CORRELATION OF DIHYDROFOLATE REDUCTASE ELEVATION WITH GENE AMPLIFICATION IN A HOMOGENEOUSLY STAINING CHROMOSOMAL REGION IN L5178Y CELLS

BRUCE J. DOLNICK, RONALD J. BERENSON, JOSEPH R. BERTINO, RANDAL J. KAUFMAN, JACK H. NUNBERG, and ROBERT T. SCHIMKE

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510; and the Department of Biological Sciences, Stanford University, Stanford, California 94305

ABSTRACT

A methotrexate (MTX)-resistant murine lymphoblastoid cell line has been obtained by serial passage in increasing concentrations of MTX which is >100,000-fold resistant to MTX (L5178YR) and has dihydrofolate reductase (DHFR) levels 300-fold higher than the parental line. The L5178YR cell line synthesizes ~10–11% of its total soluble cell protein as DHFR regardless of growth phase, as measured by direct immunoprecipitation with a monospecific antiserum. Molecular hybridization of a purified [^{3}H]DNA probe complimentary to DHFR specific mRNA with cellular DNA and RNA indicates that DHFR coding sequences are elevated several hundred fold in both nucleic acid species in the mutant cell line. Giemsa-banding studies of the diploid mutant line indicate the presence of a large homogeneously staining region on chromosome No. 2. *In situ* molecular hybridization studies indicate that the DHFR genes are localized in this homogeneously staining region. The homogeneously staining region probably consists of tandom repeats of a basic segment ~800 kilo base pairs long.

KEY WORDS dihydrofolate reductase · gene amplification · homogeneously staining region

Resistance to the folate antagonist, methotrexate (MTX),[1] can be readily induced in cells propagated in vitro by exposing cells to increasing levels of this drug. The most common cause of this resistance has been an elevation of the MTX target enzyme, dihydrofolate acid (DHFR) (7–9, 16, 22, 23). Recently, it has been demonstrated that increases in DHFR result solely from increased rates of enzyme synthesis (2, 10). It has also been demonstrated that this increase in rate of DHFR synthesis is proportional to the mRNA content of these cells, as demonstrated by both molecular hybridization (1) and in vitro translation utilizing a mRNA-dependent rabbit reticulocyte lysate translation system (11). Further studies demonstrated that, in addition to the elevation of specific mRNA levels as a means of obtaining increased

[1] *Abbreviations used in this paper*: ACS, Aqueous Counting Scintillant; cDNA, DNA synthesized complimentary to mRNA using reverse transcriptase; DHFR, dihydrofolate reductase; H_2PteGlu, dihydrofolic acid; HSR, homogeneously staining region; L5178YR and L5178YS, L5178Y cells resistant or sensitive to MTX, respectively; MTX, methotrexate; SSC, standard saline citrate solution.

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/11/0394/09 $1.00
Volume 83 November 1979 394–402

amounts of intracellular DHFR, the MTX-resistant cells have also amplified the genes coding for DHFR (1, 23).

In situ molecular hybridization utilizing a mouse DHFR mRNA-specific [^{3}H]cDNA (DNA synthesized complimentary to mRNA using reverse transcriptase) probe with a MTX-resistant Chinese hamster cell line indicates that the amplified genes are located on a single chromosome (19). Furthermore, this chromosome is unique in that it possesses a homogeneously staining region (HSR), as first reported by Biedler and Spengler (3, 4). We have carried out similar studies in an L5178Y cell line highly resistant to MTX in an effort to determine whether gene amplification and location of the amplified genes in a murine system are similar to that in the Chinese hamster cell line. The L5178Y line is particularly well suited for this purpose because of its diploid chromosome content and the availability of murine [^{3}H]cDNA from DHFR-specific mRNA for these purposes.

MATERIALS AND METHODS

Materials

[^{3}H]leucine (60 Ci/mmol), [^{14}C]leucine (354 mCi/mmol), [^{3}H]dCTP (22 Ci/mmol), and Aqueous Counting Scintillant (ACS) were obtained from Amersham Corp., Arlington Heights, Ill. ^{3}H[dTTP] (80 Ci/mmol), [^{3}H]dGTP (26.5 Ci/mmol) and [^{3}H]dATP (10 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. Deoxyribonucleoside triphosphates and nucleic acids were obtained from Sigma Chemical Co., St. Louis, Mo. Synthetic polyribonucleotides and oligodeoxyribonucleotides were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Oligo-dT cellulose was purchased from Collaborative Research Inc., Waltham, Mass. S_1 nuclease was obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Avian myeloblastosis virus reverse transcriptase (6.3×10^4 U/mg) was kindly provided by Dr. J. Beard of Life Sciences, Inc., St. Petersburg, Fla. Chemicals and reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, Calif. Sephadex and cyanogen bromide-activated Sepharose were obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J. MTX-Sepharose was prepared as described by the manufacturer's recommendations and was a generous gift of Dr. J. McGuire. Dihydrofolic acid (H_2PteGlu) was synthesized and purified by the method of Blakely (5). Fischer's complete medium, Fischer's medium without leucine, horse serum, Hank's balanced salts, colcemid, and Giemsa stock solution were purchased from Grand Island Biological Co., Grand Island, N.Y. Kodak nuclear-type emulsion NRB-2 and Kodak D-19 developer were obtained from Eastman Kodak Co. All other chemicals and reagents were of the highest quality obtainable.

Methods

CELL CULTURE: L5178Y murine lymphoblastoid cells were maintained by suspension culture in Fischer's medium supplemented with 10% horse serum. The MTX-resistant mutant (L5178YR) was established by growing cells in the presence of increasing amounts of MTX. Cells were initially suspended in 10^{-9} M MTX, and the concentration of MTX was elevated by ½ log increments on a weekly-to-biweekly basis as the cells appeared viable. This process was continued until the cells were growing in 10^{-3} M MTX. The cell doubling time was 12 h for the parent cell line (L5178YS) and 17 h for the MTX-resistant cell line (L5178YR).

ENZYME PURIFICATION: L5178Y cells were grown to late log ($7–9 \times 10^5$ cell/ml), harvested by low-speed centrifugation, and washed with Hank's Balanced Salts. Cells were then suspended in 5 vol of 50 mM Tris-Cl (pH 7.5), 3 mM dithiothreitol, 2.5 mM $MgCl_2$, and 150 mM KCl. The cell suspension was freeze-thawed three times in a dry-ice ethanol bath (thawed at 37°C) and centrifuged at 110,000 *g* (4°C) for 30 min. The supernate was rendered 1% (vol/vol) in streptomycin sulfate by the slow addition of 0.05 vol of 20% (wt/vol) streptomycin sulfate. The solution was stirred for 10 min at 4°C, then centrifuged at 27,000 *g* (4°C) for 10 min. To the resulting supernate an equal volume of saturated ammonium sulfate solution (pH 7.0) was slowly added, and the resulting suspension was stirred for 10 min at 4°C and centrifuged as described above. The resulting supernate was loaded onto a MTX-Sepharose column (0.7×2 cm) and washed with 20 column volumes of 1 M Tris-Cl, pH 7.5. The enzyme was eluted with 1 M Tris-Cl (pH 8.5), 14.4 mM 2-mercaptoethanol, and 1 mM H_2PteGlu. No column overloading was observed with extract resulting from up to 20 g cells (yield ~3 mg of enzyme).

The resulting DHFR was homogeneous by analysis of 11 μg of purified DHFR on SDS polyacrylamide disc gel (12.5%) electrophoresis (5, 24) and MTX titration (see below). Homogeneous ^{14}C-labeled DHFR was prepared by labeling of L5178YR cells in culture (2) and purified as described above.

DHFR was assayed by the spectrophotometric method of Osborn and Huennekens (20). The reaction mix contained, in a final vol of 1 ml: 20 μm H_2PteGlu, 50 μM NADPH, 150 mM KCl, and 100 mM Tris-Cl (pH 7.5). Reaction velocities were linear as a function of time and enzyme concentration. Titration of enzyme activity with MTX (26) revealed the enzyme to exhibit a turnover number of 5.6×10^2 min^{-1}, corresponding to a homogeneous enzyme (with one ligand binding site) of 22,000 mol wt with a sp act of 25 μmol min^{-1} mg^{-1} (37°C).

ANTIBODY PREPARATION: Antisera were elicited in two female New Zealand white rabbits (2 kg) by two biweekly injections of 500 μg of purified DHFR emulsified with Freund's complete adjuvant. Injections were given i.m. in the thighs and lower back. Rabbits were bled from the ear biweekly, and the serum was obtained by allowing the blood to clot overnight at 4°C.

RATES OF DHFR SYNTHESIS: L5178YR and L5178YS were pulse-labeled for 45 min with [^{3}H]leucine, extracted, and the [^{3}H]leucine-labeled DHFR was immunoprecipitated as described by Alt et al. (2). Purified [^{14}C]leucine-labeled DHFR (~160 cpm, 0.75–1.0 μg) was included as carrier and standard. For electrophoresis of immunoprecipitates, samples were placed in electrophoresis sample buffer (14) containing 5% SDS and immersed in a boiling water bath for 5 min immediately before electrophoresis. After electrophoresis, gels were frozen on dry ice and sliced in 3-mm sections. Sections were dissolved in 400 μl of H_2O_2: NH_4OH (10:1 vol/vol) at 60°C overnight. 10 ml of ACS was added, and vials were counted after cooling at −20°C for 30 min.

DNA, RNA, AND [^{3}H]CDNA PREPARATION: DNA and

RNA were prepared from frozen cell pellets as described (1). [^{3}H]cDNA specific for DHFR poly(A) RNA was used for molecular hybridization and DNA renaturation experiments and was prepared and purified from MTX-resistant mouse sarcoma 180 cells as described (1). [^{3}H]cDNA of high radiospecificity for *in situ* molecular hybridizations was prepared from L5178YR poly(A) RNA in a similar fashion. The reverse transcriptase reaction was performed as described (1) with total cellular poly(A) RNA, except that all four deoxyribonucleoside triphosphates were tritium labeled (dTTP 20 mCi/μmol, dGTP 4.98 mCi/μmol, dATP 5 mCi/μmol, dCTP 22 mCi/μmol). The resulting [^{3}H]cDNA transcript yielded an average radiospecificity of 39.8 μCi/μg (2.6×10^7 cpm/μg), assuming random incorporation of all four deoxyribonucleoside triphosphates.

CHROMOSOME PREPARATION: Metaphase chromosome spreads were prepared essentially as described (25). Cells (3×10^4 cells/5 ml) were incubated for 68–72 h in Fischer's medium containing 10% horse serum (37°C). Colcemid was added to a final concentration of 16 ng/ml, and after 90 min at 37°C the cells were centrifuged at low speed (3 min) and resuspended in 5 ml of 75 mM KCl (37°C) for 8 min. Cells were then centrifuged at low speed (3 min) and resuspended gently in 3 ml of methanol: acetic acid (3:1 vol/vol), centrifuged as before, and resuspended in 5 ml of the same solution. The cell suspension was then dropped on wet slides and allowed to air dry. Slides were stored at room temperature for 1 wk, heated for 16 h at 56°C, incubated in 25 mM potassium phosphate (pH 6.8; 56°C) for 8 min, and then stained with Giemsa-trypsin (6). Slides were rinsed in distilled water, air dried, and examined by light microscopy. Altered chromosomes are referred to as markers, and the nomenclature of Nesbitt and Francke was employed for karyotypic classification (18).

IN SITU MOLECULAR HYBRIDIZATION: The procedure used was essentially that described by Pardue and Gall (21). Metaphase spreads were incubated for 2.5 min in 0.07 N NaOH and then rinsed in ethanol. 1.5–2 μl ($3–5 \times 10^3$ cpm) of a [^{3}H]cDNA-containing solution in 4 × SSC (0.6 M NaCl, 60 mM sodium citrate); 100 mM Tris-Cl (pH 7.5), 100 μg/ml poly(rA); 1 mg/ml salmon sperm DNA, and 40% (vol/vol) formamide were deposited over slide areas containing metaphase spreads and covered with 3-mm^2 glass cover slips. Slides were then incubated in a moist chamber containing 4 × SSC, 40% formamide within a sealed plastic bag at 37°C for 45 h. Slides were washed extensively in 4 × SSC at 60°C and dehydrated with 70% ethanol followed by 95% ethanol. Autoradiography and Giemsa staining were performed as described by Pardue and Gall (21) using Kodak NTB-2 emulsion and Kodak D-19 developer.

OPTICS: Metaphase chromosome spreads were examined under a bright field with a greenfilter (oil immersion), using a 3,000-fold magnification. Photographs were taken with Kodak Panatomic-X (ASA 25) in a Zeiss photomicroscope Model 3 (Carl Zeiss, Inc., New York).

SCINTILLATION COUNTING: Samples containing immunoprecipitates or protein precipitates were hydrolyzed in 800 μl of 0.1 N NaOH at 60°C for 1 h. 10 ml of ACS were added and the samples were counted after chilling at −20°C for 30 min. All other samples (0–500 μl) were counted with 8 ml of ACS.

RESULTS

Mechanism of Drug Resistance

The L5178YR cell line which was viable at 10^{-3} M MTX was found to contain 2.45 ± 0.9 μmol/min/mg DHFR activity, compared to 7.5 ± 2.1 $\times 10^{-3}$ μmol/min/mg activity in crude cell extracts from the parent cell line. This represents an average increase in enzyme activity of ~300-fold in the drug-resistant cell line. Transport of MTX was found to be unaltered in the resistant as compared to the parent cell line. The increase in enzyme activity was due to an increase in enzyme levels. This was demonstrated by titration with MTX (9) and the following experiment. [^{3}H]leucine pulse-labeled L5178Y cell extracts were combined with [^{14}C]leucine pulse-labeled L5178YR cell extracts, and the mixture was subjected to polyacrylamide disc gel electrophoresis. Fig. 1 *A* shows the electrophoretic pattern obtained with crude cell extracts of [^{3}H]leucine-labeled L5178Y and [^{14}C]leucine-labeled L5178YR. The ratio of ^{14}C to ^{3}H in the electrophoretogram of the two cell extracts is plotted as a function of R_f in Fig. 1 *B*. Fig. 1 *B* demonstrates that the primary difference in pulse-labeled protein content between the two cell lines is a gross elevation in a polypeptide migrating with an R_f characteristic of DHFR (see Fig. 2).

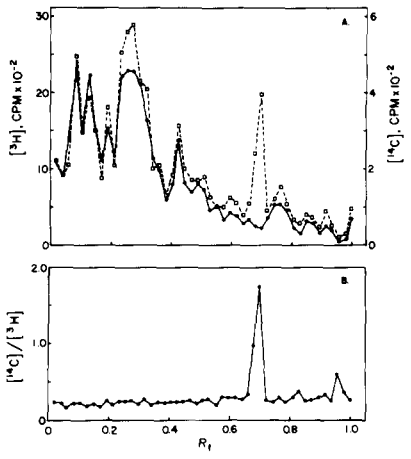

FIGURE 1 (*A*) SDS polyacrylamide (12.5%) disc gel electrophoresis of [^{14}C]leucine- (□) labeled L5178YR cell extracts (1.5×10^4 cpm) and [^{3}H]leucine- (●) labeled L5178YS cell extracts (7.5×10^4 cpm). Cells were pulse labeled for 60 min as described (Materials and Methods), except that L5178YR cells (2×10^7 cells) were incubated with [^{14}C]leucine (10 μmol) and L5178YS cells (1.7×10^7 cells) with [^{3}H]leucine (4 nmol) for 60 min at 37°C in a vol of 10 ml. Cell extracts were combined and incubated on ice for 10 min with 50 μg/ml each DNAse and pancreatic RNAse before electrophoresis. (*B*) Ratio of ^{14}C to ^{3}H counts per minute. Data are expressed as mobility relative to bromophenol blue. See Materials and Methods for details.

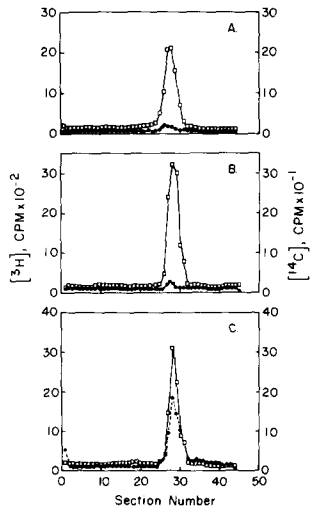

FIGURE 2 SDS polyacrylamide (12.5%) disc gel electrophoresis of immunoprecipitates from RNA-stimulated in vitro translation. Protein synthesis was performed with micrococcal nuclease-treated lysates as described (9), except that reaction vol were 480 μl and contained either no exogenous RNA (*A*) or 160 μg of total cellular RNA from either L5178Y S or R cells (*B* and *C*, respectively). After 60-min incubation at 25°C, 268 μl of a solution containing 192 μl of 0.1 M leucine and 76 μl of 10% Triton-X sodium deoxycholate was added. 5-μl aliquots were removed in duplicate to determine total protein synthesis, and 225-μl aliquots were immunoprecipitated in duplicate with DHFR-specific antiserum. 80% of the immunoprecipitated samples were solubilized, [^{14}C]DHFR was added as tracer, and the samples electrophoresed as described (Materials and Methods). (□) [^{14}C]leucine-labeled marker DHFR, (●) [^{3}H]leucine-labeled immunoprecipitates. The ^{3}H contents of immunoprecipitates A, B, C were 1,375, 3,965, and 11,141 cpm, respectively. See text for details.

The elevated levels of intracellular DHFR in the L5178YR line is primarily a result of increased rate of enzyme synthesis. This was demonstrated by immunoprecipitating cell extracts of [^{3}H]leucine-pulsed L5178Y R and S cells with a monospecific antiserum to DHFR (unpublished observations, and Fig. 2). Immunoprecipitation of the L5178YR cell extracts yielded values of 10% (±2.6 SD) for mid log cells (6×10^4 cell/ml) to 12% (±2 SD) for late log cells (6×10^5 cell/ml) as the percentage total soluble protein immunoprecipitable. Parallel samples of L5178YS cell extracts contained insignificant (0–0.1%) amounts of radioactivity in comparison.

To determine whether the increased rate of DHFR synthesis was due to increased mRNA activity coding for this enzyme, RNA prepared from MTX-sensitive and -resistant cell lines was translated in vitro with a rabbit reticulocyte lysate translation system. The reaction was terminated after 60 min (see legend to Fig. 2). The samples were immunoprecipitated. Some of the immunoprecipitates were processed for the determination of rates of DHFR synthesis while the remainder were solubilized and electrophoresed on SDS polyacrylamide disc gel electrophoresis. The results are presented in Fig. 2. Fig. 2*A* shows the results obtained upon translation in the absence of exogenous RNA while Fig. 2*B* and *C* shows the electrophoretic patterns of the immunoprecipitated material from the L5178YS and L5178YR RNA-supplemented lysates, respectively. Fig. 2 demonstrates that the anti-DHFR antiserum is monospecific and that a polypeptide of the same molecular weight as DHFR can be immunoprecipitated from L5178YR mRNA directed translated polypeptides but not from L5178YS mRNA. Quantitation of the immunoprecipitates indicated that 12% of the translated RNA was DHFR specific (2,470 cpm in immunoprecipitate, 19,800 cpm as total protein synthesis equivalent). A similar experiment conducted with poly(A) containing RNA from L5178Y R and S cells gave similar results. Translation of R-cell poly(A) containing RNA yielded an average value of 8% of total protein synthesis as DHFR (100 cpm immunoprecipitated/μg). From this experiment it was clear that the DHFR-specific mRNA activity of the L5178YR cells was elevated in comparison to the parent line.

To determine whether or not this elevation in mRNA activity for DHFR was due to an elevation of mRNA species, molecular hybridization experiments with a [^{3}H]cDNA probe specific for mouse DHFR mRNA were performed. Fig. 3 shows the results obtained when total RNA from either L5178Y R or S cells is hybridized to this purified probe. The kinetics of hybridization as illustrated in Fig. 3 show that DHFR mRNA is elevated several hundred-fold in the resistant cell line as compared to the parent cell line. Because it has been demonstrated previously (1, 19) that cells displaying elevated DHFR may do so via gene amplification, DNA renaturation studies were conducted to see whether this phenomenon occurred also in the L5178Y R or S cells (Fig. 4). The reassociation of DNA from the MTX-resistant cells as driver in the presence of purified [^{3}H]-cDNA probe displays kinetics indicative of a mod-

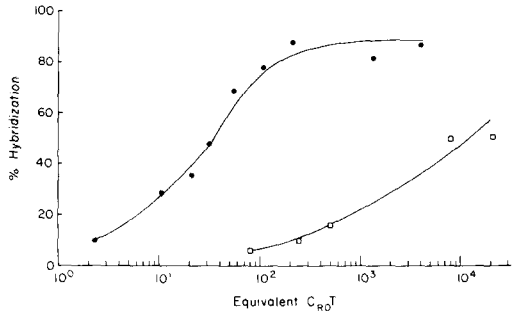

FIGURE 3 Hybridization of [^{3}H]cDNA (500 cpm, 50 pg) to cellular RNA from L5178Y R and S. Hybridizations were performed as previously described (9) in 0.6 M NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.4), 0.2% SDS at 68°C. Samples contained 12–120 μg of RNA in 10- to 100-μl reaction vessels. (●) L5178YR RNA, (□) L5178YS RNA. $C_{ro}T$ values are adjusted to standard conditions (9). See text for details.

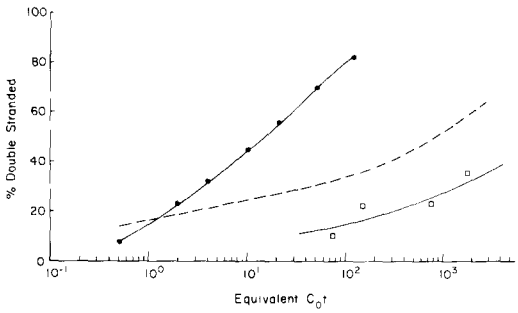

FIGURE 4 Association kinetics of [^{3}H]cDNA (500 cpm, 50 pg) with DNA from L5178YR (●) and L5178YS (□). Association experiments were performed as described previously (9) in 0.3 M NaCl, 1 mM EDTA, and 25 mM Tris-HCl (pH 7.4). C_0T values are adjusted to standard conditions. The reassociation of cellular DNA (- - -) was monitored spectrophotometrically.

erate repetitive frequency, while DNA from the sensitive cells displays kinetics characteristic of unique sequences. Thus, the L5178YR cell line contains several hundred copies of the gene for DHFR, relative to the parent cell line.

Karyotypic Studies

Karyotype analyses were next performed to determine whether any gross changes in chromosome banding patterns and morphology were evident between the two cell lines. Metaphase chromosome spreads were prepared as described (Materials and Methods). Representative Giemsa-trypsin-banded metaphase chromosome spreads appear in Fig. 5*A* and *B* of the L5178Y S and R lines, respectively. Both cell lines were essentially diploid, exhibiting a modal chromosome number of 40. As Figs. 5 and 6 illustrate, a large chromosome is present in the MTX-resistant cells which is not present in the parent sensitive cell line. This chromosome exhibits a HSR (i.e., a large region of intermediate staining intensity that does not band) and a translocation of the EFG region. Analysis of 100 Giemsa-trypsin-stained metaphase chromosome spreads showed that 90% of the resistant cells displayed one chromosome with an HSR while no HSR was observed in any of 100 sensitive cell spreads. Both cell lines were karyotypically similar in other respects, exhibiting a small degree of tetraploidy (1% in the sensitive and 2% in the resistant cell lines, respectively). The presence of the HSR and the duplication of the EFG region were the only consistent chromosomal differences between the two cell lines, and this was invariably localized to one homologue of chromosome No. 2 (Fig. 6).

Localization of DHFR Genes in L5178YR

In situ molecular hybridizations were carried out to attempt to localize the DHFR genes in the resistant cell line. Typical results of *in situ* hybridizations with the resistant cell line are presented in Fig. 7. There is specific clustering of silver grains over a region corresponding to the HSR on the long marker chromosome. Tabulation of grain distributions from 42 separate metaphase chromosome spreads yielded an average value of 25.5 ± 8.2 grains over the HSR compared to an average of 1.2 ± 0.05 grains over each of the other chromosomes. Detailed examination of eight representative spreads demonstrated the majority of nonmarker chromosomes to have either zero or one silver grain, only three out of these eight spreads displaying any chromosomes (other than the marker) with more than four grains. In those nonmarker chromosomes containing more than one silver grain, the grains are randomly distributed (unpublished observation). These results indicate that in the L5178YR line, genes coding for DHFR are primarily localized within an HSR region.

DISCUSSION

Increased intracellular accumulation of a target enzyme as a mechanism of drug resistance has been well documented for MTX as well as several

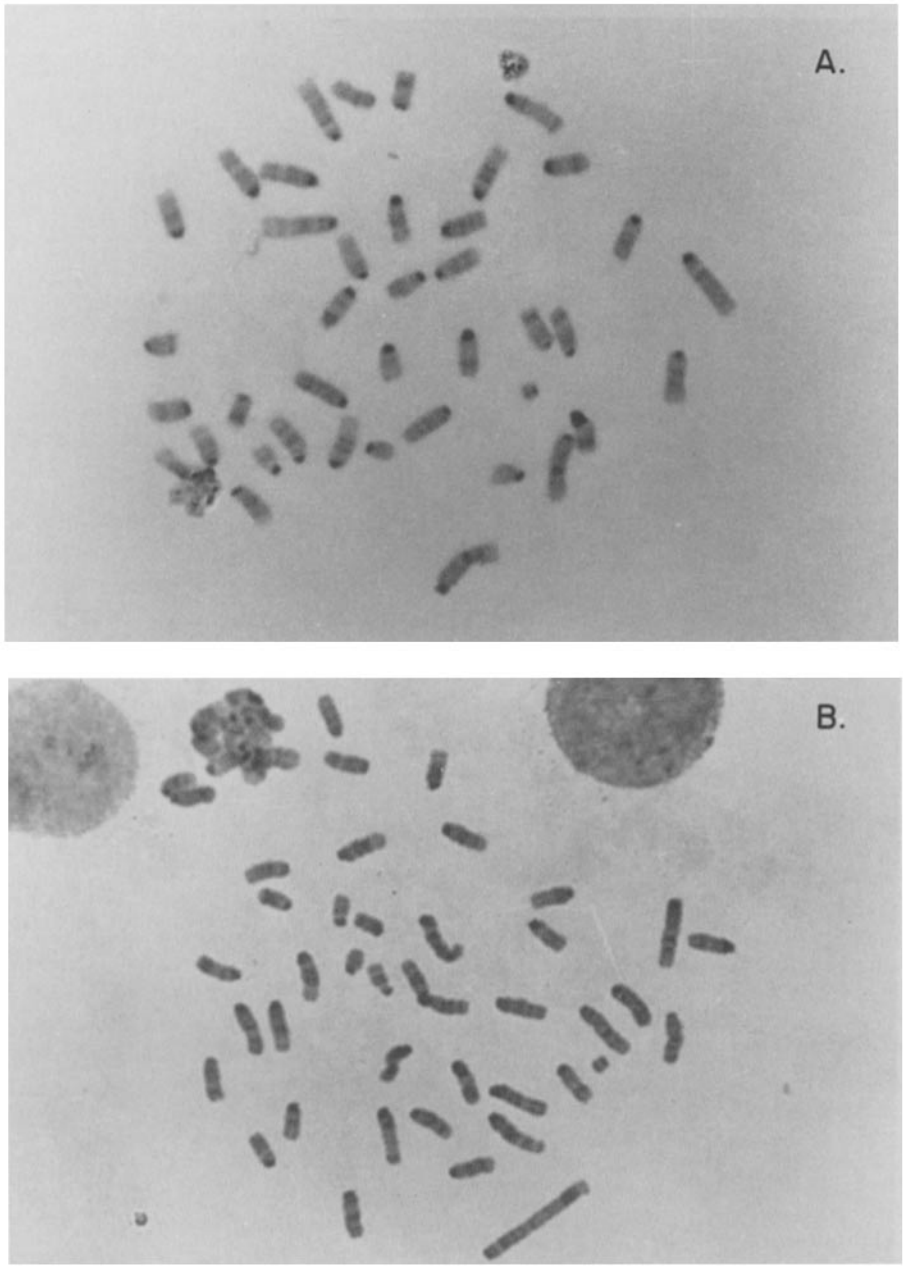

FIGURE 5 Giemsa-trypsin-stained metaphase chromosome spreads. (*A*) L5178YS. (*B*) L5178YR. See text for details.

other drugs (7–9, 13, 16, 17, 22). Until recently, the mechanism for this elevation of target enzyme as a response to drug treatment had not been elucidated. Recent studies show that in MTX-resistant murine and hamster cells in culture, the mechanism of elevated DHFR levels can be attributed to increases in rates of synthesis of that enzyme (2, 10). Furthermore, it has been demonstrated that these systems also display elevated mRNA species for DHFR coding sequences as well as propor-

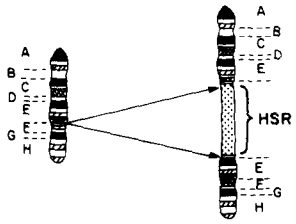

FIGURE 6 Schematic representation of HSR localization. See text for details.

tional amplification of the DHFR gene dosage (1, 23).

Even before the studies of Alt et al. (1) demonstrating gene amplification, Biedler and Spengler (3, 4) reported that the appearance of an HSR could be correlated with elevated DHFR levels in an MTX-resistant Chinese hamster lung cell line. In the absence of MTX, these cells tend to lose their elevated DHFR levels. This loss of resistance was correlated with a decrease in the size of the HSR (3). The HSR in a DHFR-elevated Chinese hamster cell line was recently reported to contain the amplified DHFR genes (19). This was demonstrated by using mouse [^{3}H]cDNA synthesized from DHFR mRNA and *in situ* molecular hybridization to metaphase chromosomes from an elevated DHFR-containing Chinese hamster ovary cell line.

In this report, a highly MTX-resistant diploid mouse lymphoblast subline (L5178YR) was found to contain an ~300-fold elevation in enzyme activity over the parent line, a direct result of an increase in enzyme concentration. By immunoprecipitation of [^{3}H]leucine-pulsed cell extracts with anti-DHFR-specific antiserum, it was determined that 10% of total soluble protein was being synthesized as DHFR at any one time. Translation of the mRNA from these MTX-resistant cells in a cell-free rabbit reticulocyte lysate translation system with specific immunoprecipitation of the [^{3}H]leucine-labeled products indicated that the mRNA activity for DHFR was elevated greatly in this cell line.

Molecular hybridization of the total cellular RNA to a purified mouse [^{3}H]cDNA specific for DHFR mRNA revealed the amount of mRNA for DHFR to be elevated several hundredfold in the resistant cell line. DNA reassociation experiments with tracer amounts of the same [^{3}H]cDNA probe indicate that the number of genes coding for DHFR is also elevated several hundredfold. Thus, the L5178YR system appears quite similar to other mouse (sarcoma 180, L1210), hamster, and a 3T6 cell line having elevated DHFR (1, 2, 10–12, 16, 23).

The L5178YR cell line, in contrast to the sarcoma 180- and L1210-elevated DHFR cell lines, is diploid. As a result of this property and its greatly elevated DHFR gene dosage, it presents an excellent homologous system for studying the correlation of biochemical and molecular changes with chromosomal morphology. The results of Giemsa-trypsin chromosome banding studies indicate that the MTX-resistant cells contained a large marker chromosome not present in the sensitive cell line and that this was the only major karyotopic difference. The discovery of a large nonbanding region of intermediate staining intensity exclusively localized to chromosome No. 2 confirms results with other MTX-resistant, DHFR-elevated cell lines (3, 4, 19).

The results of *in situ* molecular hybridization with metaphase chromosomes from the L5178YR cells with a [^{3}H]cDNA probe from homologous DHFR poly(A) containing RNA demonstrates the biological significance of the HSR. As previously demonstrated for MTX-resistant, elevated DHFR-containing Chinese hamster ovary cells (19), the probe was found to hybridize exclusively to this HSR. The clustering of silver grains over the region corresponding to an HSR on the long marker chromosome indicated that multiple DHFR gene copies are contained within the HSR. In addition, the distribution of silver grains along the entire length of the HSR suggests that genes for DHFR are present throughout the complete length of the HSR. While it is impossible to exclude the presence of one or a few DHFR genes on other chromosomes, these results indicate that the majority of DHFR genes in the L5178YR cells are localized in the HSR of marker chromosome No. 2. In the Chinese hamster ovary system, the HSR-containing DHFR genes were also found to reside on chromosome No. 2 (19). Because the HSR on the mouse chromosome represents ~5% of the total karyotype, and because there are an estimated 4.6 $\times 10^9$ base pairs in a mouse cell DNA complement (15), the HSR should contain ~2.3 $\times 10^8$ base pairs. Based upon an average amplification of 300-fold, this represents a repeat unit on the order of 800 kilo base pairs. This can be compared to a value of 500–1,000 kilo base pairs for the Chinese hamster ovary cell line (19). It appears that gene amplification phenomena may occur by similar mechanisms in different mammalian species.

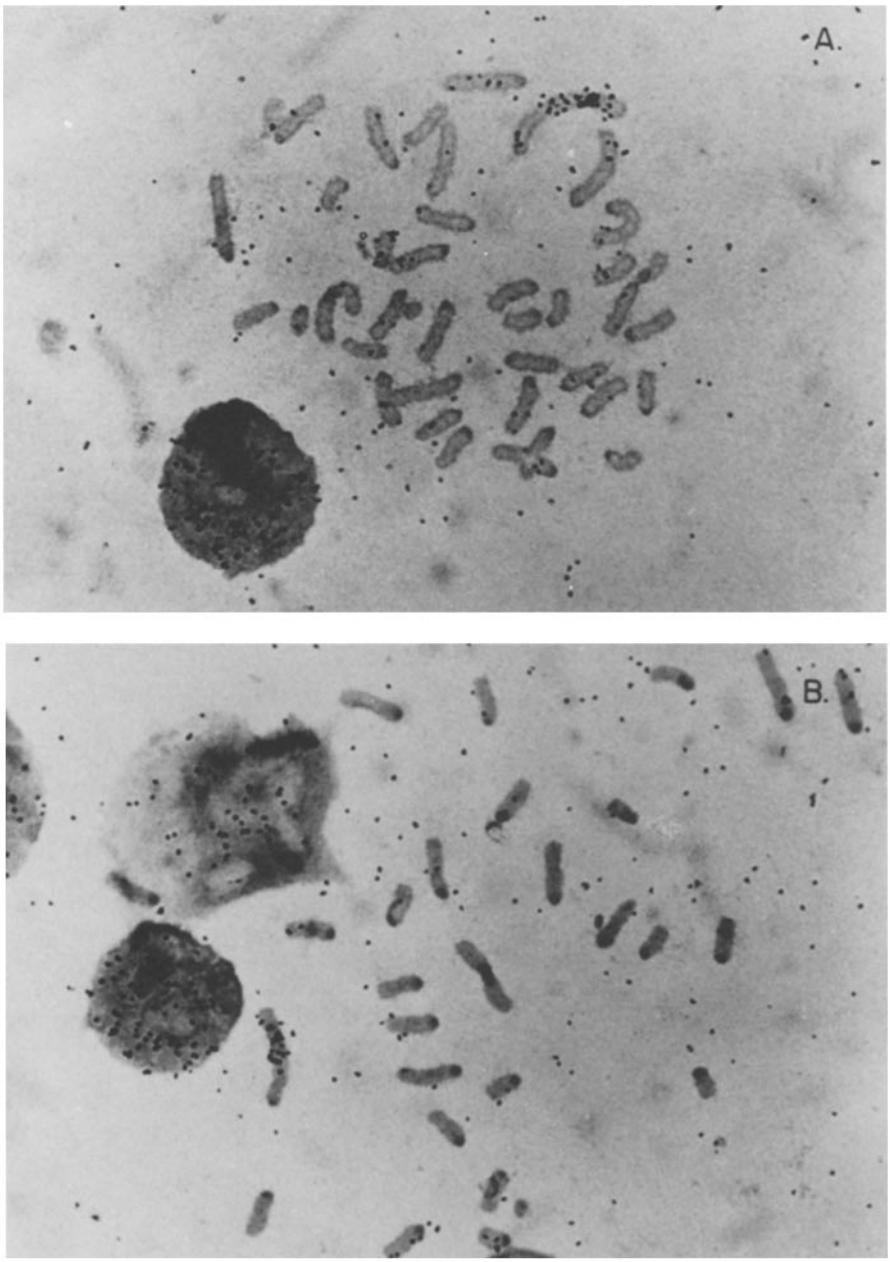

FIGURE 7 *In situ* molecular hybridization of L5178YR. Representative *in situ* molecular hybridizations of metaphase chromosome spreads were prepared as described (Materials and Methods).

The authors would like to express their special thanks to Mrs. J. Uhoch for her efforts in the development of the L5178YR cell line. The authors would like to express their gratitude to Doctors Breg, Francke, and Gall for their advice on various aspects of this work.

This work was supported by grants CA08010 and CA16318 from the National Cancer Institute (NCI) and by NCI fellowship CA06195 to B. J. Dolnick. J. R. Bertino is an American Cancer Society professor.

Received for publication 17 May 1979, and in revised form 23 July 1979.

REFERENCES

1. ALT, F. W., R. E. KELLEMS, J. R. BERTINO, and R. T. SCHIMKE. 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem.* **253:**1357–1370.
2. ALT, F. W., R. E. KELLEMS, and R. T. SCHIMKE. 1976. Synthesis and degradation of folate reductase in sensitive and methotrexate resistant lines of S-180 cells. *J. Biol. Chem.* **251:**3075–3080.
3. BIEDLER, J. L., and B. A. SPENGLER. 1976. Quantitative relationship between a chromosome abnormality (HSR) and antifolate resistance associated with enzyme overproduction. *J. Cell. Biol.* **70**(2, Pt. 2):117*a*.
4. BIEDLER, J. L., and B. A. SPENGLER. 1976. Metaphase chromosome anomaly: association with drug resistance and cell-specific products. *Science (Wash. D. C.).* **191:**185–187.
5. BLAKELY, R. L. 1960. Crystalline dihydropteroylglutamic acid. *Nature (Lond.).* **188:**231.
6. CONN, H. J., M. A. DARROW, and V. M. EMMEL. 1960. *In* Staining Procedures, 2nd ed. The Williams & Wilkins Company, Baltimore, Md. 136–137.
7. FISCHER, G. A. 1961. Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells. *Biochem. Pharmacol.* **7:**75–80.
8. FRIEDKIN, M., E. J. CRAWFORD, S. R. HUMPHREYS, and H. GOLDIN. 1962. The association of increased dihydrofolate reductase with amethopterin resistance in mouse leukemia. *Cancer Res.* **22:**600–606.
9. HAKALA, M. T., S. F. ZAKRZEWSKI, and C. A. NICHOL. 1961. Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. *J. Biol. Chem.* **236:**952–958.
10. HANGGI, U. J., and J. W. LITTLEFIELD. 1976. Altered regulation of the rate of synthesis of dihydrofolate reductase in methotrexate-resistant hamster cells. *J. Biol. Chem.* **251:**3075–3080.
11. KELLEMS, R. E., F. W. ALT, and R. T. SCHIMKE. 1976. Regulation of folate reductase synthesis in sensitive and methotrexate-resistant sarcoma-180 cells. *J. Biol. Chem.* **251:**6987–6993.
12. KELLEMS, R. E., V. B. MORHENN, E. A. PFENDT, F. W. ALT, and R. T. SCHIMKE. 1979. Polyoma virus and cyclic AMP-mediated control of dihydrofolate reductase mRNA abundance in methotrexate-resistant mouse fibroblasts. *J. Biol. Chem.* **254:**309–318.
13. KEMPE, T. D., E. A. SWYRYD, M. BRUIST, and G. R. STARK. 1976. Stable mutants of mammalian cells that overproduce the first three enzymes of pyrimidine nucleotide biosynthesis. *Cell.* **9:**541–550.
14. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680–685.
15. LEWIN, B. 1974. *In* Gene Expression, Vol. II. John Wiley & Sons, Inc., New York. 7.
16. LITTLEFIELD, J. W. 1969. Hybridization of hamster cells with high and low folate reductase activity. *Proc. Natl. Acad. Sci. U. S. A.* **62:**88–95.
17. MEUTH, M., and H. GREEN. 1974. Alterations leading to increased ribonucleotide reductase in cells selected for resistance to deoxyribonucleosides. *Cell.* **3:**367–374.
18. NESBITT, M. N., and U. FRANCKE. 1973. A system of nomenclature for band patterns of mouse chromosomes. *Chromosoma (Berl.).* **41:**145–158.
19. NUNBERG, J. H., R. J. KAUFMAN, R. T. SCHIMKE, G. URLAUB, and L. A. CHASIN. 1978. Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. *Proc. Natl. Acad. Sci. U. S. A.* **75:**5553–5556.
20. OSBORN, M. J., and F. M. HUENNEKENS. 1958. Enzymatic reduction of dihydrofolic acid. *J. Biol. Chem.* **233:**969–974.
21. PARDUE, M. L., and J. G. GALL. 1975. Nucleic acid hybridizations to the DNA of cytological preparations. *Methods Cell Biol.* **10:**1–16.
22. PERKINS, J. P., B. L. HILLCOAT, and J. R. BERTINO. 1967. Dihydrofolate reductase from a resistant subline of the L1210 lymphoma. *J. Biol. Chem.* **242:**4771–4776.
23. SCHIMKE, R. T., R. J. KAUFMAN, F. W. ALT, and R. E. KELLEMS. 1978. Gene amplification and drug resistance in cultured murine cells. *Science (Wash. D. C.).* **202:**1051–1055.
24. STUDIER, F. W. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. *J. Mol. Biol.* **79:**237–248.
25. SUN, N. C., E. H. Y. CHU, and C. C. CHANG. 1974. Staining method for G-banding patterns of tumor mitotic chromosomes. *Caryologia.* **27:**315–324.
26. WERKHEISER, W. C. 1961. Specific binding of 4-amino analogues by folic acid reductase. *J. Biol. Chem.* **236:**888–893.