried before being exposed to X-ray film at −70°C for 1 to 3 days.

BrdU substitution and isopycnic centrifugation. For density substitution experiments, isolated nuclei were incubated for 30 min in a standard reaction mixture in which 100 μM bromodeoxyuridine triphosphate (BrdUTP) was substituted for dTTP. DNA was isolated as above, digested with *Eco*RI, and resolved on 4.8-ml isopynic CsCl gradients in 15 mM NaCl–15 mM disodium EDTA–5.9 M CsCl at 39,000 rpm for 72 hr in a Beckman 50 Ti rotor as described previously (18). Gradient fractions of 200 μl were collected, diluted threefold with H_2O, adjusted to 0.25 M NaCl, precipitated with ethanol, and separated on 0.8% agarose gels in TAE buffer. Gels were rinsed, stained, photographed, dried, and exposed to X-ray film as described above. The linearity of each gradient was determined by measuring the refractive index of each sample after fractionation. Under these conditions,

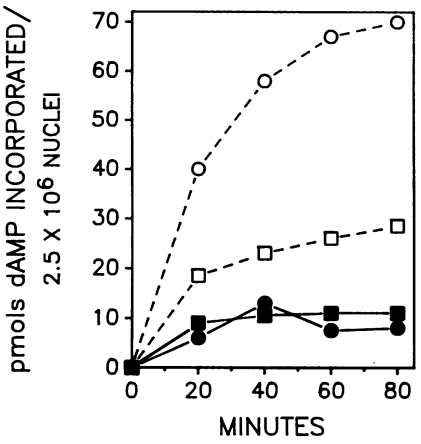

FIG. 1. Stimulation of nuclear DNA synthesis by cytoplasmic factors. Nuclei isolated from asynchronously growing log-phase CHOC 400 cultures were incubated under standard reaction conditions (50 μl) in the absence or presence of 18 μl of cytosol extract from the same cells, and the amount of acid-precipitable dAMP incorporated during the indicated time was determined. To assess the extent of synthesis due to chromosomal replication, reactions with aphidicolin (10 μg/ml) were conducted in parallel. The cytosol extract was 3.2 mg of protein per ml. Symbols: ○, with cytosol; □, without cytosol; ●, with cytosol and aphidicolin; ■, without cytosol and with aphidicolin.

unsubstituted DNA banded at 1.68 to 1.70 g/ml and hybrid density DNA banded at 1.71 to 1.73 g/ml.

Inhibitors. Aphidicolin was prepared as a 20-mg/ml stock in dimethyl sulfoxide and added directly to achieve the indicated concentration. Monoclonal antibodies to DNA polymerase alpha were originally described by Tanaka et al. (30). Hybridoma cell lines 1640 (secreting antibody SJK 132-20) and 1645 (secreting antibody SJK 237-71) were obtained from the American Type Culture Collection. Antibodies were purified from mouse acites fluid by using the Affi-Gel protein A MAPS II kit (Bio-Rad) and were a gift from W. Heiger.

RESULTS

Optimization of nuclear DNA synthesis in vitro. Refinement of conditions that promote vigorous and temporally specific chromosomal DNA synthesis in purified nuclei was accomplished in several steps. First, nuclear and cytosolic fractions from exponentially growing HeLa cells were titrated against one another in the presence and absence of aphidicolin to identify conditions that support maximal rates of aphidicolin-sensitive DNA synthesis. These titration experiments were then repeated with CHOC 400 cells. In no instance were significant differences in the rate of incorporation or the requirement for cytosol between HeLa and CHOC 400 nuclei observed. Second, autoradiography of restriction digests of the cellular DNA separated by agarose gel electrophoresis was used to evaluate the relative amounts of mitochondrial DNA (mtDNA) and nuclear DNA synthesis under various conditions. Finally, after standard conditions that supported efficient chromosomal DNA replication were developed, the temporal specificity of chromosomal replication was assessed by examining the replication of amplified sequences in nuclei prepared from synchronized CHOC 400 cells. It should be emphasized that the amplified *dhfr* region represents only 3 to 4% of the genomic DNA of CHOC 400 cells, and, therefore, examination of the synthesis of restriction fragments derived from the amplified region must necessarily be viewed against a background smear resulting from the replication of the remaining genomic DNA.

Results of reciprocal nucleus-cytosol titration experiments (data not shown) indicate that maximal incorporation of dAMP into acid-precipitable material is stimulated by the addition of cytosolic extract. In the absence of cytosol, the incorporation of dAMP plateaus by 30 min, whereas addition of cytosol stimulates the rate of incorporation of dAMP into acid-precipitable material three- to eightfold, and linear rates of synthesis are sustained for at least 60 min (Fig. 1). In the presence of optimal amounts of cytosol, nearly 90% of the incorporation of dAMP in a 60-min reaction is aphidicolin-sensitive (Fig. 1 and Fig. 2, lanes 7 to 12). This value corresponds well to the proportion of DNA synthesis (i.e., 8 to 10%) that is insensitive to aphidicolin in whole cells (data not shown). Both in vivo (N. H. Heintz, unpublished observations) and in vitro (Fig. 2, lanes 4 to 6) aphidicolin-insensitive DNA synthesis is entirely due to mtDNA replication. Interestingly, mtDNA replication is not enhanced by cytoplasmic components (Fig. 2, compare lanes 4 to 6 with lanes 10 to 12), nor is it cell cycle regulated. As described below (see Fig. 4, 5, 7, and 8), vigorous mtDNA synthesis occurs in nuclear fractions isolated from cells throughout G1 and all of the S phase. Thus, mtDNA replication serves as an excellent control for the presence of nonspecific inhibitors, nucleases, or other contaminants in the in vitro reactions.

The cytosol preparation in the absence of nuclei supports negligible replication (data not shown). Therefore, its stimulatory effect is not due to providing additional template. The single most important parameter affecting the activity of cytosol preparations from log-phase cells appears to be the protein concentration, which ranged from 1.9 to 3.2 mg/ml. Thus, maximal enhancement of DNA synthesis by log-phase

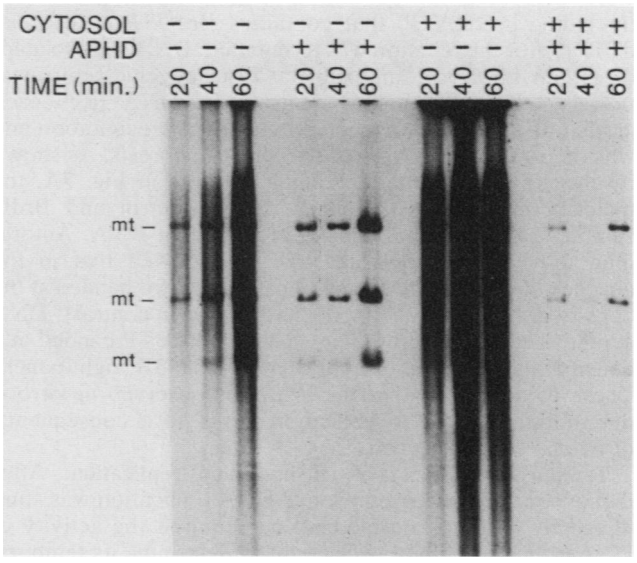

FIG. 2. Stimulation of chromosomal DNA but not mtDNA replication by cytosol. Nuclei from asynchronously growing log-phase HeLa cells were incubated under the standard replication reaction conditions (100-μl reaction mixtures) in the presence and absence of cytosol, with and without aphidicolin (APHD) as indicated. At the indicated time, nuclei were lysed, DNA was purified and digested with *Eco*RI, and 2.5 μg of the digested DNA was subjected to electrophoresis in a 0.8% agarose gel. An autoradiogram of the dried agarose gel is depicted. Abbreviation: mt, mtDNA fragments.

cytosol is observed when cells are lysed in as small a volume as possible and the ratio of cytosol to nuclei approximates or exceeds that of whole cells. In the experiments performed with synchronized cells (see Fig. 4, 5, 7, and 8), cytosol was isolated from the same cultures as were the nuclear fractions.

Requirement for DNA polymerase alpha. The experiments depicted in Fig. 1, as well as autoradiography of restriction digests separated by agarose gel electrophoresis (Fig. 2), showed that chromosomal DNA synthesis is sensitive to the inhibitor aphidicolin. Although aphidicolin is considered a specific inhibitor of DNA polymerase alpha (17), it affects the rate of DNA repair processes (8) and is an active inhibitor of DNA polymerase delta (21). To directly assess the involvement of DNA polymerase alpha in chromosomal DNA replication in vitro, we examined the effects of two monoclonal antibodies (MAbs) raised against DNA polymerase alpha, MAb SJK 132-20 and MAb SJK 237-71 (30), in the nuclear replication system. It has been shown that MAb SJK 237 does not neutralize the polymerization activity of purified polymerase alpha, whereas MAb SJK-132 inhibits the activity of the polymerase (30). Although MAb SJK-237 at concentrations up to 10 μg/ml has no effect on cytosol-dependent DNA synthesis in HeLa cell nuclei, MAb SJK-132 demonstrates a dose-dependent inhibition of dAMP incorporation such that when it is present at 10 μg/ml, the level of DNA synthesis is reduced to that observed in the absence of cytosol (data not shown). These data, along with the sensitivity of chromosomal synthesis to aphidicolin, suggest that chromosomal DNA replication in the nuclear fraction requires the synthetic activity of DNA polymerase alpha.

Nuclear synthesis in vitro represents DNA replication, not repair. To ascertain whether the observed synthesis in vitro represents authentic DNA replication, nuclei isolated from CHOC 400 cells that had been synchronized in early S phase were incubated for 30 min in a standard reaction mix (including [^{32}P]dATP) that contained BrdUTP rather than dTTP. After the reaction was terminated, DNA was isolated, digested with *Eco*RI, and subjected to isopycnic centrifugation in CsCl gradients at neutral pH. DNA from each gradient fraction was collected by ethanol precipitation and was then subjected to agarose gel electrophoresis. As shown by the ethidium bromide staining patterns in Fig. 3A, the majority of the DNA from both the control and BrdU samples banded with a density of pure light DNA. Autoradiography of the dried agarose gels showed that in the absence of BrdUTP, DNA synthesized in vitro banded at the density of light DNA (Fig. 3B, left panel). In contrast, DNA synthesized in vitro in the presence of BrdUTP banded at a density characteristic of hybrid DNA (Fig. 3B, right panel). These data indicate that the synthesis observed in vitro is due to authentic DNA replication and is not a consequence of extensive DNA repair.

Temporal specificity of chromosomal replication. After demonstrating that chromosomal DNA replication was stimulated by cytoplasmic factors and required the activity of DNA polmerase alpha, we sought to determine its temporal fidelity as a function of the cell cycle. To assess temporal specificity, we synchronized CHOC 400 cells by a combination of isoleucine deprivation and aphidicolin blockade, prepared nuclei and incubated then with their cognate cytosol in the in vitro system, and analyzed the products by agarose gel electrophoresis of restriction digests.

After incubation for 48 h in medium lacking isoleucine, CHOC 400 cells arrest in the G1 phase at a point 4 to 12 h prior to entry into the S phase (15, 32). With the addition of complete medium containing serum, CHOC 400 cells arrested in the G1 phase traverse the remainder of the G1 phase and enter the S phase in a parasynchronous wave, with the first cells entering the S phase 4 h after the addition of complete medium (6, 15). By 12 h after addition of complete medium, more than 95% of the population is in the S phase (5, 15). If cells arrested in the G1 phase are fed complete medium containing 10 μg of aphidicolin per ml, progress through the S phase is impeded and more than 95% of the population is collected at the G1/S boundary by 12 h. Fluorescence-activated cell sorting and percent labeled nuclei studies have shown that virtually the entire cell population collected at the G1/S boundary will enter the S phase within 60 min upon removal of the aphidicolin and reincubation in fresh medium (5, 15). Thus, these synchronization conditions permit collection of cells at various times in the

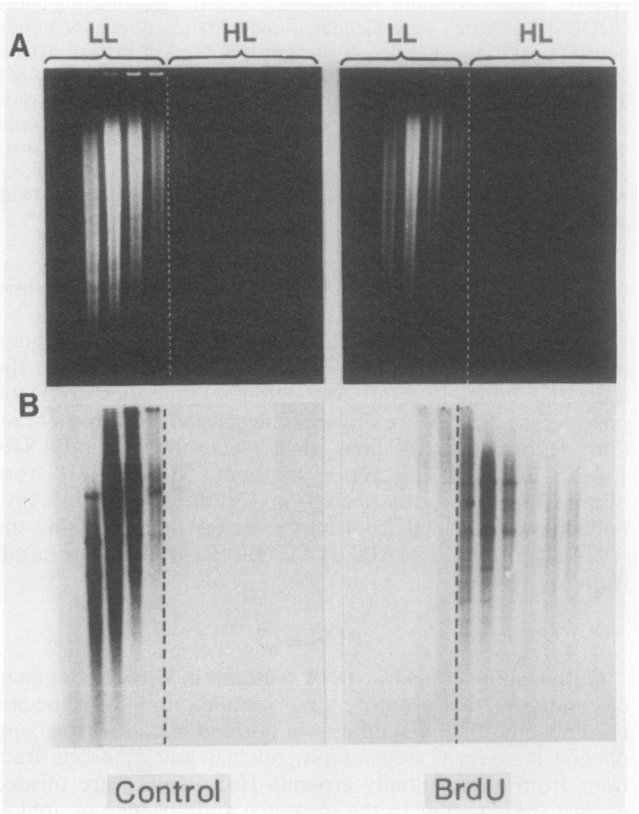

FIG. 3. Nuclear DNA synthesis in vitro represents authentic replication. Nuclei isolated from CHOC 400 cells synchronized in early S phase were incubated with [^{32}P]dATP for 30 min under standard conditions (Control) or in a standard reaction mix that contained 100 uM BrdUTP instead of dTTP (BrdU). DNA was purified from each reaction, digested with *Eco*RI, and centrifuged to equilibrium on neutral CsCl gradients. The gradients were fractionated, and the linearity of each gradient was determined by measuring the refractive index of each fraction (not shown). DNA from each gradient fraction was purified and subjected to agarose gel electrophoresis. (A) The ethidium bromide staining patterns of the regions of gels corresponding to pure light DNA (gradient fractions 7 to 11; LL) or hybrid density DNA (gradient fractions 12 to 18; HL). Under these conditions (see Materials and Methods), pure light DNA banded at a density of 1.68 to 1.70 g/ml and hybrid density DNA banded at a density of 1.71 to 1.73 g/ml. The gels were then dried and exposed to X-ray film. (B) The autoradiographic labeling pattern for the *Eco*RI digests of DNA synthesized in the absence (Control) or presence of BrdUTP (BrdU).

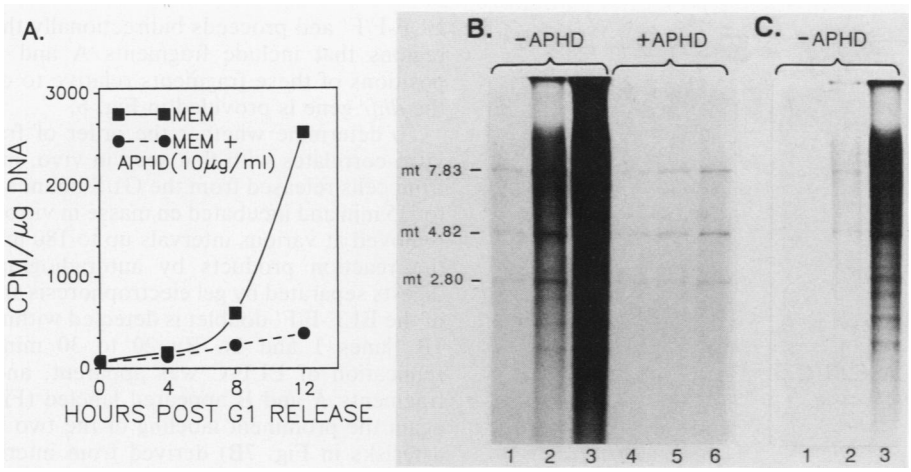

FIG. 4. Chromosomal replication in CHOC 400 nuclei as a function of the cell cycle. Replicate CHOC 400 cultures were collected in the early G1 phase by isoleucine deprivation for 48 h (0 time). Cultures were induced to traverse the remainder of the G1 phase by the addition of fresh medium with or without aphidicolin (APHD) as indicated. At the indicated time after addition of fresh medium, nuclei were prepared and incubated under standard replication reaction conditions in the presence of an optimal amount of their cognate cytosol. After 30 min, the nuclei were lysed, DNA was purified and digested with *Eco*RI, and the concentration of the DNA was determined. (A) The specific activity of each sample was measured by determining the amount of dAMP incorporated per microgram of DNA. (B) and (C) Each digested DNA sample (2.5 μg) was subjected to electrophoresis in a 0.8% agarose gel. Lanes 1 to 3 depict the labeling patterns of *Eco*RI fragments in extracts isolated from cells 4, 8, and 12 h after release from the G1-phase arrest, respectively. Lanes 4 to 6 depict the labeling patterns from parallel cultures released from the G1 block in the presence of aphidicolin. A shorter exposure of lanes 1 to 3 is provided in panel C. The sizes of *Eco*RI mtDNA fragments (mt) are indicated in kilobase pairs.

G1 phase, at the G1/S interface, and at various times in the S phase.

First, we determined whether chromosomal synthesis in vitro paralleled that observed in cells transiting the G1 phase and entering the S phase after the addition of complete medium and whether aphidicolin was effective in delaying the entry of cells into the S phase. dAMP incorporation into nuclei prepared from cells collected in the G1 phase was negligible (Fig. 4A). Autoradiography of restriction digests shows that synthesis in the nuclear fraction derived from cells deprived of isoleucine for 48 h is limited to mtDNA replication (data not shown). At 4 h after release from the G1-phase arrest, a time when cells first began to enter the S phase in vivo, synthesis of mtDNA continued to be prominent and chromosomal replication remained undetectable (Fig. 4B, lane 1). By 8 h after release from the G1-phase arrest, when approximately 35% of the cells had entered the S phase, the rate of dAMP incorporation had increased (Fig. 4A) and the replication of the entire complement of *Eco*RI fragments that make up the amplified unit was detected (Fig. 4b, lane 2). By 12 h, the entire population had entered the S phase, and high rates of chromosomal replication (Fig. 4A), as well as synthesis of all the amplified fragments, were observed (Fig. 4B, lane 3). It is important to note that replication pattern of both the 8- and 12-h samples includes the entire spectrum of amplified restriction fragments (Fig. 4C). These fragments can be observed in the ethidium bromide-stained gel because the *dhfr* region is present in high copy number (15, 25, 26) and is therefore reflected in the replication products as discrete bands.

In the presence of aphidicolin, cells transiting the G1 phase become arrested at the G1/S boundary, and replication of amplified fragments is almost completely ablated. Compare, for example, the labeling pattern of nuclei from cells incubated for 12 h without aphidicolin (Fig. 4B, lane 3) with that of nuclei incubated in the presence of the drug (Fig. 4B, lane 6). Note that very little chromosomal DNA synthesis is observed in nuclei from aphidicolin-treated cells, even at time points (i.e., 8 and 12 h) when the synthesis of ELFs might be expected. In vivo, there is a 12- to 15-min lag between the removal of aphidicolin and the resumption of DNA synthesis (15). It is not known whether this lag is due to residual effects resulting from incomplete removal of the drug or whether other events that activate replication complexes occur during this interval.

To evaluate the replication pattern of the amplified sequences in cells released into the S phase, cells collected at the G1/S boundary with aphidicolin were washed and reincubated in fresh medium at 37°C for 15, 30, 60, 120, and 240 min before nuclei were prepared. Each nuclear sample was then incubated in vitro in the presence of its cognate cytosol extract for 30 min, and the specific activity as well as the labeling pattern of replicated DNA was determined. Replication activity increased dramatically in isolated nuclei prepared from cells at progressively later points in early S phase (Fig. 5). By 240 min after release from the aphidicolin block, the replication capacity of the extracts was 10-fold higher than those prepared from cells released into the S phase for 15 min. These results correlate very well with the increased synthetic rates observed for cells entering the S phase in vivo (10). In vivo (10), and probably in vitro, these increases do not result from higher rates of replication fork movement, but rather reflect the activation of increasing numbers of replication forks as cells traverse very early S phase.

Examination of the restriction fragment labeling pattern under these conditions (Fig. 5B) reveals that in addition to the background smear resulting from the replication of the unamplified genomic sequences, staged nuclei synthesize characteristic subsets of amplified restriction fragments. Synthesis of amplified sequences in the early S-phase samples (Fig. 5B, lane 1) is limited to several faint fragments; by 60 min into the S phase, labeling of 8 to 10 fragments is observed (lane 2), with prominent labeling limited to the *Eco*RI ELFs (such as *Eco*RI ELF-F/F′ and ELF-C; see Fig. 7) that were first detected by pulse-labeling studies in whole

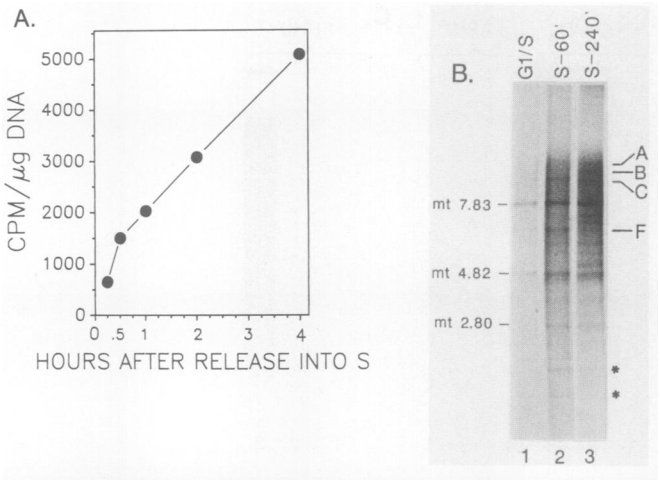

FIG. 5. Synthesis of specific subsets of amplified fragments by nuclei isolated from cells at various times in the S phase. Replicate cultures were collected at the G1/S boundary with a combination of isoleucine deprivation for 48 h and then incubated for 12 h in fresh medium containing aphidicolin. Cultures were released from the G1/S boundary by being rinsed twice with fresh medium lacking inhibitor and reincubated at 37°C. At the indicated time after release into the S phase, extracts were prepared that contained their cognate cytosol fraction and were incubated under standard reaction conditions for 30 min. (A) DNA was isolated and digested with *Eco*RI, and the amount of dAMP incorporated per microgram was measured. (B) Digests (2.5 μg) from cells released into the S phase for 15 min (lane G1/S), 60 min (lane S-60′), and 240 min (lane S-240′) were subjected to agarose gel electrophoresis, and the labeling pattern was visualized by autoradiography. To compare signals at similar intensities, the S-240′ lane was exposed for one-quarter the time of exposure of the G1/S and S-60′ lanes. A, B, C, and F indicate specific amplified restriction fragments (see Fig. 6). The sizes of mtDNA fragments are indicated in kilobase pairs. The asterisks indicate repetitive, interspersed *Eco*RI fragments endogenous to the CHO cell genome that are not derived from amplified DNA.

cells (15, 16). Also prominent in the labeling pattern of early S-phase samples are two *Eco*RI bands (denoted by asterisks in Fig. 5) which are not derived from the amplified *dhfr* domain, but rather represent highly repetitive, interspersed sequences endogenous to the CHO cell genome. Note that the amplified fragments A and B, which map distal to the *dhfr* origin (Fig. 6), are not synthesized in extracts derived from early S-phase cells. Since 60 min is required for the entire cell population to enter the S phase, it is not unexpected that the 60-min labeling pattern appears quite simple. Clearly, by 240 min into the S phase (Fig. 5B, lane 3), synthesis of many, if not all, of the amplified *Eco*RI fragments is observed.

Order of fragment replication in vitro. Pulse-labeling studies of whole, synchronized CHOC 400 cells have identified a subset of amplified *Eco*RI fragments whose synthesis precedes all others from the amplified domain (15, 16). Prominent among these ELFs are a 6.1-kb doublet, termed ELF-F/F′, and an 11.6-kb single fragment, termed ELF-C. In contrast to these prominent early-replicating fragments, two large *Eco*RI fragments, A and B, replicate later in the S phase (15). Two different types of labeling studies (5) have shown that the synthesis of ELF-F/F′ precedes that of ELF-C, which in turn replicates earlier than either A and B. On the basis of these and other data (6), it was concluded that replication of the amplified *dhfr* domain initiates from an origin of replication located within the vicinity of *Eco*RI ELF-F/F′ and proceeds bidirectionally through origin-distal regions that include fragments A and B. A map of the positions of these fragments relative to one another and to the *dhfr* gene is provided in Fig. 6.

To determine whether the order of fragment labeling in vitro correlates with that seen in vivo, nuclei were isolated from cells released from the G1/S boundary into the S phase for 15 min and incubated en masse in vitro, and aliquots were removed at various intervals up to 180 min. Examination of the reaction products by autoradiography of restriction digests separated by gel electrophoresis shows that synthesis of the ELF-F/F′ doublet is detected within 10 to 20 min (Fig. 7B, lanes 1 and 2). By 20 to 30 min, signal from the replication of ELF-C was apparent, and by 60 min both fragments A and B appeared labeled (Fig. 7C). Note once again the prominent labeling of the two bands (denoted by asterisks in Fig. 7B) derived from interspersed, repetitive sequences endogenous to the CHO cell genome. The ELF-F/F′ signal does not appear to gain additional intensity after 30 mins, indicating that replication of these sequences has ceased by this time. It is clear from the labeling patterns, as well as determinations of the specific activities of the replication products (Fig. 7A), that synthesis in vitro has plateaued by 90 min. The products of the reactions seem quite stable, since the DNA from the 180-min time point remained undegraded and the labeling pattern was nearly identical to that from the 90-min sample (Fig. 7c). This labeling pattern suggests, as found in vivo, that ELF-F/F′ replicates prior to ELF-C and that ELF-C replicates prior to either fragment A or B.

Stability of nuclear replication complexes. The differential labeling patterns observed in nuclei isolated from cells poised at various points in the cell cycle, as well as the time course labeling experiment presented in Fig. 7, provide evidence that the synthetic patterns observed in vitro result from elongation of preinitiated replication complexes assembled in a temporally and positionally specific manner in vivo. If disruption of the cell during nuclear isolation promotes random disassembly and reassembly of replication forks in the nucleus, one might expect the labeling patterns of amplified sequences to lose specificity upon prolonged incubation in vitro. To test the stability of the replication complexes, we isolated nuclear extracts from G1/S-phase cells that had been released into the S phase for 10 min. A time course labeling experiment similar to that presented in Fig. 7 was conducted immediately on half of the nuclear preparation. The remainder of the nuclear sample was stored on ice in the absence of cytosol for 3 h and then was used in an identical time course labeling experiment. Note that nuclei from early S-phase cells have approximately 10% of the replication capacity of nuclei isolated from cells in mid-S

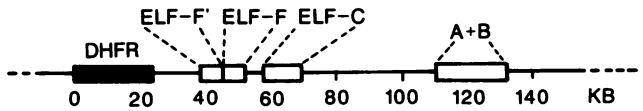

FIG. 6. Schematic representation of the amplified *dhfr* domain. The horizontal line represents a portion of one repeated amplicon from the amplified region of CHOC 400 cells. The positions of some *Eco*RI fragments relative to the *dhfr* gene are depicted. ELF-F and ELF-F′ represent the earliest-replicating portion of the *dhfr* domain that contain the *dhfr* origin of replication. Also shown are the relative map positions of amplified fragments A, B, and C discussed in the text. The *dhfr* gene is represented in a 5′-to-3′ (left to right) orientation. Abbreviation: KB, kilobase pairs. The map is a composite of those presented in references 16, 23, and 26.

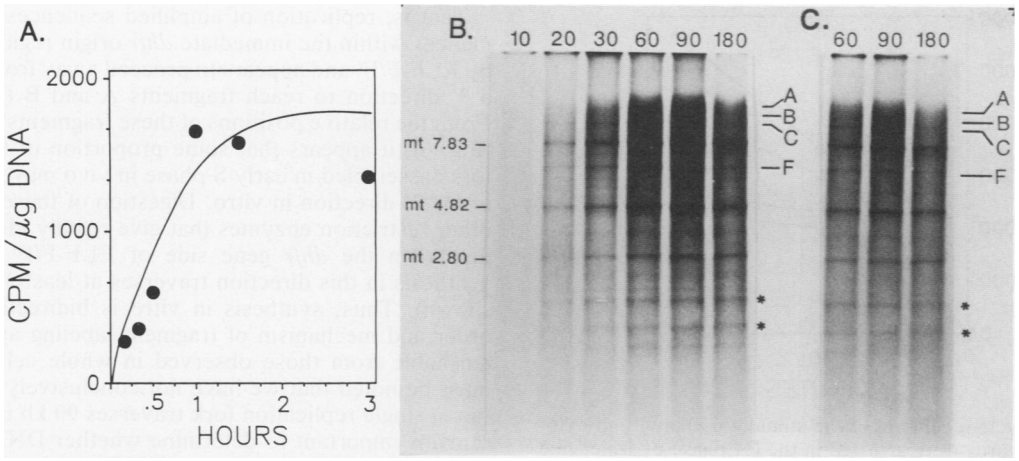

FIG. 7. Replication of amplified fragments in G1/S nuclei. Cultures were collected at the G1/S boundary and released into the S phase by being rinsed twice with fresh medium and reincubated for 15 min at 37°C. Nuclei were prepared and incubated en masse under standard replication reaction conditions in the presence of their cognate cytosol. At the indicated time, aliquots of the reaction mixture were removed, the nuclei were lysed, and DNA was prepared and digested with *Eco*RI. (A) The specific activity of each digest was determined by measuring dATP incorporation per microgram of DNA. (B) and (C) Each digest (2.5 μg) was subjected to agarose gel electrophoresis and autoradiography. The positions of amplified fragments A, B, C, and F are indicated. The sizes of mtDNA fragments are shown in kilobase pairs. The asterisks indicate interspersed, repetitive *Eco*RI fragments endogenous to the CHO cell genome that are not derived from amplified DNA. A shorter exposure of the 60-, 90-, and 180-min samples is provided in (C).

phase (Fig. 5). The labeling patterns of the two series of reactions are presented in Fig. 8.

Although the replication capacity of the stored nuclei (Fig. 8A) is diminished by 20%, it is clear that the specificity of the nuclear labeling pattern is not altered in nuclei maintained at 4°C for at least 3 h (compare Fig. 8A and B). Moreover, neither the degree of mtDNA synthesis nor the stability of the DNA template appears to be significantly diminished by storage of the nuclear extracts on ice. We have not investigated the stability of these parameters to freezing.

Cell cycle regulation of cytosol factors. The experiment shown in Fig. 1 reveals that cytosol extracts from asynchronous log-phase cells stimulate DNA synthesis in S-phase nuclei three- to eightfold. To determine whether the cytosol components that stimulate the synthetic capacity of nuclei are cell cycle regulated and to ascertain whether the specificity of labeling patterns is nuclear or cytoplasmic in origin, we prepared cytosol from cells starved for isoleucine for 48 h ($G1_0$ cytosol), from cells released from the isoleucine block for 4 h ($G1_4$ cytosol), and from cells released from the isoleucine block for 12 h (S cytosol) and compared the activities of these preparations in vitro in reaction mixtures containing S-phase nuclei. The incorporation rates and labeling patterns of nuclei from these time points in the presence of their cognate cytosol extracts are shown in Fig. 4. In Fig. 9, incorporation of dAMP by S-phase nuclei is shown to be stimulated, as before, by the addition of S-phase cytosol. In contrast, $G1_0$ cytosol preparations fail to stimulate DNA synthesis in S-phase nuclei. Interestingly, $G1_4$ cytosol preparations, which are derived from cultures just about to enter the S phase, have nearly as much stimulatory activity as do S-phase preparations. Autoradiography of restriction enzyme-digested DNA samples obtained from these reactions and separated by agarose gel electrophoresis shows that only the amount of replication, and not the qualitative nature of the labeling pattern, is affected by components of the cytosol extract (data not shown). In control experiments (data not shown), S-phase cytosol was incapable of inducing chromosomal DNA synthesis in $G1_0$ or $G1_4$ nuclei.

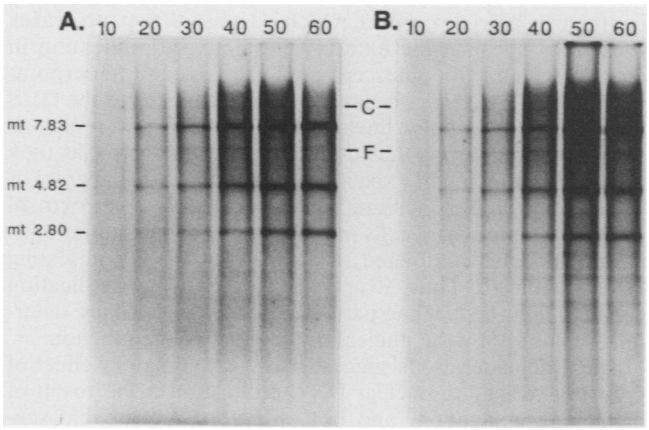

FIG. 8. Stability of the labeling pattern in G1/S nuclei. (A) Nuclei were prepared from cells collected at the G1/S boundary following a short (10-min) release into the S phase, and half the sample was stored on ice for 3 h in the absence of cytosol extract before a time course labeling experiment similar to that presented in Fig. 7 was conducted. (B) The remainder of the sample was immediately used in a time course labeling experiment. Note that G1/S nuclei that had been released into the S phase for only 10 min synthesize less DNA relative to the mtDNA fragments (mt; indicated in kilobase pairs) than do nuclei from cells released into the S phase for 15 min (Fig. 7B). The positions of amplified fragments C and F are shown.

DISCUSSION

We have adapted the conditions used for cell-free synthesis of simian virus 40 DNA (22) to the study of chromosomal DNA replication. The pellet fraction prepared by Dounce homogenization in a hypotonic buffer is enriched for nuclei and is capable of both mtDNA and chromosomal DNA synthesis in vitro. Mitochondria most probably copurify with nuclei, perhaps owing to their being trapped by cytoskeletal components still attached to the nuclei. mtDNA synthesis occurs in the pellet fraction isolated from cells at

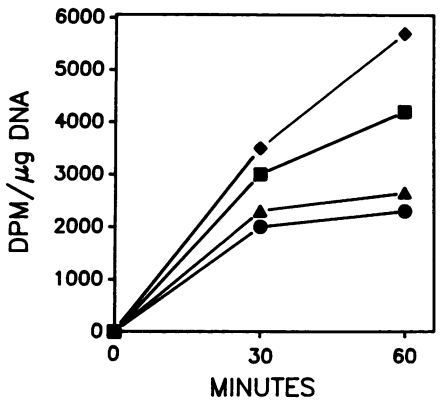

FIG. 9. Cell cycle regulation of the stimulatory activity of cytosol. Replicate cultures were arrested in the G1 phase by isoleucine deprivation for 48 h, at which time the cultures were induced to transit the G1 phase by the addition of complete medium. Cytosol extract was prepared from cells 0 ($G1_0$), 4 ($G1_4$), and 12 (S) h after the addition of complete medium and were used at equivalent protein concentrations in replication reactions with S-phase nuclei prepared from the 12-h cultures. At the indicated time, the reactions were terminated and the amount of dAMP incorporated per microgram of DNA was determined. For comparison, Fig. 4 depicts the activity of these cytosol extracts with their cognate nuclei. Symbols: ◆, S phase; ●, no cytosol; ▲ $G1_0$ cytosol; ■, $G1_4$ cytosol.

any point in the G1 or S phase, is not stimulated by factors present in the cytosol extracts, and is insensitive to either aphidicolin or antibodies against DNA polymerase alpha. mtDNA synthesis appears to be a convenient and suitable control for nonspecific inhibitors or contaminating nucleases in the in vitro reactions. Indeed, the relative amounts of mtDNA and nuclear DNA replication appear to be diagnostic for various phases of the cell cycle. Note, for instance, the differences in the ratios of mtDNA replication to nuclear DNA synthesis in nuclei isolated from cells at various times in the S phase depicted in Fig. 5.

In contrast to mtDNA synthesis, chromosomal DNA replication in isolated nuclei is highly stimulated by cytoplasmic factors, requires the activity of DNA polymerase alpha, and is temporally exact. A number of observations suggest that DNA synthesis in isolated nuclei represents authentic chromosomal DNA replication. First, we have been able to directly visualize the in vitro replication of specific restriction fragments derived from the amplified region of the CHOC 400 genome. Second, both aphidicolin and antibody inhibition experiments indicate that the synthesis of amplified fragments requires the activity of DNA polymerase alpha, a polymerase thought to be responsible, at least in part, for chromosomal replication in whole cells (17). Third, density shift experiments with BrdUTP show that the products of the in vitro reactions band in CsCl gradients at a hybrid density characteristic of fully replicated molecules. Fourth, the in vitro synthetic capacity of a fixed number of nuclei increases dramatically as a function of the position of cells in the early S phase, as found in vivo. Fifth, the synthesis of specific subsets of fragments derived from the amplified DNA is directly dependent upon the position of cells within the S phase. Thus, both the variation in synthetic capacity and the specificity of fragment labeling that occurs in whole cells are accurately reflected in these extracts. Finally, the order of fragment synthesis in nuclei isolated from cells at the G1/S boundary appears identical to that observed in whole synchronized cells.

That is, replication of amplified sequences in vitro commences within the immediate *dhfr* origin region represented by ELF-F/F′ and appears to proceed away from the origin in a 3′ direction to reach fragments A and B (Fig. 6 and 7). From the relative positions of these fragments in the genome (Fig. 6), it appears that some proportion of the replication forks assembled in early S phase in vivo may traverse 90 kb in the 3′ direction in vitro. Digestion of these samples with other restriction enzymes that give readily identifiable fragments on the *dhfr* gene side of ELF-F/F′ suggests that synthesis in this direction traverses at least 37 kb (data not shown). Thus, synthesis in vitro is bidirectional, and the order and mechanism of fragment labeling appear indistinguishable from those observed in whole cells, although it must be noted that we have not conclusively demonstrated that a single replication fork traverses 90 kb in vitro. It also remains important to determine whether DNA synthesis in purified nuclei proceeds via a semidiscontinuous mechanism and whether the DNA replicated in vitro is condensed into chromatin.

In cytosol extract titration experiments, maximal rates of chromosomal DNA synthesis are observed when the cytosol/nuclei ratios equal or exceed that of whole cells. Under optimal conditions, more than 90 to 95% of the replication activity observed in vitro can be attributed to nuclear DNA replication (Fig. 1), which correlates very well with the value reported for whole cells (9). Although the stimulatory effect of cytosol extracts on nuclear replication has been reported previously (see references 11, 18, 27, and 31 and references therein), the experiments with synchronized cells reported here provide direct evidence for an effect of cytoplasmic factors on the synthesis of specific chromosomal DNA fragments. It should be possible to fractionate the cytosol extract and identify specific replication factors, just as has been done with the simian virus 40 in vitro system (28).

Several observations suggest that it is unlikely that the cytoplasmic factors stimulating replication do so by virtue of induction of initiation. First, S-phase cytosol extracts under these conditions were not able to induce chromosomal replication in quiescent nuclei. This observation is in direct contrast to a recent report which suggested that proteases present in cytoplasmic extracts can activate replication in nuclei derived from quiescent cells (31). Second, incorporation of dAMP in nuclei isolated from cells poised at the G1/S interface is essentially linear for 60 min. If new replicons were activated during the 60-min incubation in vitro, as they are in vivo, one might expect that the replication capacity of the sample would increase with time. Moreover, cytosol extracts isolated from cells in mid-S phase, when initiation is assumed to be completed, are most active in stimulating DNA synthesis. Thus, it appears that both the replication capacity and the labeling pattern observed in vitro are determined entirely by the nuclear component of the reaction.

These considerations suggest that the stimulatory effect of cytosol extracts on nuclear DNA replication is the result of enhancing elongation, and not initiation. A probable scenario is that specific and stable initiation complexes are formed in vivo and that these can subsequently be elongated in vitro. Therefore, we conclude that elongation of replication may itself be a cell cycle-regulated event, since the simple mixing experiments discussed here (Fig. 9) indicate that cytoplasmic factors that stimulate replication are cell cycle regulated and that they are elaborated prior to initiation (i.e., are present in the $G1_4$ cytosol extracts). Thus, this system should prove particularly useful in identifying cytoplasmic factors that are required for legitimate chromosomal

DNA synthesis and perhaps in determining how these factors are regulated throughout the cell cycle. We are currently investigating whether the same cytoplasmic factors that are required for simian virus 40 DNA synthesis in vitro (28) stimulate legitimate chromosomal replication in isolated nuclei.

It is unlikely, for the reasons stated above, that the process by which new replicons are activated in whole cells occurs in the present in vitro system. A recent report suggests that whole-cell extracts from *Xenopus* eggs are able to induce chromosomal DNA synthesis in reconstituted nuclei (1). However, replication initiation did not appear to require specific DNA sequences. In contrast to the rapidly dividing *Xenopus* egg nuclei, mammalian chromosomes seem to require specific DNA sequences for initiation of DNA synthesis. Since we are able to directly differentiate between random labeling of all of the fragments derived from amplified DNA and synthesis of those few fragments replicated immediately upon entry into the S phase, it may be possible to adapt this system and evaluate various nuclear extracts or purified factors for their ability to induce site-specific initiation of DNA replication at the origin of replication of the *dhfr* amplicon.

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