High resolution analysis of the timing of replication of specific DNA sequences during S phase of mammalian cells

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ABSTRACT

A new method, utilizing selective photodegradation of 5-bromo-deoxyuridine (BUdR)-substituted DNA and flow cytometry, has been developed for analyzing the timing of replication of specific DNA sequences. Chemically synchronized Chinese hamster ovary cells were given a pulse of the deoxythymidine analogue, BUdR, at different times during S phase, and flow sorted according to DNA content, before DNA isolation. Newly-replicated, unifilarly BUdR-substituted DNA was selectively degraded by treatment with 33258 Hoechst plus near UV light followed by S1 nuclease digestion; the resistant DNA was analyzed for its content of 18s and 28s rDNA or dihydrofolate reductase (DHFR) sequences via Southern blot analysis. Both the rDNA and DHFR sequences were found to replicate almost entirely during the first quarter of S phase. The approach described should have general utility for analyzing replication kinetics of specific DNA sequences in mammalian cells.

INTRODUCTION

DNA replication in eukaryotes follows a specific temporal sequence during the S phase of the cell cycle. Initially, autoradiography was used to demonstrate that DNA from specific regions of Chinese hamster ovary (CHO) cell chromosomes replicated at distinct times during the same portion of S phase from one cell generation to the next (1,2,3). More recently, the order of replication of specific eukaryote structural genes has been investigated. These studies have generally employed 5-bromodeoxyuridine (BUdR) labelling and density gradient centrifugation to isolate newly synthesized DNA from synchronous cell populations, followed by assay for specific gene content by hybridization with RNA, cDNA, or cloned genomic probes (4,5,6). Using centrifugal elutriation to separate cells from an exponentially growing population, previously pulsed with BUdR, into discrete phases of the cell cycle, Furst et

at. (4) and Epner et al. (5) have independently demonstrated early replication of globin DNA sequences in mouse erythroleukemia cells. Also, Braunstein et al. (6) have shown a temporal order of replication of the immunoglobulin heavy chain constant region sequences $C\alpha$, $C\gamma$, and $C\mu$ which parallels their linear order in the murine genome.

Among eukaryote gene sequences, the ribosomal cistrons have been most extensively studied with respect to their time of replication during S phase (7,8,9; reviewed in 10). Amaldi et al. (7) first indicated that the rDNA genes of CHO cells replicate early in the 6.5 hour S phase, specifically between the first 1.5 to 3 hours. Early replication of rDNA in CHO cells was independently confirmed by two further studies (8,9). Of significance, these studies achieved CHO synchrony by three independent methods: double thymidine protocol (7), selective mitotic detachment (8), and isoleucine-starvation hydroxyurea block (9). Early replication of rDNA genes in CHO cells thus appears not to be an artifact caused by metabolic imbalance of the cell synchronization protocol. A different temporal order of rDNA replication has been reported for other eukaryote cell types (11,12,13). rDNA genes were found to replicate late in S phase in rat kangaroo cells (11) and throughout S phase of Hela cells (12), yeast (13), and Friend cells (5).

The structural gene for dihydrofolate reductase (DHFR) in CHO cells presumably exists as a single copy per haploid genome, as does the 31 kb DHFR sequence in the mouse (14). Early replication of amplified DHFR sequences in methotrexate-resistant Chinese hamster cells containing HSRs is suggested by detection of increased DHFR expression in the first part of S phase (15) and by autoradiographic analysis of the timing of [3 H]Tdr incorporation (16,17). However, the timing of replication of the single DHFR locus in methotrexate-sensitive CHO cells has not yet been determined.

The present study introduces a new methodology which is utilized for analyzing the time of replication of both rDNA and DHFR sequences in CHO cells. The approach employed differs from previous methodologies in two ways. First, flow cytometry has been used to sort CHO cells, according to DNA content, at different

times after release from G1/S arrest, to achieve a high degree of synchrony. BUdR was administered before cell harvest to label DNA at a given stage in replication. Second, near UV light plus the dye 33258 Hoechst has been used for selective introduction of nicks in DNA, substituted with BUdR during a narrow time interval, and these nicks were then converted to double strand breaks with S1 nuclease. DNA which is unifilarly substituted with BUdR (B-T DNA) is sensitive to ultraviolet light (18,19). This effect can be enhanced by complexing the DNA with 33258 Hoechst. Such photodegradation of B-T DNA was demonstrated cytologically by Goto et al. (20), localized to the BUdR-substituted-strand by Latt et al. (21), and quantitated by alkaline sucrose gradient centrifugation measurements by Rosenstein et al. (22). In the present study, conversion of single strand nicks in B-T DNA to double strand breaks by S1 nuclease obviates the need for density centrifugation for separation of B-T DNA from T-T DNA. Intact T-T restriction fragments were then subjected to agarose gel electrophoresis, and specific DNA sequences were assayed by Southern blot analysis (23). This methodology was utilized to determine that rDNA and DHFR sequences replicate at discrete times in early S phase of the CHO cell cycle.

MATERIALS AND METHODS

1) Cell culture and synchrony

CHO cells were grown in modified Hams F-10 medium and synchronized by an isoleucine-starvation, hydroxyurea block protocol (24) modified for monolayer cultures by Hamlin and Pardee (25).

2) Labelling of cells

G1/S-arrested CHO cells were transferred to fresh, complete medium supplemented with deoxycytidine (1 or 5×10^{-6} M), and their progress into S phase was analyzed using a FACS II fluorescence activated cell sorter. BUdR was administered as one hour pulses of 5×10^{-5} M nucleoside, a concentration providing an average of 80 $\pm$ 5% substitution of BUdR for dT in CHO cell DNA replicating throughout S phase, as estimated by CsCl density centrifugation (26). Cells trypsinized from monolayers were resuspended at 2.0×10^6 cells/ml in Hank's buffered saline

solution (pH 7.0, 4°C), 0.1% Triton X-100 and 2µg/ml 4',6-diamidino-2-phenylindole (DAPI). The fluorescence of the dye, DAPI, is not sensitive to BUdR at pH 7.0 (27).

In one experiment, cells arrested at G1/S were released in medium with or without BUdR, transferred to fresh medium at mid-S phase, and harvested by mitotic detachment.

Stained cells were vortexed, syringed from an 18 gauge needle through a 30 micron mesh to insure a single cell suspension, and stored stably at 4°C for up to 8 hours prior to flow cytometry (28).

3) Flow cytometry and sorting

The experiments utilized a Becton-Dickenson FACS II instrument equipped either with a Spectra Physics Model 164-05 argon ion laser with an output of about 100 mW in the ultraviolet (351-364 nm) or with a Coherent INOVA 90 5 watt argon ion laser with approximately twice this UV output. Fluorescence was detected after passage through a 450 nm band pass filter. DNA histograms, used to evaluate cell cycle progression, contained approximately 1000 fluorescent cells in the peak channel. Preparative experiments, sorting cells within pre-selected fluorescence intervals (windows) were performed at flow rates of approximately 3000 cells per second. Sorting windows were chosen to constrain overlap from one time point to the next and were approximately symmetric about the peak channel. Wider windows were chosen during later S phase time points.

4) DNA preparation

Mitotic cells, prepared by selective detachment, were pelleted at 1500 rpm, washed in PBS, and resuspended directly in lysis buffer (40 mM Tris-Cl, pH 7.5, 20mM EDTA, 100mM NaCl, 0.5% SDS).

Sorted cells (approximately 5×10^5 cells/ml) were pelleted by centrifugation at 11,000 rpm in a Brinkman Eppendorf centrifuge. Pellets were resuspended using a syringe and 22 gauge needle in 0.3M sucrose, 10mM Tris/HCl, pH 7.5, 5mM MgCl₂, 1.0% Triton-X100, and were vortexed, repelleted, and stored stably at -20° as pellets of nuclei until DNA preparation in lysis buffer.

Protein digestion with proteinase K (Boehringer Mannheim), 100 µg/ml, was carried out at 37°C for 4 hours. The lysate was

then extracted three times with phenol (100mM Tris-Cl, pH 8.0, saturated) and once with chloroform prior to ethanol precipitation and resuspension in TE buffer (10mM Tris-Cl, pH 7.5, 0.1mM EDTA). DNA concentrations were determined by the fluorometric assay using DAPI (29).

5) Preparation of a 10-20 kb band of T-T and B-T DNA

For those experiments assessing the number of DNA breaks, T-T and B-T DNA (200ug) were digested with HindIII restriction endonuclease to completion in 10mM Tris-Cl, pH 7.6, 50mM NaCl, 10mM MgCl₂, 14mM DTT at 37° for 3 hours in a 0.5 ml reaction mixture. An aliquot was analyzed on a 0.7% agarose gel to insure digestion. The restriction endonuclease was heat inactivated at 68° for 20 minutes, and the sample was loaded on a 18 ml 10-40% linear sucrose gradient (1M NaCl, 20mM Tris-Cl, pH 8.0, 10mM EDTA in a Beckman SW27 centrifuge tube) and centrifuged for 20 hours at 20°C, 27,000 rpm.

Fractions (0.5 ml) were collected and analyzed on an 0.7% agarose gel alongside HindIII digested bacteriophage lambda DNA molecular weight standards. Fractions containing 10-20 kb molecules were pooled and dialyzed. Dialysis was in 20mM Tris-Cl, pH 8.0, 10mM EDTA for 24 hours at 5°C. T-T and B-T broad band fractions were ethanol precipitated, resuspended in TE, and stored at 5°C, shielded from light before use in calibrating the rate of DNA strand breakage.

6) Enzyme reactions

Restriction endonuclease digestion of genomic DNA utilized approximately twice the enzyme exposure recommended by the vendor. Gel electrophoresis and Southern blot analysis were used to confirm that these conditions effected complete digestion of unifilarly-substituted (B-T) DNA, a substrate which digests more slowly than does unsubstituted DNA (30,31). Digestions carried out at 50 ug/ml DNA, were terminated by heating at 60°C for 5 minutes and addition of EDTA to 20mM.

7) Treatment with 33258 Hoechst and 365 nm light

DNA in restriction endonuclease digestion buffer was added directly to "nicking buffer". Final concentrations were 100mM Tris/HCl, pH 7.5, 100mM NaCl, 10 ug/ml DNA, 20 ug/ml yeast tRNA carrier, 20ug/ml 33258 Hoechst. For most experiments, 50 ul (1 ug

DNA) was transferred to a 400 ul capacity quartz cuvette (Coleman) and exposed to near UV radiation produced by a Blak Ray hand lamp, attenuated with a Corning 7-51 filter. This filter transmits light primarily in the 350 to 375 nm range. Peak illumination, at 365 nm, quantitated with an IL 500 radiometer, was approximately 100 erg per mm^2 per sec. Of note, 33258 Hoechst, when bound to DNA, has an absorption maximum at approximately 350 nm (32), affording efficient absorption of the applied illumination.

8) Treatment with S1 nuclease

After irradiation, the DNA in "nicking buffer" was transferred to a microcentrifuge tube and the dye was extracted three times with isobutanol (saturated with 100mM Tris-Cl, pH 7.5, 100mM NaCl). Without adequate isobutanol extraction, residual dye remains bound to the DNA, and subsequent Southern blots made with this DNA non-specifically affix the ^{32}P -labelled probe (data not shown). The DNA was ethanol precipitated, lyophilized, and resuspended in 20 ul of S1 nuclease buffer (30mM Na-acetate, pH 5.3, 75mM NaCl, 5mM ZnCl_2). DNA concentrations were again determined by the DAPI fluoremetric assay to insure equivalent DNA quantities per reaction and establish that no significant loss of DNA occurred during isobutanol extraction. Where indicated, 50 units of S1 nuclease (BRL) were added, and digestion carried out at 37°C for 2 minutes. The reaction was terminated by addition of 10 ul of S1 nuclease stop solution (100 mM Tris, pH 8.0, 2.5 mM EDTA, 50% glycerol, 0.1% bromophenol blue, 0.1% SDS) and heating at 60°C for 5 minutes. DNA was stored at -20°C or loaded directly on 0.7% agarose gels.

9) Gel electrophoresis and blot hybridization

Methods for electrophoresis, transfer of DNA to nitrocellulose filters, and hybridization of specific probes were previously described (33).

10) DNA probes and radiolabelling

A phage Charon 4A clone (MCR-1), with a 6.6 kb insert containing mouse 28s and 18s rDNA sequences (34) was a generous gift of Drs. J. Gusella and D. Housman. The insert was subcloned into plasmid pBR325 by the methods of Bolivar et al, (35) and nick translated (36) with E.coli DNA Polymerase I to a specific activity of 5×10^8 dpm/ug DNA. The plasmid (pDHR26)(37)

Figure 1. Selective breakdown of rDNA sequences in CHO cell DNA by 33258 Hoechst and near UV light treatment. DNA from CHO cells unsubstituted with BUdR (lanes 1,2,3) or synchronized and substituted (for one complete cycle) with BUdR in one polynucleotide chain (lanes 4,5,6) was digested with EcoRI, exposed to 33258 Hoechst and approximately 1.2×10^5 erg/mm² (20 minutes) (lanes 2,5) or 1.2×10^6 erg/mm² (200 minutes) (lanes 3,6) near UV light, electrophoresed, blotted, and probed with a 6.6 kb mouse rDNA segment. Untreated DNA is in lanes (1,4) while lane 7 contains ³²P-labelled lambda-HindIII size standards. The HindIII rDNA fragment in CHO DNA is about 6.5 kb. Selective degradation of BUdR-substituted DNA (lane 2 vs. lane 5 or lane 3 vs. lane 6) is apparent.

containing a mouse DHFR cDNA insert was digested with PstI to release the insert and labelled with T4 DNA polymerase (33).

RESULTS

Differential photodegradation of B-T and T-T DNA can be detected by neutral agarose gel analysis if samples are complexed with 33258 Hoechst and subjected to extensive illumination (Figure 1). A 6.5 kb B-T rDNA fragment has been degraded completely (i.e. beyond the resolution of the hybridization procedures used here) after a dose of 1.2×10^6 erg/mm² of 365 nm light has been given (lane 6).

A more rapid degradation of B-T DNA was effected by first complexing the DNA with 33258 Hoechst. S1 nuclease was used to convert light-induced nicks in the BUdR substituted strand to double stranded breaks (Figure 2). Figure 2A, lane 9, demonstrates the selective degradation of B-T DNA pretreated with dye

Figure 2. Dependence of DNA degradation on BUdR substitution, dye plus light, and S1 nuclease treatment. CHO cell DNA digested to completion by HindIII endonuclease and either unsubstituted with BUdR (T-T DNA) or substituted by one cycle of growth in BUdR (B-T DNA) was subjected to agarose gel electrophoresis with/without prior exposure to 33258 Hoechst (20 $\mu\text{g}/\text{ml}$), near UV light (5 min. at 100 $\text{erg}/\text{mm}^2/\text{sec}$) and S1 nuclease (50 units/ μg DNA for 2 minutes at 37°C). Lanes 2,6 (control); lanes 3,7 (S1 alone); lanes 4,8 (dye + light only); lanes 5,9 (dye, light, plus S1). Top) Picture of gel fluorescence after ethidium bromide staining. Bottom) Southern blot of the same gel, probed for rDNA sequences.

plus $3 \times 10^4 \text{ erg}/\text{mm}^2$ light, by a 2 minute exposure to S1 nuclease under the conditions specified in the METHODS section. A comparable reduction in molecular weight of HindIII digested genomic B-T DNA by dye plus light alone (not shown) requires $1.2 \times 10^6 \text{ erg}/\text{mm}^2$ irradiation. The Southern blot, (figure 2B) shows the partial degradation of the 20-25 kb HindIII rDNA fragment in lane 9. Little DNA in the 20-25 kb size range is apparent by ethidium bromide fluorescence, and only traces of the band hybridizing with the rDNA probe remain on the blot. Of note, T-T and B-T DNA not pretreated with dye plus light are equally resistant to S1 nuclease activity (figure 2A, lanes 3 and 7).

The increase in double strand breaks in B-T and T-T DNA generated by the standardized reaction conditions utilized (i.e. dye plus light followed by S1 nuclease) depends on the dose of 365

Figure 3. Rate of photodegradation of TT and BT CHO DNA by treatment with 33258 Hoechst plus near UV light followed by S1 nuclease digestion. A broad band of T-T and B-T HindIII digested CHO DNA, ranging from 10-20 kb, was prepared by the methods described in the text. DNA was irradiated with 365 nm light ($100 \text{ erg/mm}^2\text{-sec}$) for 0 minutes (lanes 2,3) 2 minutes (lanes 4,5), 3 minutes (lanes 6,7), and 4 minutes (lanes 8,9), treated with S1 nuclease for 2 minutes, and separated by electrophoresis on a 0.7% agarose gel alongside HindIII lambda molecular weight standards (lane 1). The negative of the photograph of the ethidium bromide stained gel (top) was scanned in a recording densitometer, the mean band position and hence size estimated, and the change in the reciprocal of this size plotted against illumination time (bottom). Slopes: (B-T DNA), $0.086 \text{ kb}^{-1}\text{min}^{-1}$; (T-T DNA), $0.0033 \text{ kb}^{-1}\text{min}^{-1}$.

nm light employed (figure 3). If the data are fit to a straight line over a light exposure interval from 6×10^3 to $2.4 \times 10^4 \text{ erg/mm}^2$, the slope, expressed in terms of the number of breaks per kilobase of double strand DNA per erg per mm^2 is 1.3×10^{-3} . The rate of B-T double strand breaks is more than 25 fold greater than is the rate of T-T double strand breaks.

For initial localization of the timing of replication of rDNA sequences, CHO cells were pulsed with BUdR during approximately the first or second half of S phase and harvested at metaphase. Progression beyond the G1/S block was assessed by flow cytometry after the cells had been stained with DAPI (figure 4).

Figure 4. Examples of DNA flow histograms of CHO cells, allowed to incorporate BUdR for approximately the first or second half of S phase. A) Logarithmically growing cells. B) Cells blocked at the G₁/S border. C) Cells released for 3-1/2 hours in control medium. D) Cells 4 hours after release in medium containing BUdR. Cells were trypsinized, permeabilized by Triton X-100 and stained with DAPI as described in the text. Indicated by vertical marks and channel numbers are G₁ and G₂ (A,B) or progression of DNA replication (C,D). BUdR was added to cells cultured as in (C), and cells cultured as in (D) were changed to medium lacking BUdR prior to continued growth, isolation at metaphase, and DNA isolation.

DNA prepared from the mitotic cells was digested to completion with EcoRI restriction endonuclease and analyzed with the rDNA probe (figure 5). Treatment with dye plus light has greater effect on diminishing the signal of early replicating B-T sequences (compare lane 7 to lane 4), suggesting that the rDNA sequences are early replicating. However, there is also some diminution of the rDNA band in lane 4 suggesting either (1) that some rDNA sequences replicate in the second third of S phase or (2) that, due to imperfect synchrony, there is some contamination of T-T DNA by B-T DNA from cells that were still in the early part of S phase.

To distinguish between these two explanations, cells were

Figure 5. Use of dye plus light sensitization to study the replication of ribosomal DNA sequences in CHO cells. DNA was extracted from cells labelled with BUdR during the second half (lanes 2,3,4) or first half (3-1/2 to 4 hours) of S phase (lanes 5,6,7). Untreated DNA is in lanes 2 and 5, while DNA treated with 1.2×10^5 erg/mm² (20 minutes) is in lanes 3 and 6 or with 1.2×10^6 erg/mm² (200 minutes) is in lanes 4 and 7. ³²P-labelled HindIII digested phage lambda DNA standards are in lane 1. Conditions are otherwise as in Figure 1. By comparing lanes 3 and 6 or lanes 4 and 7, it appears that rDNA in CHO cells (band at about 6.5 kb) replicates more in early S than in late S. However, the differences are not as extreme as those observed in Figure 1. Flow sorting experiments provide much cleaner results with higher resolution, both with regard to replication timing and tightness of synchrony.

sorted at one hour intervals during a seven hour S phase, following a one hour BUdR preharvest pulse (figure 6). The eight histograms in figure 6 depict the progression of the synchronized cell population through S phase. Important observations include: (1) The histograms are aligned vertically by the G1 and G2 fluorescence channels. (2) A fraction of G1/S arrested cells fail to enter S phase and is shown by the persistent peak of cells at the G1 fluorescence channel. (3) The sorting window chosen for each time point is represented by a bracket above the peak of the synchronized cell distribution. Although these windows increase in width over time, the ratio of the channel half width to the peak fluorescence channel was typically less than 10% (Table 1). (4) The baseline coefficient of variation (CV) of the fluorescence

Figure 6. DNA flow histograms of CHO cells allowed to incorporate BUdR for one hour intervals during S phase. Cells were arrested at G1/S and, after release, were given a one hour preharvest pulse of BUdR. (A) depicts G1/S arrested cells. Pulses of BUdR are indicated. Histograms are aligned using the G1 and G2 fluorescence channels with channel numbers indicated. Windows sorted at each time point are shown with a bracket.

activated cell sorter, defined as the ratio of the standard deviation of a distribution to that distribution's mean fluorescence is 3-4%. This value is a numerical statement of the ability of the fluorescence activated cell sorter to resolve populations of cells differing in fluorescence and hence DNA content.

The progression of S phase can be appreciated graphically (figure 7). The mean fluorescence distribution (i.e., the peak of the distribution) increases in DNA content more rapidly in mid S phase than near the start or finish of replication, indicating

Figure 7. Time dependence of progression through S phase of CHO cells release from G1/S arrest. Histograms were determined by flow cytometry and are depicted in Figure 6. S phase progression is depicted as the fraction of G2/G1 fluorescence which the population of synchronized cells has progressed. The brackets represent the fractions of S phase, centered approximately about the peak of the cell distribution, which were sorted.

that DNA replication begins at a slow rate, accelerates to a peak rate during mid S phase, and then slows down towards the end of S. Because the rate of DNA replication is variable with time in S phase and because the pulse interval chosen (one hour) was constant, the amount of B-T DNA per cell analyzed depends on the time in S at which the BUdR pulse was given.

The DNA from the cells sorted in the windows indicated in figure 6 was digested to completion with HindIII endonuclease and exposed to the standard conditions of dye plus light and S1 nuclease described above. This DNA was subjected to electrophoresis in an agarose gel, blotted, and hybridized with the rDNA probe described (figure 8). As an internal control, whole B-T DNA (lanes 15,16) was exposed in parallel in order to insure B-T selective degradation. The weak signals in lanes 2,4, and 6 reveal that all of the rDNA sequences in these cells have replicated by the end of the third hour of S phase. Most of rDNA replication is occurring during hour 1-2, as denoted by the weakest signal in lane 4. rDNA replication is barely apparent during hour 0-1. Moreover, total cellular DNA replication is barely apparent at 1 hour (figure 6B and figure 7, 1 hour time point). Therefore, by the

Figure 8. Use of dye plus light plus S1 nuclease to identify early replication of rDNA in CHO cells. CHO cells were synchronized at the G1/S interface, released, pulsed with BUdR during different intervals, harvested, and stained with DAPI. Cells from each time point were analyzed in a FACS II flow cytometer, cells in a narrow range around the modal DNA content sorted, the DNA isolated, digested to completion with HindIII endonuclease, and half of each sample treated with 33258 Hoechst, light (approximately 10 minutes, or 6.0×10^4 erg/mm², 365 nm), and S1 nuclease (50 units/ug DNA/2 minutes at 37°C). Samples (0.5 ug DNA) were then subjected to electrophoresis, blotted onto nitrocellulose, and probed with ³²P-labelled rDNA. Lane constitution: BUdR during 0-1 hour (lanes 1,2); 1-2 hours (3,4); 2-3 hours (5,6); 3-4 hours (7,8); 4-5 hours (9,10); 5-6 hours (11,12); 6-7 hours (13,14); substituted DNA prepared from one cycle in BUdR (15,16) and ³²P-labelled lambda HindIII size standards (17). Dye plus light plus S1 treatment is indicated by (+); control (S1 nuclease treated) samples are indicated by (0).

resolution offered in this technique, rDNA sequences are among the very first sequences replicated. The absence of degradation in lanes 8,10,12 and 14 shows that the rDNA genomic sequences have completed DNA replication after three hours of S phase. That is, the rDNA sequences have completed replication before the bulk (approximately 60%, figure 7) of the remaining genomic DNA has replicated.

The full length rDNA fragment intensity is greater in lanes 3,5, and 7 and diminishes in lanes 9,11, and 13. This fact provides further verification of the timing of rDNA sequence replication during early S phase. In general, one expects the relative quantity of a given DNA sequence to be a maximum at the instant it has completed its replication. As further replication proceeds this sequence will represent a decreasing fraction of the

TABLE I

Time Interval In S (hrs)	Experiment 1		Experiment 2		
	Fraction S-Phase	Fraction Sequence Degradable** rDNA	Fraction S-Phase	Fraction Sequence Degradable**	
				rDNA	DHFR
0-1	0(0-0.05) ⁺	0.65	0.12(0-0.27)	1.00	1.00
1-2	0(0.07-0.18)	0.78	0.26(0.13-0.39)	0.78	0.76
2-3	0.2(0.19-0.32)	0.59	0.53(0.26-0.80)	0.65	0.66
3-4	0.46(0.39-0.57)	0 ⁺⁺	0.73(0.58-0.88)	0.39	0.42
4-5	0.66(0.56-0.80)	0.09	0.91(0.73-1.00)	0.20	0
5-6	0.92(0.76-0.93)	0.32	1.00(0.92-1.00) ⁺⁺	0.03	0.16
6-7	1.00(0.92-1.0)	0 ⁺⁺			

⁺ Mean (range) of S phase progression of cells sorted for DNA analysis.

⁺⁺ Intensity of "treated" DNA hybridization actually slightly greater than that of "control."

⁺⁺ 5-6 1/2 hours.

** Determined by densitometric analysis of X-ray film exposures of DNA blots. The data for DHFR cDNA hybridization are derived solely from the more intense, 8kb band (figure 9).

total DNA in the cell. Equal amounts of DNA were loaded per lane, facilitating densitometric comparison of the hybridization signals used for localizing specific sequence replication to discrete intervals in S phase (Table 1).

In another experiment with CHO cells, in which the S phase was of slightly shorter duration, with less of a lag in DNA synthesis after release of cells from the G1/S block, most of the rDNA sequences have completed replication during hour 0-1, as shown by the absence of a full length rDNA band in lane 4 (figure 9, top). The variability of S phase duration and of the timing of DNA replication after release from G1/S arrest render absolute time a poor scale of measurement. These values probably vary because the time that cells require to recover from hydroxyurea block varies from synchrony to synchrony. A scale on which to localize rDNA replication more consistently is the fraction of S phase progression, i.e., the fraction average of DNA content increase over that in G1, relative to the difference in DNA contents between cells in G2 and those in G1. In both figures 8 and 9, all rDNA replication has been completed when S phase has progressed to 38% (see upper limit of 3 hour sorting window, figure 7).

The limits of resolution of the present analysis can be further defined by exploring the replication kinetics of a unique sequence probe. The bottom of figure 9 shows the blot from the

Figure 9. Replication of the CHO rDNA sequences and DHFR sequences during the early part of S phase. An experiment (#2, Table I), analogous to the sequential figures 6, 7, and 8 was performed for a synchronized CHO cell population with a 6 1/2 hour S phase. The subsequent blot was first hybridized with the DHFR₃ probe (bottom), stored for one month to allow decay of ³²P, and rehybridized with the ³²P-labelled rDNA probe (top).

top of this figure, hybridized with a cloned probe for DHFR sequences. The extensive degradation of the DHFR sequences in lane 4 shows that DHFR replicates primarily during the first hour of S phase, or alternatively, during the first 25% of S phase progression. Partial degradation of DHFR sequences in lanes 6 and 8 suggest replication of DHFR DNA as late as hour 3 in some CHO cells.

DISCUSSION

The experiments described utilize two refinements in the methodology for examining the order of replication of specific DNA sequences during the S phase of the mammalian cell cycle to determine precisely the timing of 18s and 28s rDNA and DHFR sequence replication. First, as a tool for fractionating cells at a discrete interval in S phase, the fluorescence activated cell sorter has been used. A presynchronized culture of CHO cells was given a one hour preharvest pulse of BUdR at one hour intervals during the seven hour S phase. Harvested cells were stained with the DNA specific dye, DAPI, and analyzed with the fluorescence activated cell sorter on the basis of DNA content. The progression of DNA replication was monitored by following the peak of the fluorescence distribution during S phase versus time. Sorting windows were set about the fluorescence peak for each time point. Cells were sorted from these seven discrete windows in S phase and DNA content was analyzed for newly-replicating sequences. Fluorescence activated cell sorting is particularly useful in dissecting regions of S phase during which DNA replication is most rapid (middle S phase).

This technique permits resolution of cells that had progressed into S phase by discrete increments based on DNA content. The fraction of S phase progression serves as a useful reference point when assessing the relative order of replication of individual sequences. The progression of the CHO cells in the present experiment from G1 to G2 is more rapid in mid S than in early or late S, in accord with previous data (e.g. 38). The baseline coefficient of variation (CV) of the DNA flow histograms obtained is only 3-4%. This is negligible compared with the width of the BUdR pulse employed. That is, the ratio of the increase in fluorescence during a one hour pulse of BUdR to the mean fluorescence of the cell population at the end of the pulse ranges from 8% to 20% for the seven intervals chosen (Figure 6; experiment #2, Table 1). Also, the one hour interval is large compared to the time required for nucleotide uptake, metabolism, and incorporation into DNA (a few minutes) (39). BUdR pulses shorter than one hour are limited by the baseline CV and by the lag in BUdR uptake. The minimum practical pulse interval has not

yet been determined.

The present experiments utilized agents capable of inhibiting cell cycle progression to create a presynchronized cell culture, and it is conceivable that the isoleucine deprivation-hydroxyurea block method (25) used may have altered DNA replication, particularly that immediately following release from this block. Also, release of cells from the G1/S block is often incomplete (Figure 7), in which case contamination of cells sorted in early S phase windows may be contaminated with G1 cells, lowering the fraction of degradable DNA, e.g. 0-1 hr time point of experiment #1, Table 1. Release of cells from G1/S was more complete in experiment #2 of Table 1 (data not shown), and degradation of DNA in the 0-1 hr sample is more complete. Recent studies have also shown that pharmacologic perturbation can increase the number of origins of replication and decrease the speed of DNA replication at the replication fork (40). However, there is agreement on early replication of rDNA sequences in CHO cells which were presynchronized by three independent techniques (7,8,9). In addition, while cell synchronization reduces flow sorting time, it is not absolutely necessary. That is, synchrony provides cell distributions with high concentrations of cells in any pre-determined fluorescence window. Sorting S phase cells from a logarithmically growing distribution is slow because of the comparatively few cells in S phase and the large number of cells cells by centrifugal elutriation, as an enrichment step, prior to flow cytometric analysis and sorting.

The second refinement provided by these experiments is the use of differential photodegradation of unifilarly substituted (B-T) DNA. A mixture of B-T and T-T sequences, prepared from a homogeneous population of S phase cells pulsed with BUdR, is treated with 33258 Hoechst plus 365 nm light for a few minutes. The B-T DNA is selectively nicked in the BUdR-containing strand. A subsequent two minute treatment with S1 nuclease generates double strand breaks and a selective removal of B-T sequences from the analyzable DNA pool. This subtractive technique is rapid and convenient. Newly-replicated sequences have classically been density labelled with BUdR and fractionated on CsCl gradients. Density gradients are costly, requiring 24-48 hours of

ultracentrifugation, and provide imperfect separation (6). Selective B-T photodegradation is particularly useful when working with small quantities of DNA. There is effectively a 90% recovery of intact T-T sequences from the procedure, as determined by DNA quantitation before and after dye plus light and S1 nuclease. Therefore, selective B-T photodegradation is useful in analyzing DNA from flow sorted cells ($\leq 10^6$ cells/single sequence analyzed), with DNA yields typically in the one microgram range.

The procedure employed for selective B-T photodegradation was highly reproducible. The standardized reaction conditions, including dye plus 365 nm light and S1 nuclease, provide a constant rate of B-T degradation for irradiation doses up to 24,000 erg per mm^2 . Assuming an 80% substitution of BUdR for dT in the BUdR-containing strand, this rate is 7×10^{-8} double strand breaks per BUdR residue per erg per mm^2 . It is thus possible to estimate the dose of 365 nm light required to degrade a B-T fragment of a given length. That is, the degradation of a sequence of defined length yields a distribution of fragmented molecules whose mean size is inversely proportional to the dose of near UV light. The present results are compatible with data of Rosenstein et al. (22), who used somewhat different conditions and found that treatment with 33258 Hoechst plus near UV light yielded a photolysis rate of 2.3×10^{-8} single strand breaks per BUdR residue per erg per mm^2 .

The present data are compatible with the interpretation that the 18s and 28s rDNA sequences of CHO cells initiate and terminate replication by the time the S phase has progressed to 35-40% of total DNA replication. Most rDNA begins replication within the first 10% of progression from G1 to G2. Cytogenetic studies (41) show that the expressed CHO rDNA repeats are localized to several autosomal telomeric regions which are probable sites of rDNA clusters of replicons. Autoradiographic studies (42) indicate that clusters of replicons in CHO cells initiate and terminate replication during discrete, approximately one hour intervals. Thus, it is possible that rDNA replication in CHO cells involves clusters of rDNA-containing replicons at several telomeric loci which synchronously initiate replication very early in S phase. Whether some initiation signal acts independently at each

telomeric cluster or the clusters are spatially adjacent to each other or some nuclear architecture at the time of DNA synthesis initiation is not yet known. One possible "signal" postulated for early S phase initiation is the low level of the dNTP pools at this stage in the cell cycle (42).

Consistent with the published data regarding amplified DHFR sequences (15,16,17), the unique DHFR sequence in methotrexate-sensitive cells is replicated, at least for the most part, early in S phase. In the present experiments, the DHFR sequence replicates before 27% of bulk DNA replication has occurred.^{FN} At a replication fork rate of 5 kb per minute in CHO cells (43), an individual sequence might conceivably complete replication in a window as narrow as one minute. That the DHFR sequence replicates during a larger window (i.e., two hours, in this study) suggests (1) the resolution described in this report is imperfect, (2) temporal order is not as constant in early S phase as originally postulated, or (3) DNA polymerase stalls and does not proceed with local replication at a constant rate. Repeat studies with shorter BUdR pulses and narrower, non-overlapping windows may reveal an even narrower replication interval for DHFR or other single-copy sequences.

Previous cytologic studies have shown that the timing of replication of a DNA sequence is a function of chromosome position, as defined by chromosome banding patterns (44). Very recent data (45) indicate that transcribed sequences may be found in both early and late replicating chromatin. The present work has identified two types of transcribed sequences which replicate predominantly during the first part of S phase. Additional experiments will be necessary to determine the existence and nature of mechanism(s) by which the potential of DNA sequences to be expressed might be coordinated with the control of their replication timing.

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A small amount of degradability in the DNA from cells sorted after a BuDR pulse during the fourth hour of S phase may represent cells that released from the G1/S block later than the majority of cells, and then progressed through S phase with unusual rapidity, or it may reflect experimental error. Conclusions about DHFR and rDNA sequences replicate all or for the most part early in S phase remain unchanged by this observation.

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