

Amplified Dihydrofolate Reductase Genes Are Located in Chromosome Regions Containing DNA that Replicates during the First Half of S-phase

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ABSTRACT To obtain a better understanding of the relationship between metaphase chromosome banding patterns and genome organization, attention was focused on regions of metaphase chromosomes that were found to contain the genes for a specific cellular enzyme, dihydrofolate reductase (DHFR). These studies involved the use of highly methotrexate-resistant mouse lymphoblastoid cells (L5178YR), which contain approximately 300 times the number of DHFR genes present in parental cells (L5178YS). Karyotypic analysis revealed the presence of two very large, nonhomologous, marker chromosomes that were absent in the parental line. *In situ* hybridization of ³H-labeled cloned DHFR cDNA to metaphase chromosomes of L5178YR cells was used to localize the DHFR genes to a very large Giemsa (G)-negative region on each of the two large marker chromosomes. Regional patterns of DNA replication in metaphase chromosomes were studied by autoradiographic visualization of [³H]thymidine incorporation and by fluorescent microscopic visualization of bromodeoxyuridine incorporation. Because the amplified DHFR genes were present within two prominent cytogenetic regions on two easily identifiable chromosomes, it was possible to observe the following. The amplified DHFR genes were located in chromosome regions that replicated at the same time during the first half of a 9-h S-phase. DNA replication began simultaneously and terminated simultaneously at many locations throughout each amplified region. We conclude that transcriptionally active DHFR genes are located within large G-negative regions of metaphase chromosomes and that the DNA within these regions replicates during the first half of S-phase.

The genome of animal cells is subdivided into ~30,000 replication units, or replicons, ranging in size from 50 to 330 kilobasepairs (kb) (13, 22, 23). DNA replication proceeds bidirectionally within each replicon from an origin until reaching replication forks proceeding outward from adjacent origins of replication (24, 28). Groups of replicons are activated in a defined temporal order that is maintained from one S-phase to the next (1, 11, 39, 40, 45, 47). Clusters of replicons initiate replication in synchrony and are similar in size and rate of fork movement (21, 27). This clustering of replicon activity may be responsible for the regional patterns of DNA replication observed in metaphase chromosomes (9, 12, 15, 30, 35, 38, 46). A number of reports have indicated that the DNA of specific cytogenetic loci replicate at characteristic times within S-phase.

In particular, it has been observed that heterochromatic chromosomal regions are usually the last to complete replication. These include the inactive X chromosome in females (facultative heterochromatin [8, 18, 36]) and centromeric regions (constitutive heterochromatin [11, 16]). The darkly staining G-positive bands produced by Giemsa staining procedures contain DNA that replicates late during S-phase, whereas G-negative regions contain DNA that replicates predominantly during the first half of S-phase (9, 12, 15, 30, 35, 38, 46). Thus, there is an intriguing relationship between metaphase chromosome morphology and the temporal order of DNA replication during S-phase.

To obtain a better understanding of the relationship between metaphase chromosome banding patterns, genome organiza-

tion, and regional patterns of DNA replication, we focused our attention on chromosomal regions containing the genes for a specific cellular enzyme. For these studies we used a highly methotrexate (MTX)-resistant line of mouse lymphoblastoid cells that contain approximately 300 times more dihydrofolate reductase (DHFR) genes than parental cells (10). All of these genes are presumably active since, as in other MTX-resistant cells (2, 34), the increase in DHFR gene number is accompanied by a corresponding increase in DHFR enzyme levels. Using *in situ* hybridization we localized the amplified DHFR genes to two very prominent regions on two easily identifiable marker chromosomes. Such cytogenetic regions have been previously referred to as homogeneously staining regions (or HSRs) because they do not exhibit prominent G-banding (4, 5). The intensity of staining is rather comparable to that of G-negative bands. Amplified DHFR genes have previously been localized to an HSR of a single chromosome in a MTX-resistant Chinese hamster ovary cell line (42). Because HSRs are prominent cytogenetic features, it has been possible to determine that replication of the DNA within the HSRs of two MTX-resistant Chinese hamster cell lines is restricted to the first half of S-phase (4, 5, 20). In these earlier studies, however, the localization of DHFR genes to the HSRs was not directly demonstrated. For the present report we have localized amplified DHFR genes to two prominent HSRs in a line of MTX-resistant mouse lymphoblastoid cells and have addressed the following questions: Are the amplified DHFR genes at each location replicated during a specific portion of S-phase, and, if so, when? Do the amplified DHFR genes on each of the two large marker chromosomes replicate at the same time? Does DNA replication begin simultaneously and end simultaneously at many locations throughout each HSR?

MATERIALS AND METHODS

Cell Culture

A line of MTX-resistant murine lymphoblastoid cells, designated L5178YR, was used for all experiments. The cells were previously selected in a stepwise fashion for the ability to grow in 1 mM MTX, and they contain approximately 300 times the number of DHFR genes and gene products that the parental cells (L5178YS) contain (10). Cells were maintained by suspension culture in Fisher's medium (Grand Island Biological Co., Grand Island, New York [Gibco]) supplemented with 10% horse serum and 1 mM MTX. Cells were grown at 37°C in a humidified incubator with 5% CO₂.

Chromosome Preparation and Staining

Cultures of exponentially growing cells were treated with Colcemid (Gibco; final concentration, 0.06 µg/ml) for 9 min at 37°C. Cells were collected by centrifugation at 600–800 rpm for 10 min in a swinging bucket rotor, gently resuspended in 75 mM KCl, and incubated for 10 min at 37°C. Cells were removed from the hypotonic solution by centrifugation as before, gently resuspended in methanol:acetic acid (3:1), and fixed for 20–30 min. Cells were collected by centrifugation, rinsed in methanol:acetic acid three additional times, dropped onto slides, and allowed to air-dry. Chromosomes were G-banded with Wright stain in 0.06 M phosphate buffer, pH 6.8 (51).

In Situ Hybridization

Escherichia coli strain C-600 SR1592, containing the plasmid pDHFR21, was generously provided by Robert T. Schimke (Stanford University). The plasmid is a derivative of pBR322 and contains a 1,500-bp (base pair) insert corresponding to nearly all of the DHFR mRNA (7). Plasmid DNA was prepared according to standard procedures (31) and in accordance with National Institutes of Health guidelines. *In situ* hybridization of pDHFR21 to metaphase chromosomes was carried out essentially as described by Harper and Saunders (25). Fresh chromosome preparations (1–2 wk old) were ribonuclease-treated and denatured with 70% formamide, 2 × SSC (SSC equals 0.15 M NaCl, 0.015 M sodium citrate), pH 7.0, at 70°C for 2 min. ³H-pDHFR21 DNA, labeled by nick translation (43)

to specific activity 8 × 10⁶ cpm/µg, was dissolved in 50% formamide, 2 × SSC, 10% dextran sulfate (Pharmacia Fine Chemicals, Piscataway, N. J.), pH 7.0, at a concentration of 0.2–1.0 µg/ml along with 500-fold excess sonicated salmon sperm DNA as carrier. The probe was denatured and applied to the chromosome preparations and incubation was carried out for 4–16 h at 37°C. Slides were rinsed thoroughly at 39°C, first in 50% formamide, 2 × SSC, pH 7.0, then in 2 × SSC, and exposed to Kodak NTB2 nuclear track emulsion for 3–6 d at –80°C. Preparations were developed with Kodak Dektol and then stained with Wright stain.

Regional Patterns of DNA Replication

These experiments were patterned after the retroactive labeling analysis originally described by Bostock and Prescott (6).

METHOD 1: Pulse-labeling with [³H]thymidine. Asynchronous, logarithmically growing populations of L5178YR cells were pulse labeled for 15 min with ³H-thymidine (20 Ci/mmol, 4 µCi/ml), collected by centrifugation (600 rpm for 5 min), and resuspended in the original volume of fresh medium containing unlabeled thymidine (10 µg/ml). At hourly intervals, metaphase chromosomes were prepared and the distribution of ³H-thymidine incorporation into metaphase chromosomes was determined by autoradiography as described above under *In Situ Hybridization*. Autoradiographic exposure was carried out for 5–10 d.

METHOD 2: Continuous exposure to bromodeoxyuridine. Asynchronous, exponentially growing cultures of cells were exposed to 100 µM bromodeoxyuridine (BrdUrd), 0.4 µM fluorodeoxyuridine, 100 µM deoxycytidine, and 6 µM deoxyuridine, and subsequently handled in the dark. At hourly intervals, metaphase chromosomes were prepared, stained with acridine orange, and visualized by fluorescence microscopy (37).

RESULTS

Localization of Amplified DHFR Genes

Karyotypic analysis of the highly MTX-resistant L5178YR cell line revealed the presence of two very large chromosomes that were not present in the parental L5178YS cell line (Fig. 1). The largest chromosome in L5178YR was designated marker 1 (M1) and appeared to be a derivative of chromosome 2. The second largest chromosome, designated marker 2 (M2), was a derivative of a chromosome other than number 2. Uncertainty regarding the origin of M2 was due to the extensive chromosomal rearrangements which have accumulated within the L5178Y cell lines. Although other differences were observed, such as aneuploidy both within and between the resistant and parental cell lines, the two large, nonhomologous marker chromosomes accounted for the most striking difference between the chromosomes present in the parental and MTX-resistant cells (Fig. 1). Analysis of G-banded metaphases revealed that marker chromosomes M1 and M2 each contained a large nonbanding G-negative region, referred to as an HSR. Some heterogeneity in the size of each HSR was observed. Close inspection revealed faint G-banding within the HSR regions of M1 and M2, especially within the M1 HSR, but variability in these faint banding patterns made it difficult to compare the chromosomal morphology of each HSR.

The technique of *in situ* hybridization was used to localize the amplified DHFR genes to the two large marker chromosomes, M1 and M2, of L5178YR cells (Fig. 2). Judging from the location of autoradiographic grains over marker chromosomes M1 and M2, the amplified DHFR genes were located throughout the HSRs and comprised ~30% of the length of M1 and 55% of the length of M2. Analysis of the distribution of autoradiographic grains among the chromosomes of L5178YR cells indicated that ~39% of the amplified DHFR genes were located within the HSR of M1, 52% within the HSR of M2, and 5% at a minor site on the proximal portion of chromosome 2 (Table I). Hybridization to the distal end of a third chromosome the size of a moderately large mouse chromosome was sometimes observed (Fig. 2 and Table I). How-

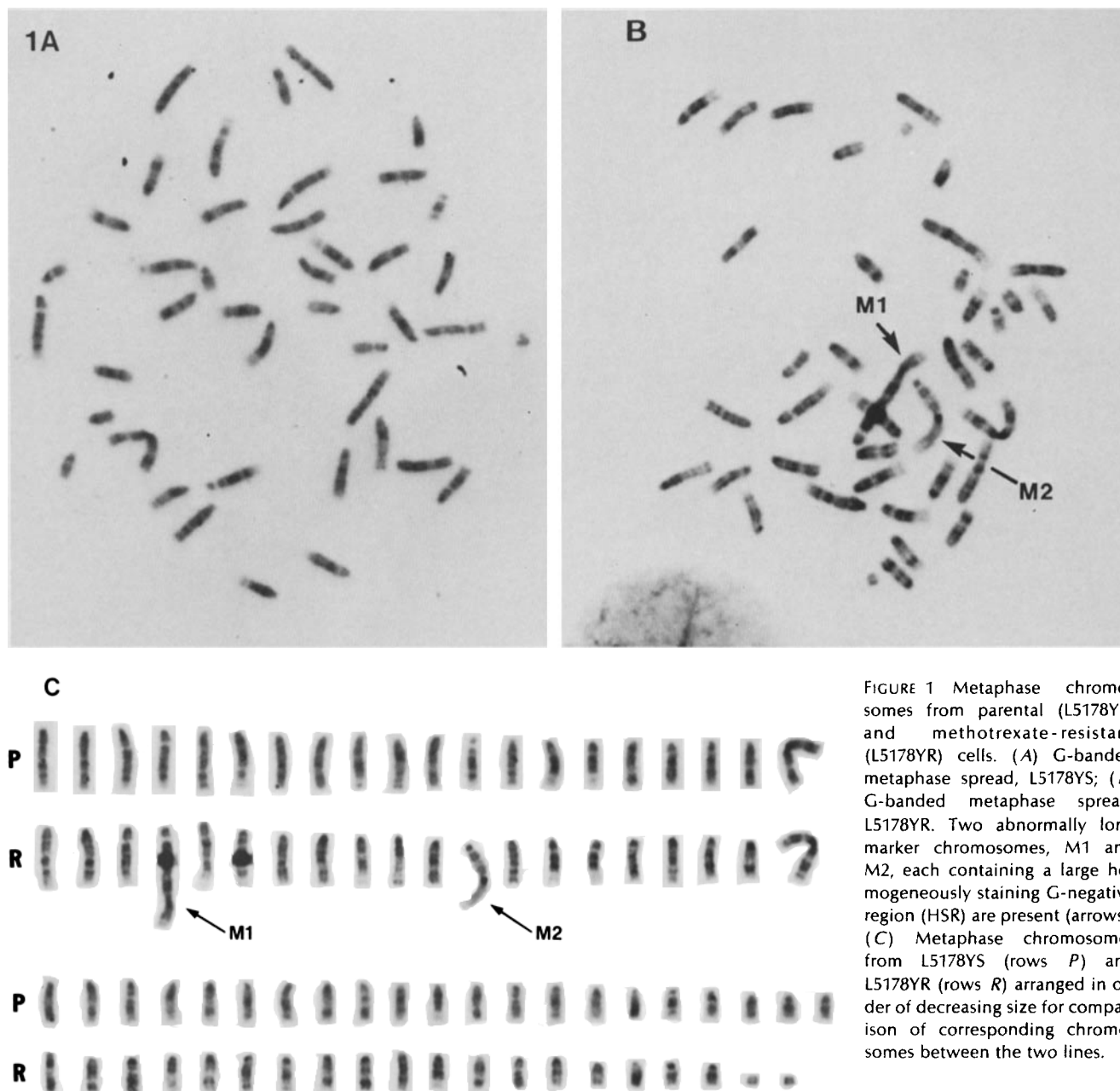


FIGURE 1 Metaphase chromosomes from parental (L5178YS) and methotrexate-resistant (L5178YR) cells. (A) G-banded metaphase spread, L5178YS; (B) G-banded metaphase spread, L5178YR. Two abnormally long marker chromosomes, M1 and M2, each containing a large homogeneously staining G-negative region (HSR) are present (arrows). (C) Metaphase chromosomes from L5178YS (rows P) and L5178YR (rows R) arranged in order of decreasing size for comparison of corresponding chromosomes between the two lines.

ever, lack of optimal G-banding of the posthybridized chromosomes did not allow the consistent assignment of these grains to one identifiable chromosome. Since previous data (10) indicate that these cells contain approximately 300 times more DHFR genes than do parental cells, our observations indicate that 250–300 DHFR genes are located at each major site of hybridization.

DNA Replication: [^3H]Thymidine Incorporation

The temporal order of metaphase chromosomal DNA replication was determined as follows. Asynchronous, logarithmically growing L5178YR cells were labeled with [^3H]thymidine for 15 min, removed from radioactive medium, and placed in fresh medium containing excess unlabeled thymidine (10 $\mu\text{g}/\text{ml}$) to prevent additional incorporation of radioactivity. At hourly intervals after the pulse, a portion of the cell population was treated with Colcemid and harvested for examination of

metaphase cells. The incorporation of [^3H]thymidine into chromosomes and portions thereof was determined by autoradiography. Those metaphase cells in which label first appeared represent cells that were at the end of S-phase at the time of the pulse. Labeled metaphase cells observed at later times after the pulse represent cells that were progressively earlier in S-phase at the time of the pulse. A composite of representative metaphase cells labeled with [^3H]thymidine during various portions of S-phase is presented in Fig. 3. Cells that were not in S-phase at the time of the pulse ($\sim 40\%$ of the population) did not incorporate a significant amount of radioactivity (Fig. 3A). Metaphase cells labeled near the end of S-phase showed incorporation into the DNA of a few specific chromosomes and chromosomal locations (Fig. 3B). Metaphase cells labeled during the middle of S-phase showed incorporation of [^3H]thymidine throughout nearly all of the genome except for the two major sites of amplified DHFR genes (Fig. 3C and D).

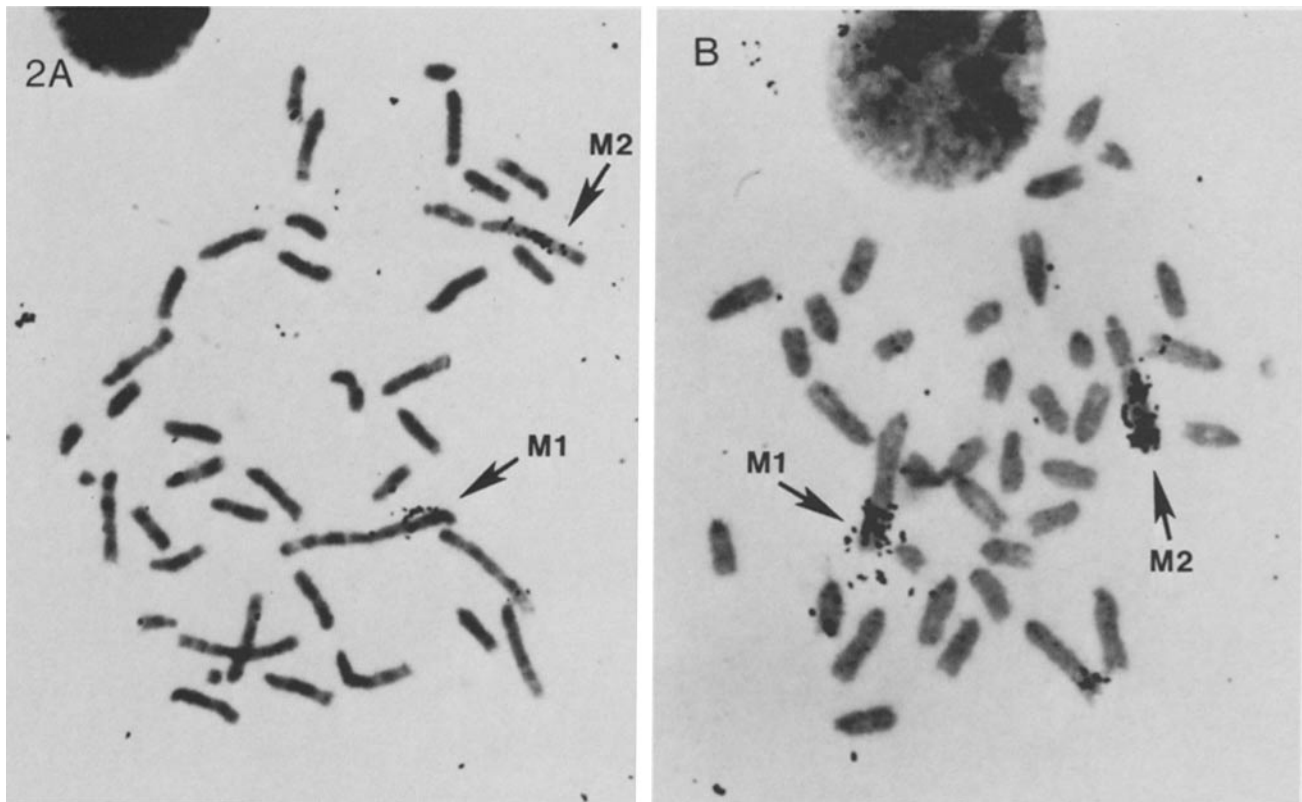


FIGURE 2 In situ hybridization of ^3H -labeled pDHFR21 to metaphase chromosomes of L5178YR cells. (A) Cell hybridized with probe at $1\ \mu\text{g}/\text{ml}$ for 8 h and exposed for 3 d. (B) Cell hybridized with probe at $1\ \mu\text{g}/\text{ml}$ for 16 h and exposed for 6 d. Silver grains are localized almost exclusively on the HSRs of M1 and M2. Note also labeling of distal portion of a third chromosome.

TABLE I
Location of Autoradiographic Grains following In Situ Hybridization of ^3H -pDHFR21 to L5178YR Cells*

	Additional sites					Total
	M1 HSR	M2 HSR	M2 (minor site)	Third chromosome†	Other§	
Percentage of total chromosomal grains	36	48	4	4	8	100
Percentage of grains on specific sites	39	52	5	4	—	100

* DNA hybridized at $1\ \mu\text{g}/\text{ml}$ for 4–12 h; 4-d exposure.

† Unidentified chromosome of size corresponding to that of moderately large mouse chromosome.

When these metaphase cells were exposed to the photographic emulsion for much longer times than those shown in Fig. 3, many more silver grains appeared over the labeled chromosomes, but incorporation of [^3H]thymidine into the regions containing amplified DHFR genes remained undetectable. A small percentage of metaphase cells (<5%) labeled during the middle of S-phase showed an intermediate level of incorporation into the regions of amplified DHFR genes. In these cases we assume that HSR DNA replication terminated at some time during the pulse, thus accounting for the reduced number of grains over the HSR regions in comparison with the rest of the chromosomes. Cells labeled during the first part of S-phase showed incorporation of [^3H]thymidine throughout virtually all regions of the metaphase chromosomes (Fig. 3 F).

Quantitative analysis of these data was performed by determining the percentage of metaphase cells labeled and the percentage of metaphase cells exhibiting label in each region of amplified DHFR DNA (Fig. 4). In preparations harvested 2 h after the [^3H]thymidine pulse, 50% of the metaphase cells were labeled, indicating that the average length of G2 plus half of mitosis was 2 h. The percentage of metaphase cells which showed incorporation of [^3H]thymidine into the regions of amplified DHFR DNA reached half-maximal values ~6 h after the [^3H]thymidine pulse and remained at maximal levels until ~10 h after the pulse. These observations indicate that the DNA in the regions of amplified DHFR DNA on chromosomes M1 and M2 began replication at the beginning of S-phase and finished an average of 4 h before the end of a 9-h S-phase. Because of variation in cell cycle times among individual cells within the population, the percentage of metaphase cells showing labeled HSRs did not reach 100%. In a very small number of cases (<5%), the HSR DNA on one marker chromosome finished replication before the HSR DNA on the other marker chromosome (Fig. 4, difference between open circles and open triangles). In these cases the HSR DNA on chromosome M2 was usually the first to finish.

DNA Replication: Continuous Labeling with BrdUrd

Regional patterns of metaphase chromosomal DNA replication were also determined by a technique based on the incorporation of the thymidine analogue, BrdUrd. By the use of this technique, portions of chromosomes containing BrdUrd-substituted DNA can be distinguished from those containing unsubstituted DNA by staining the metaphase chromosomes

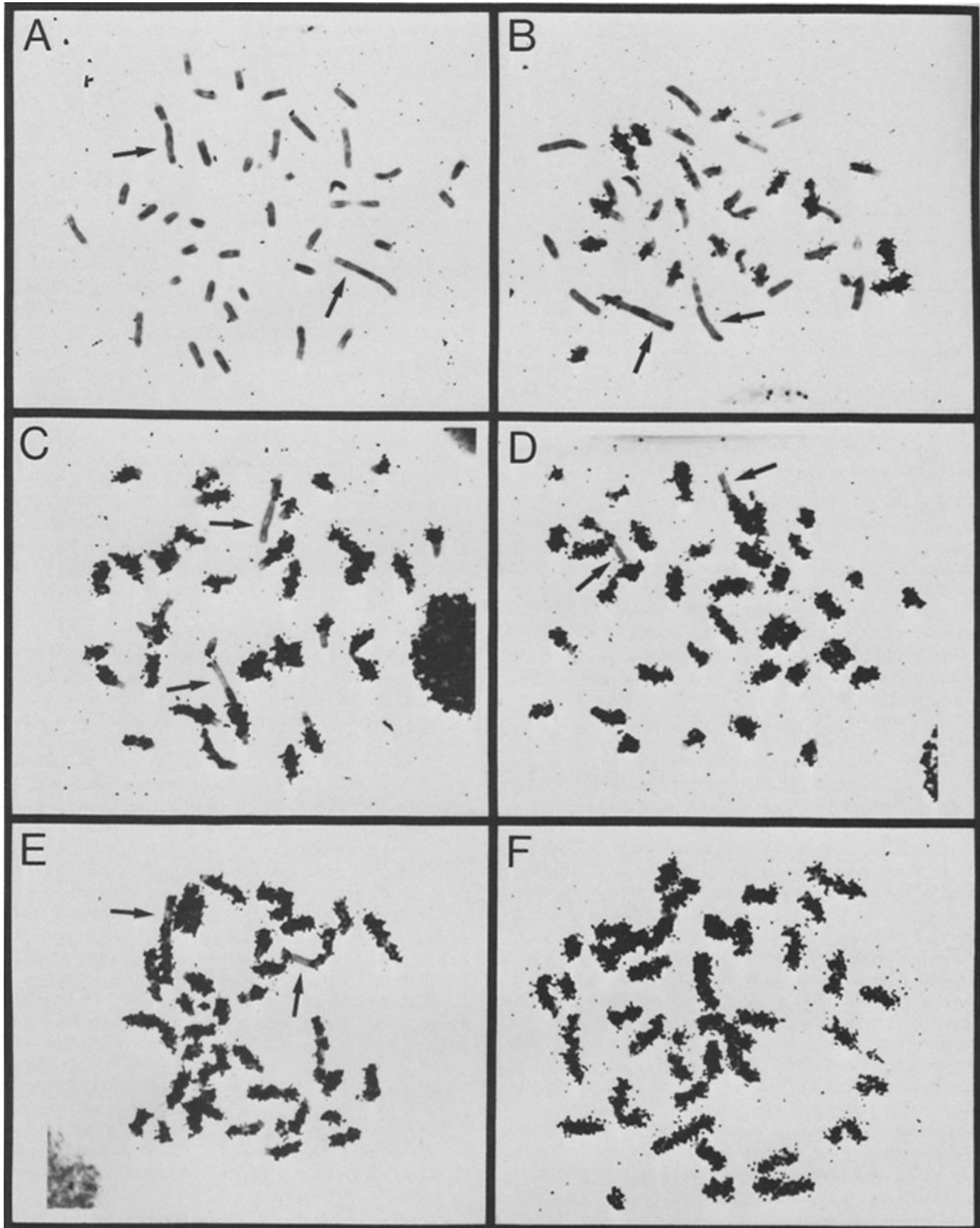


FIGURE 3 Regional patterns of [³H]thymidine incorporation during S-phase. Asynchronous, logarithmically growing populations of L5178YR cells were pulse-labeled for 15 min with [³H]thymidine (4 μCi/ml) and chased in fresh medium containing unlabeled thymidine (10 μg/ml). At hourly intervals, metaphase cells were prepared and the distribution of [³H]thymidine incorporation into metaphase chromosomes was determined by autoradiography (5-d exposure). The cytogenetic regions containing the amplified DHFR genes are indicated by the arrows. The autoradiographs consist of cells taken at the following times after [³H]thymidine labeling: A, 1 h; B, 3 h; C, 5 h; D, 7 h; E, 9 h; F, 11 h.

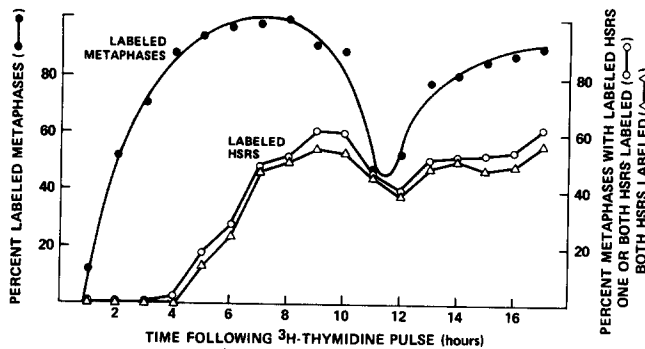


FIGURE 4 Dihydrofolate reductase DNA replication. L5178YR cells were pulse-chased with [^3H]thymidine and metaphase cells were autoradiographed as described in the legend to Fig. 3. 400 metaphase cells from three separate experiments were analyzed for most points. Since the amplified DHFR genes were present at two well-defined loci on two easily identified marker chromosomes (M1 and M2, see Figs. 1 and 2), the appearance of [^3H]thymidine at these loci was easily determined. Percentage of labeled metaphase cells (●); percentage of metaphase cells with both regions of amplified DHFR genes labeled (△); the percentage of metaphase cells with one or both regions of amplified DHFR genes labeled (○).

with the fluorescent dye, acridine orange. Portions of chromosomes containing unsubstituted DNA fluoresce brightly, whereas those portions of chromosomes containing BrdUrd-substituted DNA appear faint because the fluorescence of the acridine orange is quenched (37). An advantage of this approach over autoradiographic determination of [^3H]thymidine incorporation is that detailed regional patterns of DNA replication are not obscured by autoradiographic grains. Populations of asynchronous, logarithmically growing cells were continuously exposed to BrdUrd for various lengths of time. At hourly intervals, metaphase chromosomes were prepared, stained with acridine orange, and examined by fluorescence microscopy. Chromosomes from cells that were not in S-phase during the time of BrdUrd labeling fluoresced brightly (Fig. 5A) and showed very little banding, indicating no BrdUrd incorporation. Metaphase cells that were exposed to BrdUrd during the last hour of S-phase showed some banding (Fig. 5B). Metaphase chromosomes of cells that incorporated BrdUrd continuously during the second half of S-phase showed very prominent banding patterns consisting of brightly and faintly fluorescing areas. The cytogenetic regions corresponding to the locations of amplified DHFR genes were the largest brightly fluorescing regions in these metaphase spreads (Fig. 5C and D), indicating the relative absence of BrdUrd-substituted DNA in these regions. Chromosomes from cells exposed to BrdUrd for progressively longer portions of S-phase displayed progressively less fluorescence (Fig. 5E and F). A detailed analysis of BrdUrd incorporation into the DNA of marker chromosomes M1 and M2 (Fig. 6) indicated that the large regions containing amplified DHFR genes incorporated BrdUrd only during the first half of S-phase. The regions containing amplified DHFR DNA appeared to be among the

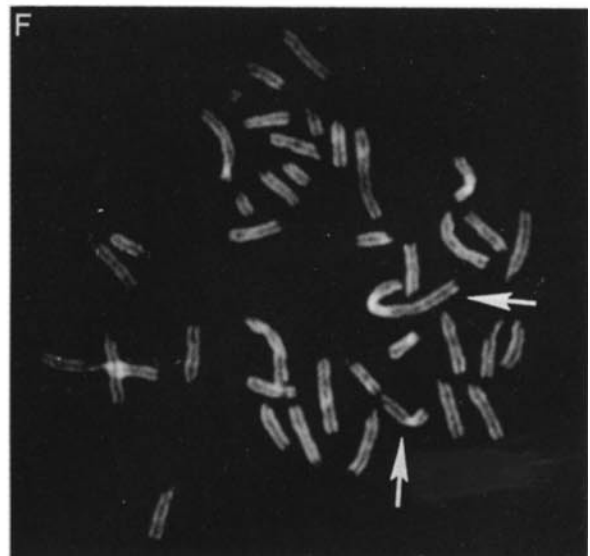
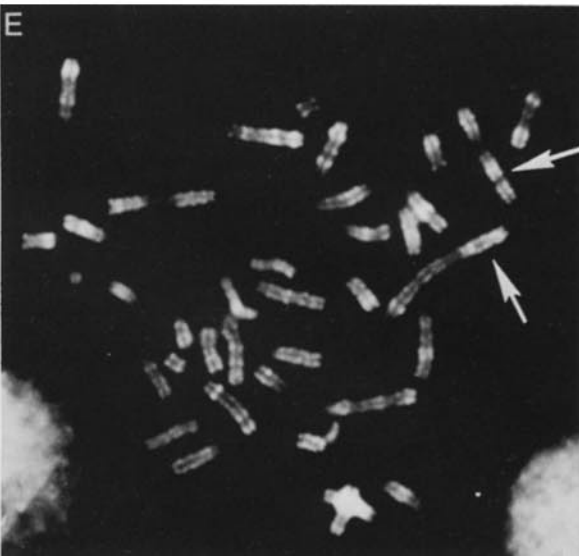
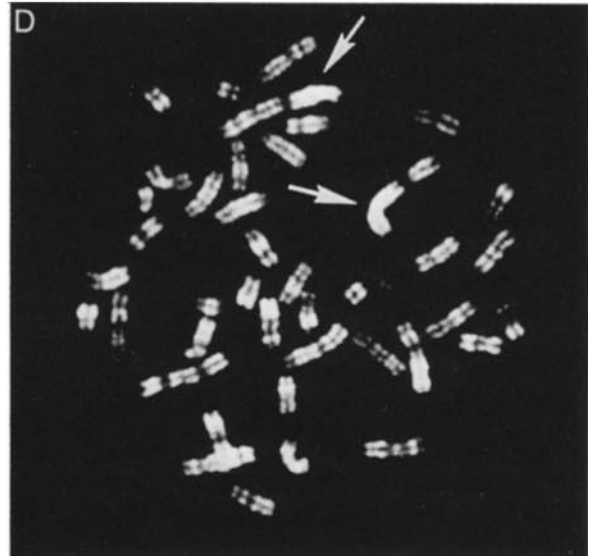
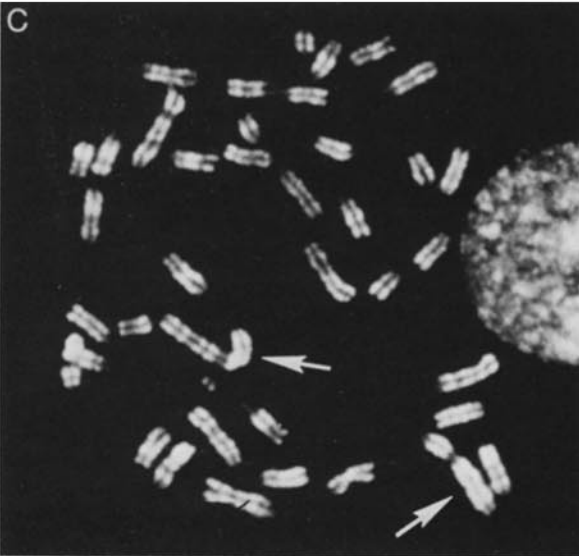
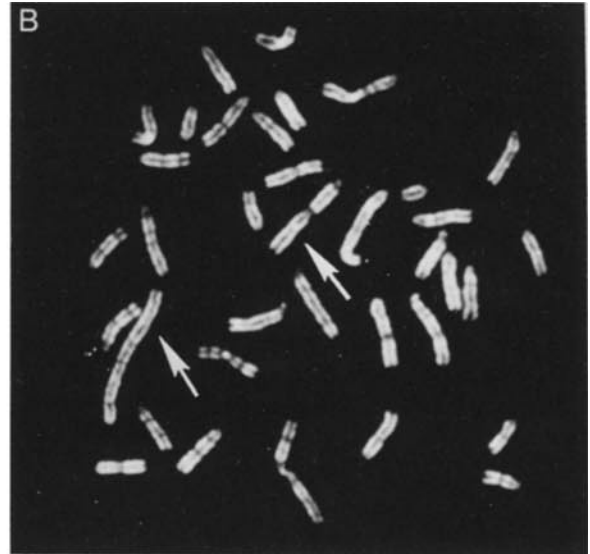
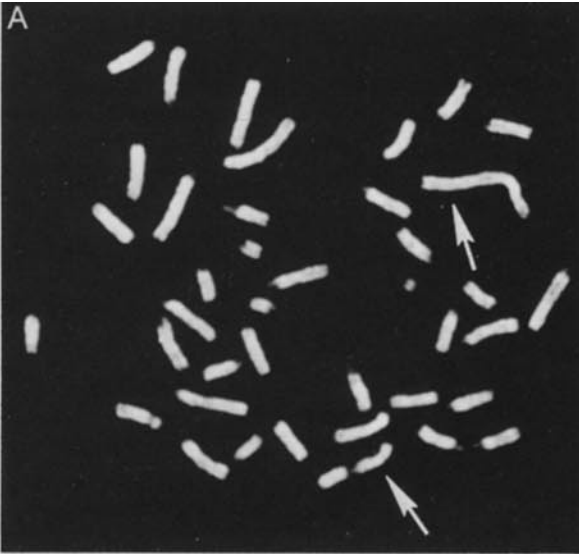
first portions of M1 and M2, and the entire genome, to finish replication during S-phase. By 10 h of continuous labeling, BrdUrd was present in virtually all regions of all metaphase chromosomes (Fig. 5F).

DISCUSSION

The MTX-resistant mouse lymphoblast cells used for these studies contain approximately 300 times the number of DHFR genes present in parental cells (10). Dolnick et al. (10) have previously localized the amplified DHFR genes to a prominent cytogenetic region on a large marker chromosome that appeared to be a derivative of chromosome 2. The cytogenetic region containing the amplified DHFR genes appeared as a large nonbanding G-negative region following trypsin-Giemsa treatment of metaphase chromosomes. Such large G-negative regions have been referred to as HSRs (4, 5). However, in general, close visual inspection usually revealed the presence of faint banding patterns within the HSRs as we have noted above. In the cell line used for the present study, the amplified DHFR genes were located within prominent G-negative regions on two large marker chromosomes. One of these chromosomes (M1) was a derivative of chromosome 2 and was presumably the chromosome identified by Dolnick et al. (10). The fact that HSRs in MTX-resistant mammalian cells are usually associated with a derivative of chromosome 2 suggests that the gene amplification originates from only one of the number 2 homologues. In this regard the hamster DHFR gene has recently been mapped to chromosome 2 (44). The presence of a large number of DHFR genes on another marker chromosome (M2) of undetermined derivation presumably resulted from a chromosomal translocation event. In addition, two other minor sites of the amplified DHFR genes (<5%) were located, one at a second site on marker chromosome M2 and another on a third unidentified chromosome of moderately large size. Assuming that the amplified DHFR genes on M1 and M2 account for nearly all of the DHFR genes in the L5178YR cells, we calculate that ~250–300 DHFR genes were present at each major location. In other MTX-resistant cell lines, amplified DHFR genes are believed to be associated with extrachromosomal structures termed double minutes (32). Whether located within chromosomal HSRs or on extrachromosomal double minutes, the increase in DHFR gene number in MTX-resistant cells is accompanied by a corresponding increase in DHFR protein, suggesting that virtually all of the amplified DHFR genes are active (2, 3, 19, 29, 33, 34, 50).

In situ hybridization of radiolabeled pDHFR21 to metaphase preparations of L5178YR cells was used to localize the amplified DHFR genes. pDHFR21 is a recombinant DNA plasmid consisting of pBR322 and a 1,500-bp insert corresponding to nearly all of the DHFR mRNA sequence (7). Use of a cloned probe as well as the addition of 10% dextran sulfate to the hybridization reaction made possible significant detection of the ^3H -labeled probe after relatively short autoradiographic exposure (2–6 d). Dextran sulfate accelerates the hybridization

FIGURE 5 Regional patterns of BrdUrd incorporation during S-phase. Asynchronous, logarithmically growing L5178YR cells were continuously exposed to BrdUrd for various periods of time, and metaphase chromosomes were prepared, stained with acridine orange, and visualized by fluorescence microscopy. Portions of metaphase chromosomes containing DNA that did not incorporate BrdUrd during the labeling period fluoresce very brightly. The cytogenetic regions containing amplified DHFR genes are indicated by the arrows. The fluorescence micrographs consist of cells that had been exposed continuously to BrdUrd for the following times: A, 1 h; B, 3 h; C, 5 h; D, 7 h; E, 9 h; F, 11 h.



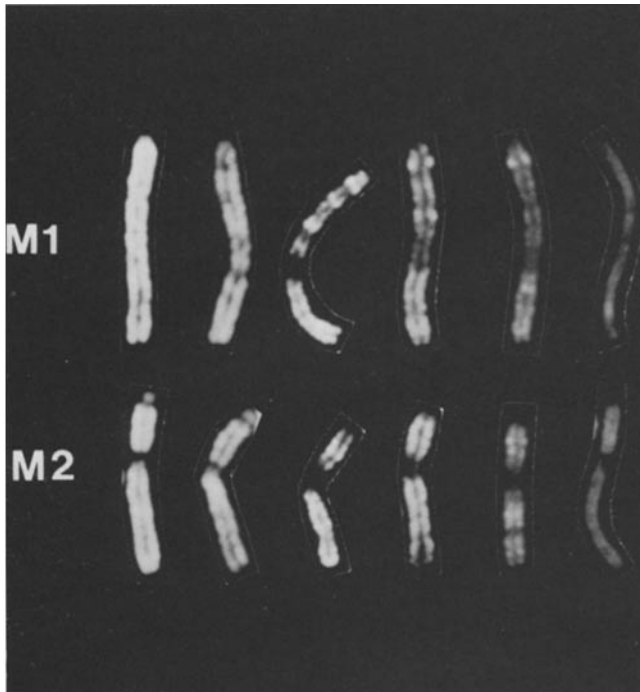


FIGURE 6 Regional patterns of BrdUrd incorporation into the DNA of marker chromosomes M1 and M2. Asynchronous, logarithmically growing L5178YR cells were grown continuously in the presence of BrdUrd for various periods of time and the distribution of BrdUrd incorporation into M1 and M2 determined by fluorescence microscopy as described under Materials and Methods. Each M1:M2 set is taken from a metaphase spread derived from a cell that had been exposed continuously to BrdUrd for the following times: (from left to right) 1, 3, 5, 7, 9, and 11 h.

rate of DNA (49), promoting the formation of DNA networks between single-stranded, randomly cleaved molecules (48). Such a population of DNA molecules is obtained upon radiolabeling by nick translation (43) followed by denaturation. Moreover, pDHFR21 contains pBR322 vector sequences that cannot hybridize to the chromosomal DNA but are free to participate in network formation, increasing the amount of radioactivity deposited at each specific chromosomal site. Since the resolution of tritium autoradiography is $<0.5 \mu\text{m}$ and since the silver grains of the photographic emulsion were localized over a region larger than several microns, we conclude that the amplified DHFR genes are distributed throughout each homogeneously staining region. The cytogenetic regions containing the amplified DHFR genes each comprise $\sim 2\%$ of the length of the entire karyotype. Since a diploid mammalian genome contains 6×10^9 bp of DNA, each HSR contains $\sim 1.2 \times 10^8$ bp, assuming that DNA packaging within an HSR does not differ substantially from the average DNA packaging density throughout the metaphase chromosomes. 300 copies of the DHFR gene (42 kb per gene [41]) within each HSR account for only 10–20% of the DNA present within that cytogenetic region. Thus, the DNA located between DHFR genes accounts for the majority of DNA present within each HSR. It is not known whether the non-DHFR DNA is transcribed, whether it consists of reiterated sequences, or whether the same non-DHFR DNA is found in each HSR.

Because the amplified DHFR genes constitute prominent cytogenetic regions on two easily identifiable chromosomes, it was possible to use a retroactive method of cell cycle analysis

(6) based on examination of metaphase chromosomes to determine when, during S-phase, HSR DNA replicated. This approach has two major advantages: (a) the use of synchronized cell populations is not required, and (b) the replication of amplified DHFR genes on each large marker chromosome can be examined separately. We have found that the DNA of each cytogenetic region containing amplified DHFR genes replicated during the first half of a 9-h S-phase. Replication began simultaneously (relative to the 15-min labeling period used) at many locations throughout each region and, after an average of 5 h, ended simultaneously throughout each region. The DNA present within each HSR was replicated over the same interval of S-phase and only rarely did the DNA from one HSR finish replication before the other. A remarkable feature about HSR DNA replication was that the regions containing amplified DHFR genes were the largest continuous segments of the genome that replicated simultaneously. While we conclude from these data that DHFR genes are located in chromosome regions that replicated during the first half of S-phase, the fact that DHFR genes very likely account for only a small portion of the DNA within these regions makes it difficult to rule out the possibility that some of the DHFR genes replicated late in S-phase.

Earlier studies using MTX-resistant Chinese hamster cells showed that a single HSR associated with a number 2 chromosome replicated during the first half of S-phase (4, 5). Although it is reasonable to assume that DHFR genes were present within the HSR region examined, this was not shown. For the present report, we directly localized amplified DHFR genes to two prominent cytogenetic loci in a line of lymphoid mouse cells and examined the temporal order of replication of the DNA at each cytogenetic locus. Together, these studies show that the chromosomal HSRs associated with MTX resistance in hamster and mouse cells contain DNA that replicates only during the first half of S-phase. Factors governing the time required to replicate large segments of the genome presumably include the number of replication origins and the rate of fork movement. These parameters are known to vary from one part of the genome to another but are remarkably similar for adjacent parts of the genome, thus giving regional patterns of DNA replication (21, 27). Another factor responsible for regional patterns of DNA replication is control of the initiation of DNA replication. Our observations and those of others (4, 5, 20) indicate that the replication of DHFR DNA throughout the HSRs is under regional control. Replication throughout each region was initiated at the beginning of S-phase and terminated by mid-S-phase.

Other studies have suggested that transcriptionally active genes are located in the G-negative regions of metaphase chromosomes (26, 52) and that the DNA of G-negative regions replicates early in S-phase (9, 12, 15, 30, 36, 38, 46). Additional studies have shown that globin genes replicate early in S-phase (14, 17). We have shown that transcriptionally active DHFR genes are located in large G-negative regions of metaphase chromosomes and that the DNA within these regions replicates early during S-phase. These observations provide additional evidence that the structural organization of metaphase chromosomes, as judged by chromosome banding procedures, is related to certain functional properties such as transcriptional activity and the temporal order of DNA replication.

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Note Added in Proof: While this manuscript was in press, Milbrandt et al. reported that amplified DHFR DNA in MTX-resistant Chinese hamster ovary cells also replicates early during S-phase (J. D. Milbrandt, N. H. Heintz, W. C. White, S. M. Rothman, and J. L. Hamlin. 1981. Methotrexate-resistant Chinese hamster ovary cells have amplified a 135-kilobase-pair region that includes the dihydrofolate reductase gene. *Proc. Natl. Acad. Sci. U. S. A.* 78:6043-6047).

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