S phase-specific synthesis of dihydrofolate reductase in Chinese hamster ovary cells

(cell cycle/methotrexate-resistance/fluorescence-activated cell sorter)

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ABSTRACT We investigated the cell cycle modulation of dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase, 7,8-dihydroxyfolate:NADP⁺ oxidoreductase, EC 1.5.1.3) levels in methotrexate-resistant Chinese hamster ovary cells synchronized by mitotic selection. DNA content and DHFR concentration were analyzed throughout the cell cycle by standard biochemical techniques and by double fluorescence staining utilizing the fluorescence-activated cell sorter. We found an S phase-specific period of DHFR biosynthetic activity. Commencing within hour 2 of S phase and continuing throughout the duration of S phase, there is a 90% increase in DHFR specific activity. This results from an ≈ 2.5 -fold increase in the level of DHFR, while total soluble protein increases 50% during the same period. This increase is the result of new synthesis of DHFR molecules initiated after the cell is physiologically committed to DNA replication. This increase in DHFR activity through S phase parallels the increasing rate of [³H]thymidine incorporation during the same interval. The maximum peak of DHFR activity is coincident with the maximum rate of DNA synthesis, both activities occurring during the bulk of DNA replication within the last stages of the 6.5-hr S phase.

Control of specific protein synthesis in the framework of the cell cycle represents a fundamental form of regulation. Numerous enzyme activities have been studied as a function of S phase in mammalian cells. The activities of DNA polymerases (review in ref. 1) and the enzymes necessary for the provision of deoxyribonucleoside triphosphates (review in ref. 2)—i.e., thymidine kinase (3), thymidylate kinase (4), thymidylate synthetase (5, 6), dihydrofolate reductase (7), ribonucleotide reductase (8), and deoxycytidine monophosphate deaminase (9)—follow a general pattern of increasing through S phase and attaining a maximum near the S/G₂ interface. We investigated one enzyme involved in the integrative process of growth regulation: dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase, 7,8-dihydroxyfolate:NADP⁺ oxidoreductase, EC 1.5.1.3).

DHFR is necessary for the production of tetrahydrofolate, a key intermediate in one-carbon transfer reactions. Thus, DHFR activity is temporally coupled with the maintenance of sufficient thymidylate pools necessary to support DNA synthesis. Normally, the intracellular concentration of a “housekeeping” enzyme such as DHFR is extremely low—0.1% of total protein (7). The low concentration of DHFR limits any study exploring the biochemical parameters involved in regulation. This study takes advantage of a methotrexate (MTX)-resistant Chinese hamster ovary cell line, K₁B₁₁0.5, which contains elevated levels of DHFR corresponding to an amplified number of genes encoding the information for DHFR production, the target enzyme for methotrexate inhibition (10).

A previous report (11) has centered on the modulation of

DHFR content in MTX-resistant mouse 3T6 fibroblasts when serum-deprived cells were induced to reenter the cell cycle as a result of serum replenishment. Although this phenomenon of a phase transition from a metabolically quiescent state to a state of active proliferation has clear physiological significance (1), our investigation focused on the modulation of DHFR levels within the framework of a single, physiologically continuous cell cycle.

We achieved precise cell cycle synchrony by the selection of mitotic cells from exponentially growing monolayers. We determined the specific activity of DHFR throughout the cell cycle, using the standard [³H]folic acid reduction assay (12). The fluorescence-activated cell sorter (FACS) was used to simultaneously quantitate the levels of DHFR in parallel with precise DNA content determination in exponential and synchronous cell populations that were doubly labeled with fluorescent Hoechst 33342 and fluorescein-methotrexate (MTX-F). We also examined the pattern of new DHFR biosynthesis in [³⁵S]methionine-labeled synchronous cultures processed by NaDodSO₄/polyacrylamide gel electrophoresis.

The data shows that the concentration of DHFR remains constant throughout the G₁ period and into hour 1 of S phase. DHFR synthesis initiates within hour 2 of S phase and continues through the DNA replicative phase. The number of DHFR molecules more than doubles in S phase, with maximum enzymatic specific activity coincident with maximum DNA replication in late S phase.

MATERIALS AND METHODS

Cells and Culture Conditions. Chinese hamster ovary cells were maintained in medium I (Ham's F₁₂ without glycine, hypoxanthine, and thymidine; GIBCO). The medium was supplemented with 10% (vol/vol) dialyzed fetal calf serum (GIBCO) and 100 units of penicillin and 100 μ g of streptomycin per ml. The parental, MTX-sensitive Chinese hamster ovary (CHO) cell line CHO-K₁ was provided by L. Chasin (Columbia University). K₁B₁₁0.5 is a clone of CHO-K₁ derived in this laboratory by R. Kaufman (10) and is stably resistant to 0.5 μ M MTX. K₁B₁₁0.5 cells were maintained in medium I with 0.5 μ M MTX. The MTX was removed four generations before each experiment. The CHO-K₁ cell line and the MTX-resistant derivative K₁B₁₁0.5, when grown in either the presence or absence of 0.5 μ M MTX, have identical 12-hr generation times based on exponential growth kinetics. Cell line K₁B₁₁0.5 has been selected stepwise for resistance to MTX (10), contains 50 times the

Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; MTX-F, fluorescein methotrexate; FACS, fluorescence-activated cell sorter; CHO, Chinese hamster ovary.

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DHFR specific activity, and is resistant to 50 times the concentration of MTX that kills 50% of the sensitive parentals (10 nM). Cell lines with much higher resistance have been selected in our laboratory, but these lines are unsuitable for precise cell cycle analysis because the generation times of these high-resistance variants increased due to a lengthening of the G_1 period. Because the sensitive parentals were controls in all experiments, we used a resistant cell line, $K_1B_{11}0.5$, that did not deviate from normal cell cycle kinetics with respect to the CHO- K_1 background.

Mitotic Cell Selection. Cultures were synchronized by mitotic selection by a modification of the method as described (13). Exponential cultures of CHO- K_1 and $K_1B_{11}0.5$ were grown in 150-cm² tissue culture flasks (Costar Plastics, Cambridge, MA). The medium was drained, 5–10 ml of fresh prewarmed medium I was added, and the flasks were tapped four times on the sides with the palm of the hand. This medium, containing dislodged mitotic cells, was removed and replated in a 25-cm² or 75-cm² flask. At 30 min after selection, the culture medium was gently removed, filtered through a 0.45- μ m Nalgene filter, and added back to the culture. This ensures the elimination of dead, interphase, and mitotic cells that fail to plate out.

[³H]Thymidine Labeling. To determine the rates of [³H]thymidine incorporation during the S phase of the CHO cell cycle, synchronous populations of both CHO- K_1 and $K_1B_{11}0.5$ grown in T-25 flasks were labeled for 15 min at 37°C at hourly intervals throughout the cycle with 1 ml of medium containing 2.0 μ Ci (1 Ci = 3.7×10^{10} becquerels) of [³H]thymidine per ml ([methyl-³H]thymidine, 6.7 Ci/mmol; New England Nuclear). Rates of [³H]thymidine incorporation were determined by terminating the labeling with the addition of ice-cold Hanks' balanced salt solution with unlabeled thymidine (10 μ g/ml). Cells were digested with trypsin, pelleted, and then resuspended in 10 mM Tris-HCl, pH 8.0/0.01 M EDTA/0.5% NaDodSO₄ lysis buffer at 22°C. Trichloroacetic acid (10%, wt/vol; 4°C) was added, and the extracts were kept on ice for 30 min. Trichloroacetic acid-precipitable material was collected on Whatman glass fiber filters (GF/C) and washed with 5% trichloroacetic acid. Filters were dried and subjected to liquid scintillation counting in "Liquiscent" (National Diagnostics, Somerville, NJ). Rates of [³H]thymidine incorporation were expressed as total precipitable radioactivity incorporated per 10⁶ cells.

Percentage of Labeled Nuclei in Interphase Cells. Synchronized populations of CHO cells were plated onto acid-cleaned, sterilized coverslips. Using the same conditions and procedures as before to determine rates of [³H]thymidine incorporation, we labeled these monolayers also for 15 min at 37°C with [³H]thymidine, incorporation of which was terminated by three washes with Hanks' solution at 4°C. Monolayers were fixed in methanol/acetic acid, 3:1 (vol/vol), and prepared for autoradiography as described (14). The percentage of labeled nuclei were scored from at least 500 cells. Control cultures treated with hydroxyurea were used to determine random background levels of grain appearance over non-S-phase nuclei.

Chromomycin A₃ Staining. Cell populations were analyzed for DNA content on the FACS by using the DNA fluorochrome chromomycin A₃ (15). Exponential and synchronous cell populations were harvested by treatment with trypsin, fixed in 70% ETOH, and stained in 15 mM MgCl₂ with 20 μ g of chromomycin A₃ per ml for 1 hr at 22°C as described (15).

Hoechst 33343 Staining. Exponential and synchronous cultures were prepared for DNA content analysis on the FACS by the addition of Hoechst (1 mM) 33342 (Calbiochem-Behring) (16) to the culture medium to a final concentration of 10 μ M. Cultures were incubated for 1 hr at 37°C prior to cell harvesting and FACS analysis.

MTX-F Labeling. Exponential cultures of CHO cells from which mitotic cells were selected were incubated for 24 hr at 37°C with the fluorescein derivative of MTX at 1 μ M. All aspects of MTX-F labeling and cell preparation for FACS analysis have been described (17).

FACS Analysis. The FACS II (Becton-Dickinson FACS Division, Mountain View, CA) in the laboratory of L. Herzenberg (Stanford University School of Medicine) was used for all quantitative fluorescence analyses and cell sortings (18). UV excitation (355 nm) for Hoechst 33342 and visible excitation for both MTX-F (488 nm) and chromomycin A₃ (457 nm) were generated by a Spectrophysics argon ion laser. For doubly labeled cells (MTX-F and Hoechst), DNA analysis was done first, followed by DHFR analysis after the laser had been switched to the proper wavelength mode. Cells were sorted on the basis of DNA content into G_1 , S, and G_2 subpopulations by setting the appropriate fluorescence windows with respect to fluorescence intensity after scanning the exponential population. From each cell cycle-phase subpopulation, 100,000 cells were sorted into 0.25 ml of dialyzed fetal calf serum at 4°C.

Determination of DHFR Specific Activity and Total Protein Content. Preparations of cell extracts and quantitation of DHFR specific activity were as described (19). Total soluble protein was measured by the method of Lowry (20). All assays are done in duplicate or triplicate.

A more sensitive quantitation of total soluble protein content throughout the cell cycle was obtained with the steady-state radiolabeling procedure as described (21).

[³⁵S]Methionine Labeling of Protein and Polyacrylamide Gel Electrophoresis. To examine new protein synthesis at various stages of cell cycle transit, synchronous populations were labeled for 30 min with [³⁵S]methionine at 100 Ci/ml (1140.0 Ci/mmol, New England Nuclear) in 1 ml per T-25 flask at 37°C. Labeling was terminated by the addition of ice-cold Hanks' solution with unlabeled methionine (10 mM) and cycloheximide (50 μ g/ml). Monolayers were scraped with a rubber policeman and collected by centrifugation. Pellets were resuspended in 500 μ l of 10 mM potassium phosphate (pH 7.0), and soluble protein extracts were prepared as for the enzymatic assays.

NaDodSO₄/polyacrylamide gel electrophoresis was performed with 12.5% (wt/vol) separating gels and 4% (wt/vol) stacking gels. Electrophoresis buffer consisted of 14.4 g of glycine, 3.0 g of trizma base, and 1.0 g of NaDodSO₄ per liter of H₂O. Electrophoresis was run for 12–14 hr at 80 V. For fluorography, gels were impregnated with EN³HANCE (New England Nuclear). Exposures of the dried fluorographs were carried out for 24–72 hr with XR-1 film (Kodak) at –80°C.

RESULTS

Cell Cycle Synchrony by Mitotic Selection. The degree of cell cycle synchrony is shown in Fig. 1A. Cells selected in mitosis plated out within 20 min of selection and completed cytokinesis by 45 min. The first increase in cell number began at 10.5 hr and was complete 2.5 hr later. By 12 hr after mitotic selection, 50% of the population had divided. These data were identical to the division cycle profile for the parental CHO- K_1 (data not shown). With this synchrony method, 98% of the mitotically selected cells proceeded through mitosis, and 95–100% of these cells proceeded through the next round of division, based on viable cell number quantitation and FACS analysis.

Rates of [³H]Thymidine Incorporation Through S Phase and Percentage of Labeled S-Phase Cells. The first increase of [³H]thymidine incorporation above G_1 levels was observed in cells entering hour 4 of the cell cycle (Fig. 1A). The rate of [³H]thymidine incorporation increased steadily until a peak value was reached at hour 9, in agreement with Klevecz *et al.*

FIG. 1. Cells synchronized by mitotic selection. (A) Cell numbers were determined with a Coulter Counter and expressed relative to the number of cells plated out within the first hour (●). Rates of [^3H]thymidine incorporation are expressed relative to 10^6 cells (■). The percentage of cells incorporating [^3H]thymidine was determined by autoradiography of monolayers (▲). (B) DHFR specific activity was determined in "soluble" protein extracts (■) as described (19). Total protein content was determined by growing exponential cultures in [^3H]leucine (5.0 $\mu\text{Ci}/\text{ml}$) for 48 hr prior to mitotic selection and measuring [^3H]leucine incorporation (●). Equal numbers of synchronized cells were plated per flask. Each value is the average for duplicate plates per time point.

(13). The rate of incorporation decreased from this point as cells progressed into G_2 and eventually into mitosis at hour 12. Based on this data, the S phase of $K_1B_{11}0.5$ cells comprised 6.5 hr (hours 4–10.5) with G_2/M occupying the final 1.5 hr. The G_1 period was 4 hr.

To assess independently the behavior of the population during the entry into S phase, autoradiography was carried out on synchronous monolayers in parallel cultures at each of the cycle time points (14). In this manner, the percentage of cells in S phase could be established at each hour in the cycle. At 3 hr 5% of the nuclei were labeled, and at 4 hr (Fig. 1A) the entry into S phase by the total population was evident, with 86% of the cells showing labeled nuclei. The autoradiographs (data not shown) showed the uniformity and specificity of nuclear labeling. By hour 10 the first evidence of synchrony decay was seen. Although a large proportion of the cells was still in S phase, the grain density was no longer uniform, and 20% of the cells showed no [^3H]thymidine incorporation during the 15-min pulse.

DNA Histograms of Synchronous and Exponential Populations Utilizing the FACS. In the DNA histogram of an exponentially growing population of $K_1B_{11}0.5$ cells stained with chromomycin A_3 (Fig. 2A), a definitive G_1 population is present with a relative fluorescence intensity of 100, and the G_2/M population is present at a fluorescence intensity value of 200. The cells falling in between these two peaks represent cells progressing through S phase with variable DNA content (22).

The progress of cells through the cell cycle can be analyzed by determining the DNA content in synchronous populations. Fig. 2 B and C show a series of histograms of $K_1B_{11}0.5$ cells spanning the 12-hr cell cycle. The majority of the mitotically selected cells completed cytokinesis within 30 min and constituted a new G_1 population, whereas a small proportion of cells

still retained the G_2 content of DNA. Populations at hours 1–3 had a G_1 phase DNA content (data not shown). Although the 4-hr population commenced [^3H]thymidine incorporation (Fig. 1A), there was no detectable increase in DNA content. The 5-hr cells showed a broadening and skewing of the DNA content per cell toward a higher value. There was a progressive increase of DNA content per cell in subsequent hours with a maximum value reached by the G_2 populations at hours 11 and 12. Between hours 8 and 9, the increase in DNA content per cell was greater than in the previous stages of S phase, and this corresponds to the peak rate of [^3H]thymidine incorporation (Fig. 1A). DNA histograms during hours 11 and 12 contained a complex population of cells at various positions in the cycle, late S phase, G_2/M , and second cycle G_1 cells. By hour 12, a substantial proportion of the cells had completed one full generation.

FACS Analysis of Exponential and Synchronous Populations Doubly Fluorescence-Labeled for DNA Content and DHFR Concentration. We utilized MTX-F, which binds with high affinity to the active site of the DHFR molecule (17). The intensity of MTX-F fluorescence is proportional to the total DHFR content within living cells (17). FACS analysis of cellular DHFR concentration requires the maintenance of viable cellular structure. To analyze DNA content in viably labeled MTX-F cells, we have made use of the DNA-specific fluorochrome Hoechst 33342 (16). Hoechst 33342 at 10 μM is readily permeable to mammalian plasma membranes in a 1-hr incubation at 37°C. We utilized this unique property to double-label exponential and synchronous populations to examine DHFR concentration in relation to DNA content.

Fig. 3A represents a DNA histogram of an exponential population of $K_1B_{11}0.5$ cells. Specific cell cycle-phase populations

FIG. 2. DNA histograms of exponential and synchronized $K_1B_{11}0.5$ cells stained with chromomycin A_3 and analyzed on FACS. (A) DNA histogram of exponentially growing cells. (B and C) DNA histograms of synchronized populations. Cell cycle time in hours is indicated on each ordinate. Vertical dashed lines represent G_1 and G_2 fluorescence values based on the 30-min population.

FIG. 3. Double fluorescence labeling of exponentially growing $K_1B_{11}0.5$ cells with MTX-F and Hoechst 33342 and cell sorting on FACS. (A) DNA histogram of exponentially growing cells. Cells sorted from the G_1 , S, and G_2 phases. (B) Fluorescence intensity profiles of sorted subpopulations based on MTX-F binding to intracellular DHFR.

were sorted from this exponential population. The DNA histogram of the exponential population is a composite of individual DNA profiles of each of the sub-populations (22). There is appreciable overlap at the interface regions of the three major cell cycle-phase populations, G_1 , S, and G_2 .

Direct comparison of DHFR content in 10,000 cells from each of the three phase-specific populations is presented in Fig. 3B. The G_1 versus S and G_1 versus G_2 plots show an increase in DHFR concentration approaching 2-fold. The comparison of the S phase population, which contains a contribution of cells from all temporal stages of DNA replication, and the G_2 population shows no substantial increase in DHFR content per cell in G_2 . Thus, maximum DHFR concentration is obtained within the S phase of the cell cycle.

A more direct approach to correlate the increase of DHFR

FIG. 4. Double fluorescence labeling of synchronized cultures of $K_1B_{11}0.5$ cells with MTX-F and Hoechst 33342 and analysis on FACS. (A) DNA histograms of half of each synchronized population at each hour in the cell cycle as shown on the ordinate. (B) MTX-F fluorescence histograms of the second half of each synchronized population are based on DHFR content. Dotted lines in each panel represent the mode of each histogram at hour 1.

concentration through the cell cycle was the use of synchronous populations (Fig. 4). The DNA-binding properties of Hoechst 33342 is considered to be due to the specific interaction with A-T-rich regions of genomic DNA (16). This interaction is qualitatively and quantitatively different from the DNA-binding properties of chromomycin as used in Fig. 2. Chromomycin has a high-affinity, noncovalent interaction with G-C regions of the primary DNA sequence, apparently interacting with the 2-amino group of guanine residue (23). The DNA histograms generated by each staining procedure would not necessarily be identical. However, comparison of the 4- and 5-hr Hoechst DNA histograms in Fig. 4A shows the shift in DNA content per cell after hour 1 of S phase. From hours 5 through 9 there is a steady increase in DNA content per cell as expected from the progression of the population through S phase.

Analysis of DHFR concentration per cell in these synchronized populations is represented in the series of histograms in Fig. 4B. Sequential comparison of these plots shows that the G_1 content of DHFR remained constant throughout the pre-DNA replication phase. It was not until hour 6 that an increase in DHFR was detectable; this point corresponds to 1 hr after the initial shift in DNA content. From this cell cycle position within hour 2 of S phase, the concentration of DHFR steadily increased to a late S phase value at hour 9 ≈ 2.5 -fold greater per cell than the original G_1 value.

Determination of DHFR Specific Activity in Synchronous Populations. Fig. 1B shows the activity of DHFR throughout the 12-hr cell cycle. During the first 5 hr, including the entire G_1 period and the initial hour of S phase, there was a steady decrease in specific activity. This can be accounted for by the fact that the total content of DHFR per cell remained constant, whereas there was an increase of total soluble protein (Fig. 1B). From hour 5 through 10, the DHFR specific activity increased approximately 90%. This increase of enzymatic activity corresponds to the S phase-specific increase in fluorescence intensity per cell (Fig. 4B). During this same S phase interval, the total soluble protein per cell increased an additional 50% beyond the initial G_1 increase. By taking these two values into account, the actual increase in the number of DHFR molecules approached a factor of 2.7. The G_2 /M phase showed a decrease in activity,

FIG. 5. Fluorogram of NaDodSO₄/polyacrylamide gel electrophoresis of [³⁵S]methionine-labeled proteins from CHO-K₁ and $K_1B_{11}0.5$ cells. Exponentially growing CHO-K₁ cells (lane a) and MTX-resistant $K_1B_{11}0.5$ cells (lane b) were labeled for 60 min at 37°C with [³⁵S]methionine (200 μ Ci/ml). Protein extracts were prepared as described (19); 100,000 cpm were loaded per lane. Exponentially growing $K_1B_{11}0.5$ cells (lane Exp) and synchronized populations (lanes 2–10) were labeled for 30 min at 37°C with [³⁵S]methionine (200 μ Ci/ml). The lane numbers indicate the hour in the cycle at the time the labeling was terminated; 50,000 cpm were loaded in Exp lane and 30,000 cpm were loaded in each of lanes 2–10. Molecular weight markers are shown $\times 10^{-3}$.

possibly due to the fact that S phase-controlled synthesis of DHFR molecules had declined, whereas other cellular proteins continued to be produced in preparation for cell division.

[³⁵S]Methionine Labeling of Protein Synthesis in Synchronized Populations and NaDodSO₄/Polyacrylamide Gel Electrophoresis. The 50-fold increase in DHFR levels in the K₁B₁₁0.5 cell line in relation to methotrexate-sensitive cells allowed the detection of the DHFR protein band in the fluorograph from exponentially growing cultures (Fig. 5, lane b). This M_r 21,000 protein co-migrated with purified mouse DHFR and was absent from the labeled proteins of exponentially growing MTX-sensitive CHO-K₁ cells (Fig. 5, lane a). Lanes 2–10 show the synthesis of proteins during 30-min labeling at hourly points through the first 10 hr of the cell cycle. The fluorograph shows a period of low DHFR synthetic activity through the first 5 hr. Commencing at hour 6, DHFR synthesis was detected. This synthesis increased through hour 7 and remained through the duration of S phase. This pattern of DHFR synthesis correlates with the time course of increasing DHFR specific activity (Fig. 1A) and with the increase in DHFR concentration detected with FACS analysis (Fig. 4B).

DISCUSSION

Studies by this (7, 19) and other laboratories (11) have shown that the acquisition of resistance to methotrexate through selective gene amplification in mouse (24) and hamster cell lines (25) does not alter the normal growth phase and cell cycle pattern of DHFR content with respect to the sensitive parental cell lines. We have combined this property of increased DHFR enzyme levels in our CHO line K₁B₁₁0.5 with the property of precise cell cycle synchrony achieved by mitotic selection (13) to study cell cycle-specific enzyme regulation.

The combined use of the FACS, as an analytical and preparative tool to dissect and quantitate cell populations on the basis of specific macro-molecular content, along with the standard biochemical assays for cell cycle synchrony, DNA synthesis, and specific enzymatic activity have led to the observation of an S phase-specific increase in DHFR synthetic and enzymatic activity. Comparison of the FACS data for the double-labeled fluorescence experiments (Figs. 3 and 4) with the data for the rates of [³H]thymidine incorporation and DHFR enzyme activity (Fig. 1) demonstrates the temporal and functional relationship between DNA replication and production of an enzyme whose activity is essential for the progression and completion of DNA synthesis (26).

It is significant that the initial increase in DHFR levels (Figs. 1B and 4B) due to synthesis of new enzyme molecules (Fig. 5, lane 6) is detected at hour 6, 1 hr after the initiation of [³H]thymidine incorporation. Because hour 1 of S phase represents low levels of [³H]thymidine incorporation (Fig. 1A), the need for new tetrahydrofolate synthesis is probably not rate limiting at this point. However, the physiological commitment to complete DNA replication once S phase has been entered may initiate, or work in parallel with, a chain of regulatory events resulting in increased DHFR levels to coincide with the major portion of DNA replication in late S phase. It is in this time interval, hours 5–10, that the increase in DHFR content more than doubles while total soluble protein increases only 50% (Fig. 1B), suggesting a regulatory window for specific protein production that possibly involves other enzymes with related

functional significance to DNA replication (6). The increase of DHFR during S phase in the methotrexate-resistant cells that we have studied is similar to that observed in methotrexate-sensitive cells (data not shown), which contain less enzyme by a factor of 50. Thus, it seems unlikely that S phase regulation of DHFR can be ascribed to effects of immediate metabolic products of this enzyme; hence, a more indirect mechanism involving a number of parameters of S phase regulation is more attractive.

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