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Amplification and Organization of Dihydrofolate Reductase Genes in a Human Leukemic Cell Line, K-562, Resistant to Methotrexate†

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ABSTRACT: A subline of human leukemia cells (K-562), highly resistant to methotrexate, was developed by stepwise selection in the presence of increasing concentrations of this drug. The ED_{50} of these resistant cells was 1 mM compared to 10 nM for the parental line. Comparison of certain folate-requiring enzymes from crude extracts of the parent and resistant cells showed a 240-fold elevation of dihydrofolate reductase activity in the resistant cells with no significant increase in the levels of the other enzymes. Purified dihydrofolate reductase from the resistant cells had the same physical and kinetic properties as the enzyme from the sensitive cells. Southern blot analysis showed a marked increase in the number of dihydrofolate reductase genes in the resistant line. The genomic organization of the human dihydrofolate reductase gene was determined by hybridization with specific cDNA sequences from a human cDNA to DNA fragments from K-562 cells generated by restriction endonucleases. The human dihydrofolate reductase gene contained at least four intervening sequences and was approximately 30 kb in size. Northern blot studies demonstrated an increase of dihydrofolate reductase mRNA species; the predominant message was 3.8 kb. Karyotype analysis revealed three elongated marker chromosomes, derived from chromosomes 5, 6, and 19 which contained homogeneous staining regions, which were not present in the parent cell line.

Methotrexate is an important drug used in the clinic for the treatment of several human malignancies (Johns & Bertino, 1973). Development of drug resistance, however, occurs frequently and limits its further use. An understanding of the mechanism(s) of resistance to this drug in human cells might provide some insights into strategies to prevent resistance or to develop new therapies that selectively kill resistant cells (Bertino, 1979).

Amplification of the dihydrofolate reductase (DHFR)[1] gene is a known mechanism of resistance to methotrexate (MTX) in several mammalian cell lines (Alt et al., 1978; Bostock & Clark, 1980; Dolnick et al., 1979; Melera et al., 1980; Milbrandt et al., 1981). The presence of a homogeneous staining region (HSR) on a chromosome(s) has been associated with stable or slowly reversible drug resistance (Berenson et al., 1981; Biedler et al., 1980; Dolnick et al., 1979; Nunberg et al., 1978) whereas unstable amplification has been associated with the presence of the DHFR genes on double minute chromosomes (Kaufman et al., 1979).

To determine whether human cell lines could also develop resistance by virtue of DHFR gene amplification, K-562 cells, derived from a pleural effusion of a patient with chronic

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[1] Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); FAH_2, dihydrofolic acid; FAH_4, tetrahydrofolic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; dUMP, deoxyuridine monophosphate; pCMB, *p*-(chloromercuri)benzoic acid; HSR, homogeneous staining region; kb, kilobase; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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myelocytic leukemia in blast crisis (Lozzio & Lozzio, 1973), were grown in progressively higher concentrations of MTX over a period of months eventually yielding a population of cells which were able to grow in 100 μM MTX. Characterization of this resistant cell line demonstrated an increase in DHFR activity which was correlated with an increase in DHFR mRNA species and gene copies for this enzyme. The DHFR enzyme from the resistant and sensitive cells has been purified and characterized, and the karyotypes of these cells have been determined. The genomic organization of this human DHFR gene is also discussed.

Experimental Procedures

Materials. Dihydrofolate (FAH_2) and [3′,5′,7,9-$^3H_2$] folate were synthesized by the method of Blakley (1960). [5-^{3}H]-dUMP (12 Ci/mmol) was purchased from Moravek Biochemicals, City of Industry, CA. Cyanogen bromide activated Sepharose, Sephadex G-25, and Sephadex G-150 were purchased from Pharmacia Fine Chemicals. MTX was a gift from Dr. Harriet Kiltie of Lederle Laboratories. All other chemicals were the highest purity available.

Cell Growth. The human cell line, K-562, was obtained from Dr. B. B. Lozzio (Lozzio & Lozzio, 1973) and grown in RPMI 1640 medium containing penicillin (100 units/mL) and streptomycin (100 μg/mL) supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY). From this parent line, a cloned subline, $K\text{-}562_C$, was grown in progressively higher concentrations of MTX, starting with a nanomolar concentration and using about 2-fold increments of drug, over a period of months eventually yielding a heterogeneous population of cells which were able to grow in 100 μM MTX. This cell line was designated K-562/R4.

Enzyme Assays. The cells from both lines, K-562 and K-562/R4, were harvested at mid-logarithmic growth, centrifuged at 400*g*, and washed 3 times with 0.9% NaCl. The cells were resuspended in 4 volumes of buffer I which consisted of 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10% glycerol, and 10 mM 2-mercaptoethanol. The cells were disrupted on ice by using a standard microtip on a Branson Sonifier, set at 40 W in four 15-s bursts. The preparation was checked microscopically for complete lysis. After centrifugation at 37000*g* for 30 min in a Sorvall RC-2 refrigerated centrifuge, the supernatant was used for determination of enzyme activities or was used for purification of DHFR.

Dihydrofolate Reductase (EC 1.5.1.3). This enzyme was assayed spectrophotometrically (Bertino et al., 1965). The decrease in absorbance at 340 nm which occurred when NADPH and FAH_2 were converted to NADH and FAH_4, respectively, at 37 °C was recorded at 5-s intervals with a Gilford 2000 attachment to a Beckman DU spectrophotometer. The assay contained in a final volume of 1.0 mL the following: 100 μmol of Tris-HCl, pH 7.0 or 8.5, 150 μmol KCl, 0.1 μmol NADPH, enzyme, and deionized water. The reaction was initiated by the addition of 0.05 μmol of FAH_2 containing 0.5 μmol of 2-mercaptoethanol.

Enzyme kinetics were studied with the radioassay of Hayman et al. (1978) by using tritiated FAH_2. In this assay, the blank (a tube without enzyme) was less than 5% of the total radioactivity incubated, and 25–50% of the substrate was converted to FAH_4. The inhibition constants were measured noncompetitively by incubating varying amounts of MTX with constant amounts of enzyme and NADPH on ice for 10 min prior to the addition of labeled FAH_2. Typically, DHFR was 2 nM per reaction.

Thymidylate synthetase (EC 2.1.1.45) was assayed by the method of Roberts (1966) as modified by Rode et al. (1980). The reaction mixture contained in a final volume of 40 μL the following: 2.0 nmol of [5-^{3}H]dUMP, 18 nmol (dL) of FAH_4, 30 nmol of formaldehyde, 0.4 μmol of 2-mercaptoethanol, 2 μmol of NaF, 2 μmol of phosphate buffer, pH 7.5, and enzyme at 20 μL. The assay minus enzyme was used as a control. The reaction was initiated by the addition of enzyme and was terminated after a 30-min incubation at 37 °C by the addition of 200 μL of a suspension of charcoal (Norit; 100 mg/mL) in 2% trichloroacetic acid. After centrifugation for 30 s on a Beckman microfuge B, 100 μL of the supernatant was added to 400 μL of water and 4.0 mL of Aquasol (New England Nuclear, Boston, MA) and counted in a Beckman LSC7000. The activity is expressed as nanomoles tritium released per hour per milligram of protein.

Serine hydroxymethylase (EC 2.1.2.1), formyltetrahydrofolate synthetase (EC 6.3.4.3), methylenetetrahydrofolate reductase (EC 1.1.1.68), and folylpolyglutamate synthetase were assayed by using the methods described by Taylor & Weissback (1965), Bertino et al. (1962), Kutzbach & Stokstad (1967), and McGuire et al. (1980), respectively.

Preparation of MTX–Sepharose Column. The affinity resin for the purification of DHFR was prepared according to the method of Rode et al. (1979) except that the 10-formyl-5,8-dideazafolic acid which was used as the affinity adsorbent for the purification of thymidylate synthetase was replaced with MTX.

Purification of Dihydrofolate Reductase. To the crude supernatant, as described above, was added slowly solid ammonium sulfate to a final concentration of 35% (200 g/L). After mechanical stirring for 30 min at 4 °C, the solution was centrifuged as before. To this supernatant, was added slowly more ammonium sulfate to 70% saturation (278 g/L) and stirred for 1 h at 4 °C. After centrifugation, the 35–70% pellet was dissolved in a minimum volume of buffer I. This solution was applied to the MTX–Sepharose column.

MTX–Sepharose was slurry packed in a 3-mL plastic syringe, fitted with a glass wool plug, to a height of 1 mL. A thin layer of G-25 was placed on top to prevent perturbation of the surface of the Sepharose. The column was equilibrated with 20 mL of buffer I, washed with 20 mL buffer II (buffer I containing 300 mM KCl), followed by 20 mL of buffer III (50 mM Tris-HCl, pH 8.5, and 10 mM 2-mercaptoethanol), and then reequilibrated with buffer I. The 35–70% ammonium sulfate fraction was then sorbed onto the column. One-milliliter fractions were collected. The column was then washed with buffer I until the absorbance at 280 nm was the same as the buffer. The column was then washed with 30 mL of buffer II, followed by 20 mL of buffer III supplemented with 200 μM FAH_2. Fractions were assayed spectrophotometrically for DHFR activity. The fractions which contained activity were pooled and aliquots frozen at −20 °C.

FAH_2 was removed from the enzyme as needed by passage through Sephadex G-25. At 4 °C, a 10-mL plastic pipet was slurry packed with G-25 and equilibrated in 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100. An aliquot of 200 μL of the MTX–Sepharose-purified enzyme was applied to the column, eluted with buffer, and collected in 0.5-mL fractions. The fractions were read spectrophotometrically at 282 nm for FAH_2 and radioassayed for DHFR activity.

Gel Electrophoresis. Portions of each purification step for both cell lines were analyzed on PAGE by using 12% running gel as described by Laemmli (1970).

Protein Determination. Protein concentrations in the crude extracts were determined by the biuret method. The absorbance at 280 nm (1.0 absorbance unit = 1 mg/mL) was used

for the ammonium sulfate steps. It was not possible to obtain accurate protein concentrations of the pure enzymes since FAH_2 interfered with the method of Bradford (1976) and Triton interfered with the method of Waddell (1956).

Effects of Salts on Enzyme Activity. The effect of KCl on activity of both purified enzymes was measured spectrophotometrically as described by Bertino (1962), the effect of pCMB as described by Perkins & Bertino (1964), and guanidine hydrochloride and urea as described by Kaufman (1963).

Karyotype Analysis. Logarithmically growing K-562 and K-562/R4 cells were treated with colcemid (0.1 μg/mL) for varying time intervals from 0 to 87 min. The cells were harvested and centrifuged in 50-mL plastic centrifuge tubes at 1000 rpm for 5 min. Each pellet was suspended in 10 mL of 75 mM KCl (initially at 37 °C), triturated 3 times, and transferred to 15-mL round bottom centrifuge tubes. After 11–12 min, the tubes were centrifuged at 1000 rpm for 5 min, and the supernatant was carefully removed by aspiration and discarded. Ice-cold fixative, freshly made MeOH–glacial acetic acid (3:1) was added slowly dropwise to the cells with agitation. The fixative was changed 3 times over 1 h and the pellet resuspended in a small volume of fixative. Three drops of cell suspension were dropped onto clean, chilled microscope slides from a height of 25 in. Prior to use, the slides were stored in distilled water at 4 °C. A film of water covered the slides when the cell spreads were made. To facilitate drying, the slides were placed on a warming plate. The slides were stored at room temperature for 2 days and then baked at 95 °C for 15 min. Chromosomes were banded by using sequential trypsin–Giemsa (Wright's) staining as described by Francke et al. (1978). Metaphase cells were karyotyped by using a Nikon biological microscope (type 104, Nippon Kogaku, K.K., Tokoyo, Japan) equipped with a Microflex AFX photomicrographic attachment (Nikon). Photographs were made of representative cells.

Southern Blot Analysis. For restriction enzyme analysis, the DNAs were isolated from cells by the procedure of Blin & Stafford (1976). The restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and Boehringer Mannheim Biochemicals (Indianapolis, IN) and were used according to the directions of the manufacturers. ^{32}P-end-labeled λ *Hin*dIII fragments were used as markers to determine the size of DHFR specific restriction fragments. The digested DNAs were electrophoresed on 0.8% agarose gels in Tris–acetate–EDTA buffer (Hayward & Smith, 1972). Following depurination and neutralization, the DNA was transferred to gene screen (New England Nuclear) according to the procedures developed by Southern (1975) with modifications suggested by the manufacturer. DHFR specific restriction fragments were identified initially by using ^{32}P nick-translated cDNA probes $pDHFR_{21}$ and $pDHFR_{26}$, kindly provided by Dr. R. T. Schimke (1978). However, as human DHFR cDNA was available at the later stages of this work, the Southern blots were also analyzed by using ^{32}P nick-translated human DHFR cDNA, pHD84, kindly provided by Dr. G. Attardi (1982).

For mapping the DHFR gene, specific restriction fragments from pHD84 labeled with ^{32}P were used as hybridization probes. The separation and isolation of the desired restriction fragments from polyacrylamide gels were done by using the procedures described by Maxam & Gilbert (1977).

Northern Blot Analysis of mRNA. Total cytoplasmic RNA from sensitive and methotrexate resistant cell lines prepared according to Kellems (1976) was further purified by the cesium chloride–guanidinium thiocyanate sedimentation procedure (Chirgwin et al., 1979) as modified by Setzer et al. (1980). Poly(A)-containing mRNA was prepared by oligo(dT)–cellulose chromatography as described by Aviv & Leder (1972). Total cytoplasmic and poly(A) RNA were electrophoresed on 1% agarose gels containing 10 mM methyl mercury hydroxide. After electrophoresis, RNA was transferred to diazobenzyloxymethyl paper (Wahl et al., 1979) and hybridized with DHFR cDNA following essentially the procedure described by Setzer et al. (1980).

Table I: Level of Dihydrofolate Reductase and Other Folate Coenzyme Mediated Reactions in K-562 Parent and R/4 Resistant Subline

	μmol h^{-1} (mg of protein)$^{-1}$ [a]	
	parent	R/4
dihydrofolate reductase	0.20	46.5
thymidylate synthetase	0.03	0.01
serine hydroxymethylase	0.05	0.03
formyltetrahydrofolate synthetase	2.1	1.0
methylenetetrahydrofolate reductase	0.1	0.2
folylpolyglutamate synthetase [b]	2.0	0.8

[a] The extracts from the sensitive and resistant lines were assayed as described under Experimental Procedures. [b] Picomoles of [^{3}H]Glu incorporated per hour per milligram × 10^3.

Results

Characterization of the MTX-Resistant Subline. Stepwise exposure of a clone of K-562 cells in increasing concentration of MTX resulted in a cell line highly resistant to MTX. The resistant cell line obtained was propagated in 0.1 mM MTX and had a generation time of 41 h, as compared to a 25-h generation time of the parent line. The ED_{50} for MTX of the resistant cells was 1 mM while the ED_{50} of the parent line was 10 nM.

As shown in Table I, DHFR activity in crude extracts of the resistant line was about 240-fold higher than that from the sensitive line. Since it was possible that one or more other folate enzyme genes were closely linked to the DHFR gene and amplified, in particular serine hydroxymethylase and thymidylate synthetase, the activities of these as well as some other folate enzymes were determined. As noted in Table I, the activity of these folate enzymes was not increased in the resistant cell line as compared to the sensitive line. The increase in DHFR activity noted in the resistant line was further demonstrated by the presence of a more dense protein band corresponding to the molecular weight of DHFR (ca. 20 000) when crude lysates from the resistant and sensitive cells were analyzed on PAGE (Figure 1, lanes 2 and 3). This was the only difference in the patterns observed from these cell lines.

Purification of DHFR from Sensitive and Resistant Lines. DHFR from the parent and resistant cell lines was purified by using ammonium sulfate and chromatography on MTX–Sepharose column, recoveries of 57% and 88% were calculated for the enzyme activities from the sensitive and resistant cells, respectively. On PAGE, essentially homogeneous DHFR was obtained after this purification procedure for the resistant cells (Figure 1). Both enzymes had similar molecular weights as determined by their mobility on this gel (ca. 20 000).

Table II provides the data on the kinetic characteristics of the sensitive and resistant purified enzymes. Similar K_m values for the cofactor NADPH and the substrate, FAH_2, were found for both enzymes. The slightly higher K_m for FAH_2 noted for the enzyme from the resistant line is not thought to be significant, since both enzymes demonstrate some instability at

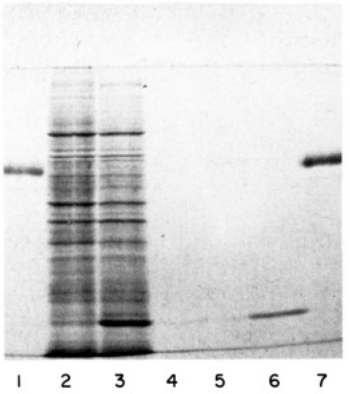

FIGURE 1: PAGE of K-562 sensitive and resistant enzymes. Lane 1, 2.5 μg of bovine serum albumin; lane 2, 100 μg of crude sensitive K-562; lane 3, 100 μg of crude K-562/R4; lane 4, unloaded; lane 5, purified sensitive enzyme from MTX–Sepharose; lane 6, purified resistant enzyme from MTX–Sepharose; lane 7, 4 μg of serum albumin. The bands in lanes 2 and 3 are similar except for the heavy band exhibited in lane 3 at about M_r 20000. Other protein standards were run as markers (data not shown). Lanes 5 and 6 show single bands, with the band in 6 estimated at 2 μg of protein in comparison to the load in lane 1 of 2.5 μg of serum albumin. Details of electrophoresis are described under Experimental Procedures.

Table II: Kinetic Properties of Dihydrofolate Reductase from K-562 Parent and R/4 Cell Lines

	parent[a]	R/4[a]
$K_m(FAH_2)$	1.0 μM	1.5 μM
K_m(NADPH)	7.1 μM	6.7 μM
turnover no.	487/min	660/min
K_i(MTX)	0.13 nM	0.15 nM

[a] The kinetic constants were determined by using the method of Hayman et al. (1978) on purified enzyme extracts from which the FAH_2 had been removed as described in the text.

4 °C after removal of FAH_2 as described above. The K_i values for MTX were 0.13 and 0.15 nM for enzymes from the sensitive and resistant cells, respectively; thus, there was no evidence that decreased binding of DHFR from the resistant line was a factor in the resistance of this line to MTX. Other data strengthened the conclusion that DHFR from the resistant subline was not qualitatively different from the sensitive line; these data include similar pH–activity profiles (Figure 2) and equivalent activation by chaotropic agents. KCl (450 mM) increased enzyme activity 2-fold; guanidine hydrochloride (300 mM) stimulated both enzymes 2.5-fold and urea (1.7 M) 2-fold, while pCMB (500 μM) had no effect on either enzyme (data not shown).

Uptake of MTX in the Sensitive and MTX-Resistant Lines. Two subclones of the resistant K-562/R4 line were used to compare the uptake of MTX to the sensitive line (Nahas et al., 1972). Similar initial rates of uptake were noted. As expected due to the markedly increased level of DHFR in the resistant lines, the plateau value of MTX uptake achieved was much higher in the resistant sublines (data not shown). From these results, it was concluded that MTX uptake was not decreased in the resistant subline.

Karyotype Analysis. In view of the karyotypic changes noted in several other mammalian cell lines highly resistant to MTX (Nunberg et al., 1978; Biedler et al., 1980; Berenson et al., 1981; Kaufman et al., 1979), the chromosomes of the resistant and sensitive lines were examined by chromosome banding techniques. Optimal results for K-562 cells were obtained with no exposure to colcemid and for K-562/R4 cells with 15-min exposure. Banding was optimal when the cell

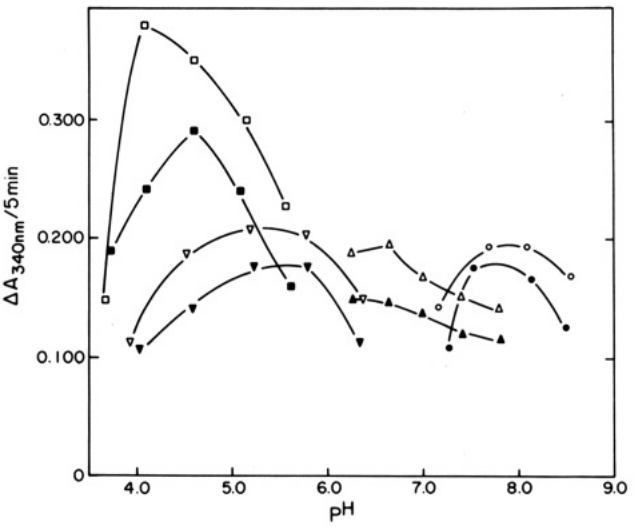

FIGURE 2: pH–activity profile for K-562 enzymes from sensitive and resistant K-562 cells purified from MTX–Sepharose. The assay contained in a final volume of 1.0 mL the following: 100 μmol of buffer as indicated, 150 μmol of KCl, 0.1 μmol of NADPH, enzyme, and deionized water. The reaction was initiated by the addition of 0.05 μmol of H_2 of folate containing 0.5 μmol of 2-mercaptoethanol. Appropriate blanks minus H_2folate were run. The solid symbols represent enzyme from the sensitive cells and the open symbols, enzyme from the resistant cells. The buffers were (○) Tris-HCl, (△) phosphate, (□) acetate, and (▽) citrate. The pH was measured at the end of each reaction period.

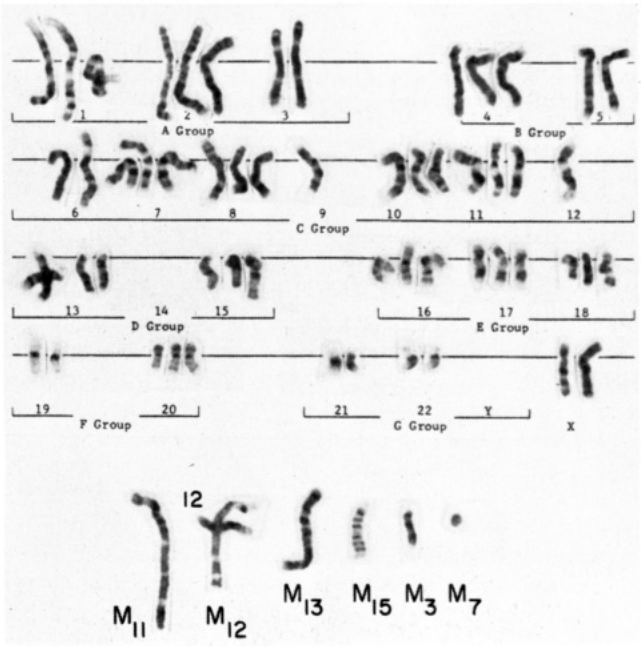

FIGURE 3: Karyotype of a single K-562/R4 cell. Ten marker chromosomes were found in the parent line, K-562 (M_1–M_{10}). Of these 10, 4 were found in the resistant strain, K-562/R4 (M_2, M_3, M_4, M_7). In addition, five new markers were found in the resistant cells (M_{11}–M_{15}). This cell contained six of the nine marker chromosomes found in the R/4 line. Two of the marker chromosomes seen in this cell, M_{11} and M_{12}, contained HSRs and were seen in 90% of the cells karyotyped. The third marker chromosome, M_{14}, containing an HSR was not found in this cell but occurred in 60% of the cells karyotyped.

spreads were treated with trypsin for 40–50 s and covered with Wright's stain for 55–60 s.

Chromosomes from 40 K-562 cells were counted. The modal number of chromosomes was 65. Eleven cells were karyotyped, and 10 marker chromosomes were found. One hundred K-562/R4 cells were counted and 16 karyotyped.

Table III: Restriction Enzyme Analysis of Human DHFR Gene[a]

*Eco*R1		*Hin*dIII		*Pst*1		*Bam*H1		*Bgl*II	
human	mouse	human	mouse	human	mouse	human	mouse	human	mouse
14	14.8	22	12.9	9	3.9	28	>20.5	5.2	7.9
5.8	6.0	5.4	3.8	8	2.8		17.6	3.9	4.4
4.0	5.4	4.2	3.4	4.5	2.0		4.8	3.7	4.2
1.8	3.4		3.4		1.6			3.5	0.55
1.7					1.6			1.9	0.30

[a] Restriction fragments containing DHFR coding sequence sizes in kilobases. Data from the human DHFR gene, Figure 5. Mouse DHFR restriction fragment sizes are from Crouse et al. (1982) except for *Pst*1, taken from Nunberg et al. (1980).

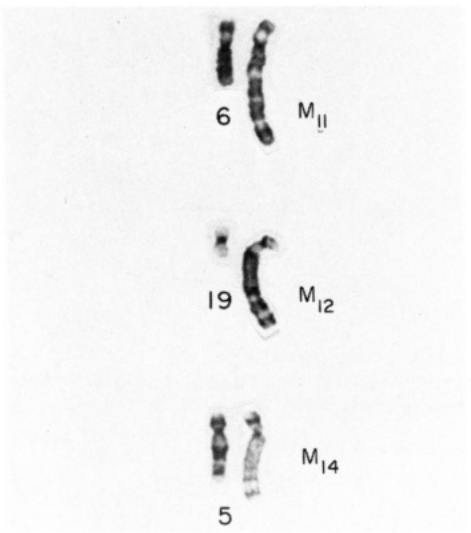

FIGURE 4: Marker chromosomes containing HSRs in K-562/R4 cells. The marker chromosomes containing HSRs (M_{11}, M_{12}, M_{14}) were taken from different cell preparations. These markers were derived from chromosomes 5, 6, and 19. M_{11} derived from chromosome 6 contained separate HSRs.

The modal number was 66. Three very long marker chromosomes, not seen in the parent line, were observed to contain homogeneous staining regions in the long arms. These markers were derived from chromosomes 5, 6, and 19 (Figures 3 and 4). M_{11}, derived from chromosome 6, contained separate HSRs. Double minutes were not identified in the R4 line or in the parent line.

Southern Blot Analysis of the DHFR Gene in Sensitive and MTX-Resistant K-562 Cells. The restriction enzyme digestion pattern of the DHFR gene in the sensitive and MTX-resistant K-562 cells was examined with five different restriction enzymes and the human cDNA probe, pHD84 (Figure 5). The intensity of the hybridization bands when restriction patterns from the resistant line (Figure 5B) are compared to the sensitive line (Figure 5A) clearly demonstrates the amplification of DHFR sequences in the resistant cells. There were no other differences noted in the restriction patterns between the resistant and sensitive lines. The restriction enzyme digested fragments from the human DHFR gene were compared to the mouse DHFR restriction patterns (Table III). The data revealed differences not only in size but also in the number of fragments generated by restriction enzyme digestions. *Hin*dIII digestion generated three fragments in the human K-562 cells and five fragments in mouse S-180 cells containing DHFR sequences. Restriction endonuclease *Bam*H1 generated only one fragment of size 28 kb in the human DNA in contrast to four fragments from the mouse DHFR gene. Only about 600 bases are required to code for human DHFR with a molecular weight of 20 000; the size and number of restriction fragments containing coding sequences indicate that the human

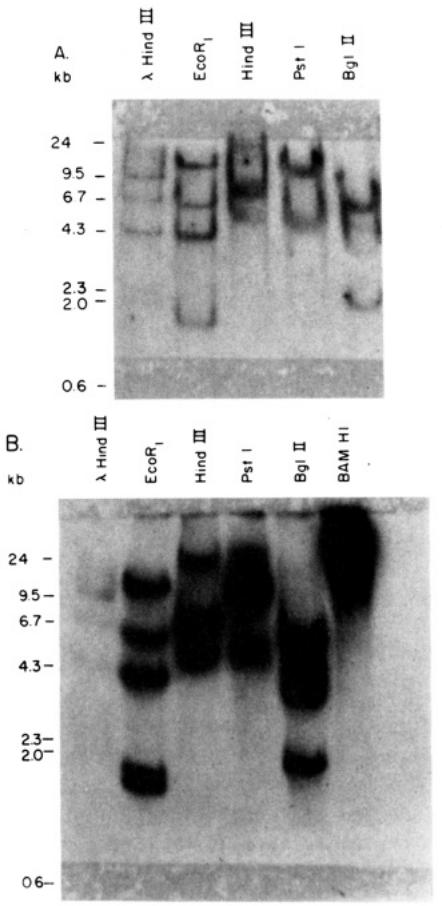

FIGURE 5: Hybridization of human DHFR cDNA to genomic DNA from sensitive (A) and methotrexate-resistant (B) K-562 cell lines. Restriction enzyme digestions were performed to completion with 25 μg of DNA from sensitive and 2.5 μg of DNA from MTX-resistant cells. The exposure times of autoradiograms for (A) and (B) were 48 and 6 h, respectively.

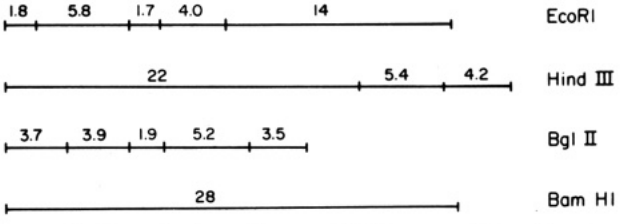

FIGURE 6: Organization of human DHFR gene. The order of restriction fragments containing DHFR sequences was determined by hybridization with specific ^{32}P-labeled fragments obtained from human DHFR cDNA (pHD84). The diagrammatic representation of the cDNA components and hybridization pattern of *Eco*R1-, *Hin*dIII-, and *Bgl*II-digested genomic DNA from K-562/R4 cells are shown in Figure 7.

DHFR gene is approximately 30 kb. This is about the same size as the DHFR gene from mouse sarcoma cells (Crouse et al., 1982).

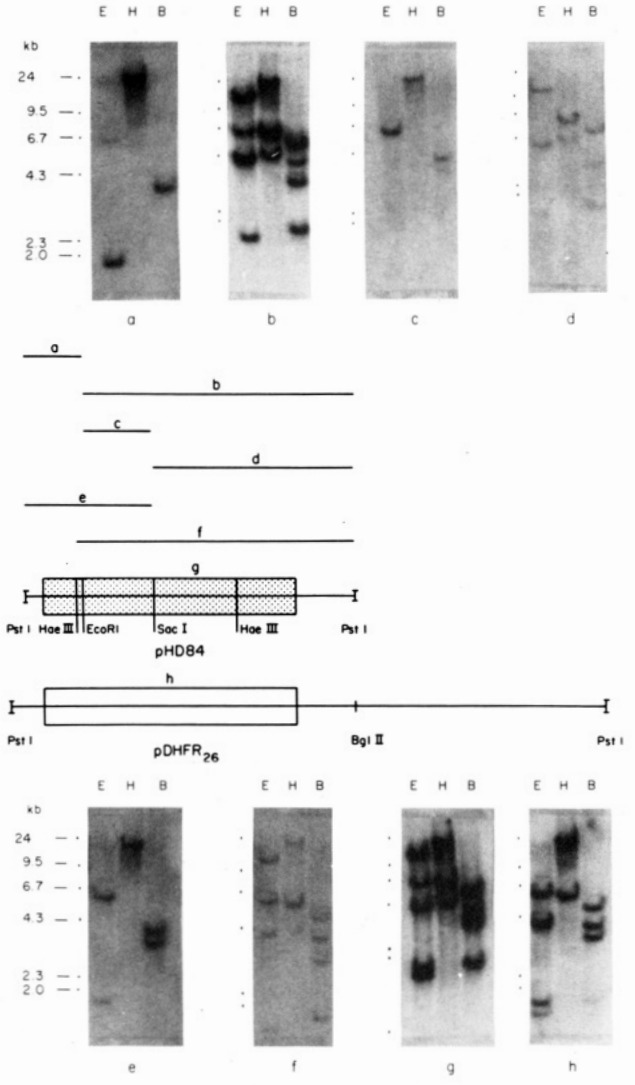

FIGURE 7: Hybridization of specific fragments (a–f) of human DHFR cDNA pHD84 (g) to restriction enzyme digested genomic DNA from K-562/R4 cell line. The genomic DNA from K-562/R4 cells was digested with *Eco*R1 (E), *Hin*dIII (H), and *Bgl*II (B). As a comparison, mouse DHFR cDNA (h) was also used as a hybridization probe.

Organization of the Human DHFR Gene. The organization of the human DHFR gene from MTX-resistant K-562 cells was determined by the use of probes prepared from specific restriction fragments of human cDNA (Figure 6). The data used to generate this map are shown in Figure 7a–f. The hybridization patterns revealed that 1.8-kb *Eco*R1, 24-kb *Hin*dIII, and 3.6-kb *Bgl*II fragments resulted from the 5′ end of the gene (Figure 7a), while the 14-kb *Eco*R1, 4-kb *Hin*dIII, and 3.5-kb *Bgl*II fragments resulted from the 3′ end of the DHFR gene (figure 7c,d). The fragments used in these experiments to map the human DHFR gene are shown in Figure 7.

Differences in restriction patterns were observed when mouse and human cDNA probes were used in the MTX-resistant K-562 cells (Figure 7g,h). Restriction fragments representing the 3′ end of the gene (14-kb *Eco*R1, 4-kb *Hin*dIII, and 3.5-kb *Bgl*II) were not readily observed when mouse cDNA probe, $pDHFR_{26}$, was used instead of the human pHD84 probe. Similarly, the 3′ end of mouse DHFR gene was not readily visible when the human cDNA probe was used (data not shown). This may be due to differences in the sequences of the 3′ end, especially in the noncoding regions of the human and mouse DHFR gene.

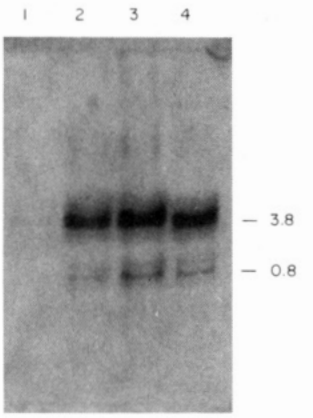

FIGURE 8: Northern blot analysis of DHFR-specific polyadenylated mRNA species in K-562-sensitive and K-562/R4 cells. RNA fractionated in 1% agarose gel containing CH_3HgOH was transferred to DBM paper and hybridized with ^{32}P-labeled mouse DHFR cDNA plasmid probe, $pDHFR_{26}$. Lanes 1 and 2 represent 10 and 50 μg of polyadenylated mRNA from K-562 sensitive cells. Lanes 3 and 4 represent 1 and 0.5 μg of polyadenylated mRNA from K-562/R4 cells. Autoradiograms were exposed at −70 °C with intensifying screens for 12 h.

Northern Blot Analysis of mRNA. The hybridization pattern of cytoplasmic polyadenylated mRNA from the K-562/R4 line obtained with the mouse $pDHFR_{26}$ cDNA probe is shown in Figure 8. Similar results were obtained with the human pHD84 cDNA probe. Two predominant species, 0.8 and 3.8 kb, and one minor species, 1.2 kb, of DHFR mRNA in both sensitive and MTX-resistant cells were found. It is also clear from the intensity of hybridization that MTX-resistant cells contain elevated levels of DHFR specific mRNA over that present in the sensitive cells.

Discussion

The data presented indicate that the mechanism of resistance of K-562 cells obtained in these studies by stepwise selection in MTX was due to an increased level of DHFR activity. This increased activity was associated with an increase in DHFR mRNA, as well as an increased number of DHFR gene copies. The resistant and parent lines were examined for MTX transport and differences in properties of DHFR, but no significant differences were noted. The resistant subline was found to contain three chromosomes with homogeneous staining regions (HSRs) identified as derived from chromosomes 5, 6, and 19. Other mammalian sublines resistant to MTX have also been found to contain HSRs, i.e., in mouse and Chinese hamster cells (Nunberg et al., 1978; Dolnick et al., 1979).

Multiple forms of mRNA that hybridized with a mouse DHFR plasmid probe were found. The predominant mRNA species detected by Northern hybridization was 3.8 kb in size; additional species of 1.2 and 0.8 kb were also detected by these techniques. Similar size mRNAs have been described by Morandi et al. (1982) in another MTX resistant human cell line, also characterized by an increase in DHFR gene copies. Multiple forms of mRNA from mouse cell lines and Chinese hamster lung lines have also been described (Setzer et al., 1980; Dolnick & Bertino, 1981; Lewis et al., 1981). The significance of these multiple messages for this enzyme activity is not clear.

Restriction analysis of the DNA from the sensitive and resistant cells indicated that the human DHFR, like the mouse gene, was large in size, when compared to the protein synthesized. The human gene is estimated to be approximately 30 kb, which is comparable to the size of the mouse DHFR

gene (31.5 kb). Recently the coding sequence for human DHFR has been shown to contain a site for the restriction enzyme *Eco*R1 (Morandi et al., 1982; Chen et al., 1982). Since in Southern blot analysis *Eco*R1-digested DNA generates five fragments containing DHFR coding sequences, it appears that at least four intervening sequences are present in the gene. The differences in size and number of restriction fragments generated from the DHFR gene between mouse and human might be due to divergence in the intervening sequences of the gene in the two species. The data also indicate that the 3′ end of the mouse and human DHFR gene are different. The restriction fragments containing the 3′ end of the DHFR gene are readily detectable only with homologous and not with heterologous cDNA probes. It is also interesting that *Bam*H1 restriction enzyme digestion of genomic DNA from human cells results in one fragment 28 kb in size which may contain the entire coding sequence of the DHFR gene.

At this time it is not known whether the *Bam*H1 site at the 3′ end of the 28-kb fragment represents the *Bam*H1 site on the 3′-noncoding region of human cDNA (about 2.4 kb from the 5′ end of corresponding 3.8-kb size mRNA). The restriction enzyme analysis of genomic DNA from other DHFR elevated MTX resistant human cell lines will reveal whether the *Bam*H1 pattern is unique to the K-562 cell line. It should be pointed out that the human DHFR cDNA (pHD84) used in the restriction enzyme analysis of genomic DNA from K-562/R4, although containing the complete coding sequence, represents only about 750 bp at the 5′ end of the 3.8-kb DHFR mRNA. Therefore, restriction fragments containing 3′-noncoding sequences of the DHFR gene could be missed in the restriction map.

Recently, Chen et al. (1982) have obtained genomic clones for DHFR from human fetal liver and adult spleen libraries. These authors also found pseudogenes or intronless genes, which they suggested may have resulted from in vivo reverse transcription of mRNA from DHFR. They also suggested that the intronless gene could be functional if it has an active promotor at the 5′ end. Genomic cloning and sequencing of the DHFR gene in these human K-562 cell lines will determine whether the intronless genes, if present, undergo amplification along with the normal gene.

The resistant subline contained three chromosomes with HSRs. These have been identified as derived from chromosomes 5, 6, and 19. Karyotypic analysis from human lines with HSRs associated with resistance have shown that the site of the HSR is chromosome 10 in KB cells (Wolman et al., 1982) and chromosome 5 in RAJI/MTX-R cells (Diddens et al., 1982). The location of the unique gene for DHFR, and its relationship (if any) to the observed locations of HSRs in MTX-resistant human cell lines, is not clear at this time. By examining various stages in the development of resistance to MTX, we hope to trace the origin of the HSRs. In situ hybridization studies are planned in order to demonstrate whether or not the three chromosomes that contained HSRs contain amplified genes, as has been described for mouse and CHO lines resistant to MTX (Dolnick et al., 1979; Biedler et al., 1980).

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Registry No. Methotrexate, 59-05-2; dihydrofolate reductase, 9002-03-3; thymidylate synthetase, 9031-61-2; serine hydroxymethylase, 9029-83-8; formyltetrahydrofolate synthetase, 9023-66-9; methylenetetrahydrofolate reductase, 9028-69-7; folylpolyglutamate synthetase, 63363-84-8.

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Immunochemical and Molecular Differentiation of 43 000 Molecular Weight Proteins Associated with *Torpedo* Neuroelectrocyte Synapses†

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ABSTRACT: Synaptic membranes, highly enriched in nicotinic receptor, contain three 43 000 molecular weight (M_r) peripheral proteins (distinctive in their peptide mapping profiles and earlier designated ν_1, ν_2, and ν_3) as well as the receptor $\alpha_2\beta\gamma\delta$ integral membrane subunits. Of the three proteins, only ν_1 is copurified with the membrane-bound receptor, while ν_2 and ν_3 are prominent cytosolic proteins, which are retained at significant levels in receptor-rich membranes during multistep centrifugation and affinity partitioning purification procedures [Gysin, R., Wirth, M., & Flanagan, S. D. (1981) *J. Biol. Chem. 256*, 11373–11376]. Peptide mapping analysis of *Torpedo* ν_3 and rabbit skeletal actin indicates that the two proteins are closely related. The enzymatic activity, creatine phosphokinase (EC 2.7.3.2), copurifies with ν_2 during chromatofocusing fractionation of the cytosol. The *Torpedo* electroplax form of creatine phosphokinase has an electrophoretic mobility identical with that of the mammalian skeletal muscle form of the enzyme. Upon release of the membrane-bound forms of ν_1, creatine phosphokinase, and actin by the action of mild alkali, ν_1 remains in a high molecular weight form. Dissociation of ν_1 into lower molecular weight species requires urea or sodium dodecyl sulfate ($NaDodSO_4$). Preparation of essentially pure ν_1 was achieved by eluting the ν_1 protein spots directly from $NaDodSO_4$–isoelectric focusing gels loaded with alkali extracts derived from membranes highly enriched in nicotinic receptor. Amino acid compositions of the purified fractions indicate that ν_1 and *Torpedo* creatine phosphokinase have distinct amino acid compositions from each other and from that of actin. In order to determine whether the observed differences in the peripheral protein peptide mapping and amino acid composition profiles are reflected as well in their antigenic properties, we have prepared an antiserum against an electrophoretically purified fraction highly enriched in ν_1. This antiserum is essentially monospecific for ν_1, detecting as little as 15 ng of ν_1 present in alkali extracts of affinity-purified membranes. The immunochemical analysis serves to emphasize the conclusion that, although ν_1 overlaps in both molecular weight and isoelectric focusing parameters with ν_2 (creatine phosphokinase) and ν_3 (*Torpedo* actin), they display no evolutionary interrelationships, having distinct antigenic sites, solubility properties, and amino acid compositions.

The subsynaptic region of the neuromuscular junction contains nicotinic acetylcholine receptor (nAChR)[1] molecules at such high densities (Fertuck & Salpeter, 1974) that there is little room within the plane of the membrane bilayer for macromolecular components other than the known $\alpha_2\beta\gamma\delta$ or core pentameric integral membrane subunits (Weill et al., 1974; Lindstrom et al., 1980a; Raftery et al., 1980). One explanation for this dense packing and restricted distribution of receptor is that the nAChR could form higher order polymers of the core pentameric integral membrane subunits via the formation of disulfide bonds between the δ-subunits (Chang & Bock, 1977; Hamilton et al., 1977, 1979). But dimers of the core pentameric structure ($\alpha_2\beta\gamma\delta$–S–S–$\delta\gamma\beta\alpha_2$) are the predominant species purified from *Torpedo*, and thus far, only the core pentameric structure itself has been purified from *Electrophorus* and mammalian muscle (Lindstrom et al., 1980b; Conti-Tronconi et al., 1982; Gotti et al., 1982; Einarson

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[1] Abbreviations: BSA, bovine serum albumin; CPK, creatine phosphokinase (EC 2.7.3.2); HSS, high-speed supernatant; nAChR, nicotinic acetylcholine receptor; $NaDodSO_4$, sodium dodecyl sulfate; TBS, Tris-buffered saline; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)ammomethane; alkaline (pH 11) extracts were from purified *Torpedo* electroplax membranes.

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