MOLECULAR AND CELLULAR BIOLOGY, Mar. 1982, p. 275–285
0270-7306/82/030275-11$02.00/0

Vol. 2, No. 3

Overproduction of Dihydrofolate Reductase and Gene Amplification in Methotrexate-Resistant Chinese Hamster Ovary Cells

WAYNE F. FLINTOFF,[1]* MARY K. WEBER,[1] CHARLES R. NAGAINIS,[1] A. KARIM ESSANI,[1] DEBRA ROBERTSON,[2] AND WINSTON SALSER[2]

Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1,[1] and The Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California, 90024[2]

Received 30 June 1981/Accepted 2 October 1981

Stable isolates of Chinese hamster ovary cells that are highly resistant to methotrexate have been selected in a multistep selection process. Quantitative immunoprecipitations have indicated that these isolates synthesize dihydrofolate reductase at an elevated rate over its synthesis in sensitive cells. Restriction enzyme and Southern blot analyses with a murine reductase cDNA probe indicate that the highly resistant isolates contain amplifications of the dihydrofolate reductase gene number. Depending upon the parental line used to select these resistant cells, they overproduce either a wild-type enzyme or a structurally altered enzyme. Karyotype analysis shows that some of these isolates contain chromosomes with homogeneously staining regions whereas others do not contain such chromosomes.

Resistance to the antifolate drug methotrexate (Mtx) has been studied in a variety of cell culture systems (5, 9, 17, 21, 24, 30). The resistance developed under various selection conditions has been ascribed to one or more of three different mechanisms. These include alterations to the structure of dihydrofolate reductase (DHFR) such that it has reduced affinity for binding Mtx (1, 17, 18, 24), alterations affecting the ability of the cells to take up Mtx (16, 17, 41), and an overproduction of the DHFR enzyme (3, 10, 26, 34, 35). This latter type of alteration is generated by a stepwise or multistep selection process. In resistant cells generated in this manner, the overproduction of enzyme is accompanied by a parallel increase in the synthesis of the enzyme (3, 35), an increase in the cellular concentration of DHFR mRNA (2, 10, 26, 34), and an amplification of the DHFR genes (2, 34, 37, 40). In most cases, the enzyme overproduced is biochemically indistinguishable from the enzyme synthesized by sensitive cells (3, 22); however, in the Chinese hamster lung system it has been shown that some isolates synthesize a reductase protein of decreased molecular weight (34). In a majority of the systems examined to date, karyotypic alterations accompany and appear to correlate with the development of high levels of resistance to the antifolates. In some cases, there is the presence of large homogeneously staining regions (HSR) located on several chromosomes (6, 9, 13, 37), whereas in other systems double minute chromosomes have been identified (25).

Previously, we have shown that, in the Chinese hamster ovary (CHO) cell system, a second-step selection on cells containing an altered DHFR (class I) produced a cell line (class III) with an increased reductase activity (17). In this report, we have shown that such cells overproduce the reductase protein and contain increased copies of the reductase gene. Furthermore, we have shown that by using stepwise selections to Mtx resistance on wild-type or the class III cells stable, highly resistant isolates which contained further increased amounts of DHFR and increased copies of the reductase gene could be obtained. Some of these isolates contained HSR chromosomes whereas others did not.

MATERIALS AND METHODS

Materials. Mtx was purchased from Nutritional Biochemicals Corp., and NADPH was from Sigma Chemical Co.

[*G*-^{3}H]folic acid (specific activity, 5 Ci/mmol) was obtained from Amersham Corp., and L-[^{35}S]methionine (specific activity, 839 Ci/mmol), [^{3}H]leucine (specific activity, 60 Ci/mmol), and [α-^{32}P]dCTP (specific activity, 600 to 800 Ci/mmol) were from New England Nuclear Corp. The restriction endonuclease *Eco*RI was purchased from Bethesda Research Laboratories.

Cell lines and cell culture. The wild-type CHO cell lines and several of the Mtx-resistant isolates used in this study have been previously described (17). Addi-

tional isolates were obtained by using either single-step selections (for the RIV and RV phenotypes) or multistep selections (for the RVI and RVII phenotypes) under similar conditions, as previously described (17). Figure 1 illustrates the cell lineage of the various lines used in this study.

An Mtx-resistant cell designated Pro$^-$3 Mtx^{RVI} describes the Mtx-resistant cell with properties of the class VI mutants. Clone "X" derived in the selective medium as a subclone of the Pro$^-$3 Mtx^{RVI} 1 line is described as Pro$^-$3 Mtx^{RVI} 1-X. Isolates of classes IV, V, and VII are similarly designated.

Cells were routinely maintained in suspension culture at 37°C in complete alpha medium (45) supplemented with 10% fetal bovine serum (Microbiological Associates).

Selection of resistant cells was carried out in monolayer culture (17) in alpha special medium (32) supplemented with 40 μg of proline per ml, 10% dialyzed fetal bovine serum, and various concentrations of Mtx. This medium is referred to as selection medium.

Determination of cellular resistance. The cellular resistance to the cytoxicity of Mtx was determined by relative plating efficiencies as previously described (17). The resistance is expressed by the D_{10} value, the drug concentration that reduces cell survival to 10%.

Extract preparation and enzyme assay. The preparation of cell extracts for the determination of DHFR was as previously described (17). DHFR activity was measured with [^{3}H]folic acid as substrate as previously described (20). One unit of DHFR activity is defined as the amount of enzyme necessary to reduce 1 nmol of folate in 15 min under standard assay conditions. The resistance of the enzyme to inhibition by Mtx is expressed as the I_{50} value, the drug concentration inhibiting the enzyme by 50%.

Protein was determined by the method of Lowry et al. (31), using bovine serum albumin as a standard.

Growth rate. Cells were seeded in suspension culture in various media as described in the text. At daily intervals, duplicate samples were diluted into phosphate-buffered saline and counted in a Particle Data Electrozone/Cellscope cell counter (Particle Data, Inc., Elmhurst, Ill.).

Preparation and purification of [^{3}H]DHFR. A 100-ml portion of either Pro$^-$4 Mtx^{RVI} 1-6 cells or Pro$^-$3 Mtx^{RVII} 1-2 cells in suspension culture at 4×10^6/ml were labeled for 7 h in leucine-free medium with 20 μC of [^{3}H]leucine per ml. Cells were washed with cold phosphate-buffered saline, harvested, and pooled with 5×10^8 unlabeled cells of the appropriate line. Cell extracts were prepared and DHFR was purified as described previously, using affinity chromatography (20). The purified enzyme from either line was electrophoretically homogeneous. The labeled enzyme from the RVI cells had a specific activity of approximately 1,500 cpm/μg of protein, and the reductase from the RVII line had a specific activity of approximately 350 cpm/μg of protein.

Preparation of antibody. Purified DHFR from Pro$^-$4 Mtx^{RVI} 1–6 (2 mg/ml in 50 mM potassium phosphate, pH 7.0) was emulsified with an equal volume of complete Freund adjuvant. Two rabbits were each injected with this emulsion, 1 ml intramuscularly and 1 ml subcutaneously, at several sites in the back. At 2-week intervals for 3 months, each rabbit was boosted subcutaneously with 1 mg of antigen emulsified as described above. Periodically, the rabbits were bled

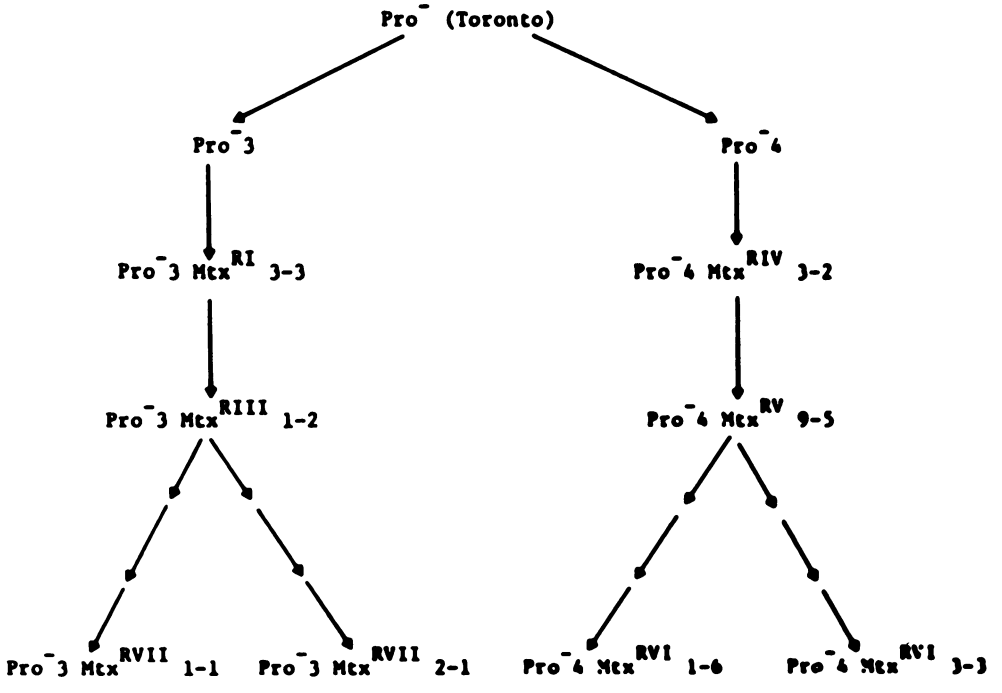

FIG. 1. Lineage of Mtx-resistant CHO cells.

from the ear vein, and the sera were tested for the ability to precipitate labeled reductase. One month after the final injection, the rabbits were bled by cardiac puncture, and the sera were collected, pooled, and frozen. This crude serum was used in immunoprecipitation experiments. One unit of absorbancy at 280 nm of anti-reductase serum would precipitate 0.1 μg of either wild-type or altered reductase at the equivalence point.

Figure 2 illustrates the ability of this serum to precipitate reductase protein from ^{35}S-labeled cell extracts (see below).

Labeling of cells. Exponentially growing cells were washed two times in methionine-free medium and suspended at 10^6/ml in the same medium supplemented with 10% dialyzed fetal bovine serum. After 90 min of incubation at 37°C, the cells were pelleted and resuspended in fresh methionine-free medium containing 10% dialyzed fetal bovine serum and 10 μCi of [^{35}S]methionine per ml. After 90 min of incubation, the cells were pelleted and washed three times with ice-cold phosphate-buffered saline containing 10 mM methionine. The cell pellet was sonically disrupted, and an extract was prepared as described above. Total protein was determined by the method of Lowry et al. (31), and incorporation of radioactive label into high-molecular-weight material was determined by trichloroacetic acid precipitation (19).

The cell extract was used for either immunoprecipitation or polyacrylamide gel electrophoresis.

Immunoprecipitation. The immunoprecipitation reaction was performed essentially as described by Alt et al. (2). Immunoprecipitation reactions contained 1% Triton X-100, 1% sodium deoxycholate, 0.5 μg of carrier [^3H]DHFR, 0.2 ml of antireductase serum, 50 mM potassium phosphate (pH 7.0), and various amounts of labeled cell extract. The tubes were incubated at 4°C overnight to ensure maximal precipitation. For all experiments, multiple immunoprecipitations were performed, using several concentrations of cell extract (antigen) to ensure antibody excess. The inclusion of radioactively labeled reductase as carried permitted the quantitation of precipitation in each reaction.

Since the antiserum precipitates nonreductase proteins (see Fig. 2), it was necessary to solubilize the immunoprecipitates and electrophorese them in sodium dodecyl sulfate (SDS)-polyacrylamide gels. The immunoprecipitates were solubilized in dissociation buffer (see below) and electrophoresed in SDS-polyacrylamide gels as described below. The protein bands were visualized by staining the gel in 0.1% Coomassie blue in 50% trichloroacetic acid and destaining in 7% acetic acid. After drying the gel, the band corresponding to DHFR was cut out, and the protein was solubilized in 1% ammonium bicarbonate, pH 8.0, and 50 μg of trypsin per ml at 37°C. The samples were then counted in Protosol-Omnifluor-toluene scintillation fluid, using a Beckman LS-350 liquid scintillation system with restricted channels. Corrections were made for overlap of the ^{35}S counts into the ^3H channel. Using this solubilization technique, in control experiments the recovery of counts varied between 25 and 70%.

One-dimensional gel electrophoresis. Samples for one-dimensional SDS-polyacrylamide gel electrophoresis were solubilized in dissociation buffer (5% β-mercaptoethanol, 2% SDS, 1% Nonidet P-40, and 10% glycerol), and the proteins were separated by electrophoresis in 4% stacking gels and 11.5% separating gels as described by Laemmli (27) and modified by Studier (46).

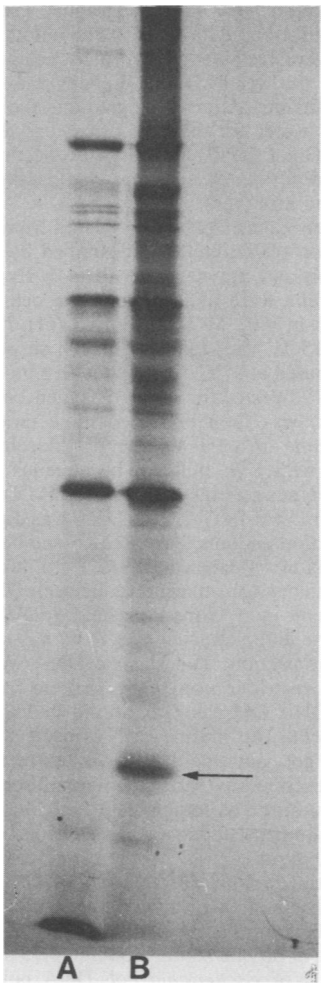

FIG. 2. Immunoprecipitation of ^{35}S-labeled cell proteins. Approximately 10^7 Pro$^-$4 Mtx^{RV} 9-5 cells were labeled with [^{35}S]methionine as described in the text. An extract was prepared and treated with immune (B) or preimmune (A) serum. The immunoprecipitate was solubilized and electrophoresed on an SDS-polyacrylamide gel at 25 mA. The gel was stained, impregnated with PPO, dried, and exposed to X-ray film as described in the text. The arrow indicates the position of marker-purified DHFR run in an adjacent lane.

Two-dimensional gel electrophoresis. Samples for two-dimensional gel electrophoresis were dissociated with the O'Farrell urea–Nonidet P-40–β-mercaptoethanol lysis buffer (36). Isoelectric focusing of the proteins was as previously described (19), using a 3:2 mixture of ampholytes of pH 2/11 and pH 5/7. For electrophoresis in SDS, an 11% acrylamide gel was used.

Fluorography and autoradiography. Protein spots were visualized by fluorography and autoradiography. The gels were destained, dehydrated, and impregnated with 20% (wt/wt) PPO (2,5-diphenyloxazole) in dimethylsulfoxide. After impregnation, the gels were soaked in water for 60 min, dried, and exposed to Kodak X-Omat (RP/R) films at −70°C according to the methods described by Bonner and Laskey (8) and Laskey and Mills (28).

DNA extraction. DNA was isolated from cells by a modification of the methods described by Berns and Thomas (4) and Stavnezer et al. (44). Exponentially growing cells were pelleted, and the cell pellet was suspended in 0.02 M Tris-chloride (pH 7.0)–0.01 M EDTA–0.15 M NaCl (STE). The cell suspension was then incubated at 37°C overnight with 1 mg of pronase per ml (self-digested for 2 h at 37°C) and 1% SDS. The suspension was then extracted three times with an equal volume of a 1:1 mixture of chloroform-phenol saturated with STE buffer. The aqueous phase was dialyzed versus several changes of 5 mM Tris-chloride (pH 7.0)–0.1 mM EDTA. This DNA was digested with the restriction endonuclease *Eco*RI (see below).

Synthesis of ^{32}P-labeled cDNA. A ^{32}P-labeled DNA complementary to a murine reductase cDNA, which was present in a cloned plasmid (pDHFR26) and kindly supplied by R. Schimke (10), was synthesized by nick translation. The plasmid DNA was digested with the restriction enzyme *Pst*I to remove the inserted DNA and electrophoresed in 1% Seaplaque agarose gels. The murine cDNA insert was excised from the gel, and the DNA was extracted by the method described by R. Higuichi (manuscript in preparation) and used as template. A typical nick translation reaction consisted in a volume of 50 μl of 0.2 μg of DNA, 500 μg of each of the deoxynucleoside triphosphates with [α-^{32}P]dCTP, 2.5 μmol of TES [*N*-tris(hydroxymethyl)methyl-2-aminomethanesulfonic acid]·K (pH 7.8), 0.25 μmol of $MgCl_2$, 0.05 μmol of dithiothreitol, 2.5 μg of bovine serum albumin, 0.75 ng of DNase I, and 5 U of *Escherichia coli* DNA polymerase I. After a 1-h incubation at 15°C, the reaction mixture was extracted once with an equal volume of a 1:1 mixture of chloroform-phenol, and the aqueous phase was applied to Bio-Gel P60 contained in a Pasteur pipette and equilibrated with 0.01 M Tris-chloride–0.001 M EDTA, pH 8.0. The excluded fractions were pooled and precipitated with 2 volumes of 95% ethanol. The specific activity of the DNA was 1.1×10^8 cpm/μg.

The cDNA synthesized in this manner hybridized only to sequences present in the original plasmid and not to any sequences present in the cloning vector (pBR322) (data not shown). Furthermore, this cDNA recognized the same restriction fragments, using DNA isolated from normal mice or from the Mtx-resistant mouse line 3T6RI (unpublished data), as did the original murine reductase cDNA, as described by Nunberg et al. (36).

Restriction analysis and Southern blot hybridization. High-molecular-weight DNA was isolated as described above and digested to completion with *Eco*RI according to the method described by the supplier. To monitor digestion, a sample of the main reaction was added to 0.5 μg of λ DNA. The main reaction was stopped by the addition of 10 mM EDTA, and the DNA was precipitated with 3 volumes of 95% ethanol. The DNA was solubilized in 3 mM EDTA, applied to a 0.7% horizontal agarose gel, and electrophoresed for 16 h at 50 V in 0.4 M Tris–0.02 M sodium acetate–0.018 M NaCl–0.002 M EDTA, pH 8.0. After staining with ethidium bromide, the gel was blotted onto a nitrocellulose filter membrane according to the method of Southern (42) and baked in vacuo for 2 h at 80°C.

For hybridization, the paper was pretreated for 16 h at 40°C in hybridization mix (3× SSC [1× SSC is 0.15 M NaCl plus 1.5 mM citrate], 50% formamide, 0.2 mg of yeast tRNA per ml, 20 μg of salmon sperm DNA per ml, and 1× Denhardt buffer [12]). Hybridizations were carried out in the same buffer containing 3×10^6 cpm for 2 days at 40°C. Filters were rinsed, sequentially washed with 2× SSC, 0.1× SSC containing 0.1% SDS, and 0.1× SSC, air dried, and exposed to X-ray film (Kodak XR-5 film) with Dupont intensifying screens at −70°C.

For quantitation, each lane contained 50 pg of the plasmid DHFR26 DNA digested with the restriction endonuclease *Eco*RI to control for differences in blotting and transfer, since preliminary experiments had indicated that no fragments containing DHFR sequences were present in the 7-kilobase region. The bands in the autoradiograms were scanned, quantitated by determining the area under each peak, and normalized to the plasmid band control.

Karyotype analysis. The preparation of chromosome spreads and trypsin banding techniques were as described by Farrell and Worton (15). To designate the chromosomes, the nomenclatures of Ray and Mohandas (39) and Worton et al. (49) were used. Short arms of chromosomes are designated p and long arms are designated q, with + and − signs used to indicate additions or deletions of chromosomal material.

RESULTS

Selection and properties of highly resistant isolates. Previously, we have demonstrated that in single-step selections for resistance to Mtx in the CHO cell system, two phenotypes could be obtained: class I cells containing a structural alteration in DHFR (17) and class II cells defective in the ability to take up the drug. More interesting, however, was the observation that in second-step selections cells could be obtained which had increased activity of the reductase protein. These cells were termed class III and were selected from the class I isolates. More recently, we have been able to obtain cells in a two-step selection process, termed class V, which contain an increased activity of wild-type enzyme and are approximately 300-fold resistant (see below).

Our preliminary experiments indicated that, to obtain cells which were highly resistant to Mtx, a single-step selection process could not be used. Therefore, to obtain such cells a multistep or continuous selection system was necessary with the class RIII or class RV cells as parents. Initially, approximately 10^6 cells of either line were exposed to a drug concentration that was two- to fivefold higher than that necessary to result in a 10% survival. Surviving colonies

generally appeared after 2 to 3 weeks and were picked, cloned in the presence of the selecting concentration of drug, and subjected to another round of selection with an increased drug concentration. This procedure was repeated until a final drug concentration of 10^{-4} M was obtained. The entire selection process took approximately 6 months. After the final exposure to drug, two of the cloned isolates from each selection were maintained in continuous culture in the absence of drug for at least 15 months. Lines derived from the Pro$^-$4 Mtx^{RV} 9-5 line were given the designation R^{VI}, and lines derived from the Pro$^-$3 Mtx^{RIII} 1-2 line were given the designation R^{VII}.

To determine the level of resistance of these isolates, various concentrations of cells were added to various amounts of drug as described in Materials and Methods, dose response curves were constructed, and the concentration of drug reducing the cell survival to 10% was determined. Table 1 summarizes the results of such an analysis and compares the resistance levels of several isolates. The isolates obtained in the multistep selection process (classes RVI and RVII) are highly resistant to Mtx, being in the order of 10^4 less sensitive than the wild-type cells. The isolates obtained in single-step selections and used as the immediate parental lines (RIII and RV) are, as expected, less resistant, being in the order of 200 to 300 times less sensitive to Mtx than the wild-type cells.

The growth rate of the highly resistant isolates under either selective or nonselective conditions was somewhat slower than the wild-type or class III and V cells. The RVI resistant cells had a doubling time of 24 to 28 h and the RVII lines had a doubling time of 22 to 25 h compared with the wild-type, RIII, or RV cells, which had doubling times of approximately 15 h. These highly resistant isolates have maintained their growth properties and resistant characteristics in the absence of selective pressure for at least 17 months.

TABLE 1. Cellular resistance and reductase activity

Cell line	Relative resistance to Mtx[a]	Relative DHFR activity[b]
Pro$^-$ 3	1	1
Pro$^-$ 3 Mtx^{RIII} 1-2	200	13
Pro$^-$ 4 Mtx^{RV} 9-5	300	20
Pro$^-$ 4 Mtx^{RVI} 1-6	20,000	150
Pro$^-$ 4 Mtx^{RVI} 3-1	20,000	ND[c]
Pro$^-$ 3 Mtx^{RVII} 1-1	10,000	50
Pro$^-$ 3 Mtx^{RVII} 2-2	10,000	ND

[a] Relative resistances are expressed as the ratio of the D_{10} value (drug concentration reducing cell survival to 10%) for the resistant lines to that for the wild-type line. The D_{10} value for the wild-type line was 1.5×10^{-8} M.

[b] Crude cell extracts were prepared and assayed for DHFR as described in the text. Relative activity is expressed as the ratio of the activity in the resistant cell extract to the wild-type activity (2.6 U/mg of protein).

[c] ND, Not determined.

DHFR of resistant isolates. In other systems, cells highly resistant to Mtx have been obtained and have been shown to contain an increased activity of the DHFR (5, 30, 40). This seemed a likely possibility with the CHO cells described here since it had been previously shown that the RIII line used as a parental line contained about 10 times the reductase activity of the wild-type cells (17). To examine this activity in the highly resistant isolates, cell extracts were made and the DHFR was assayed. The class VI isolates contained from 100 to 200 times the wild-type reductase activity, whereas the class VII isolates had about 50 times the wild-type reductase activity (Table 1). The cells of lower resistance, class III and V, contained about 10 to 20 times, respectively, the reductase activity present in the wild-type cells.

Since the class III cells contained a reductase that had decreased affinity for binding Mtx (18), which is reflected in a decreased sensitivity to inhibition by the drug (17), it was of interest to determine the nature of the enzyme being expressed in the highly resistant isolates. To examine this, cell extracts were prepared and DHFR was assayed as a function of the Mtx concentration. Figure 3 shows a titration of DHFR from

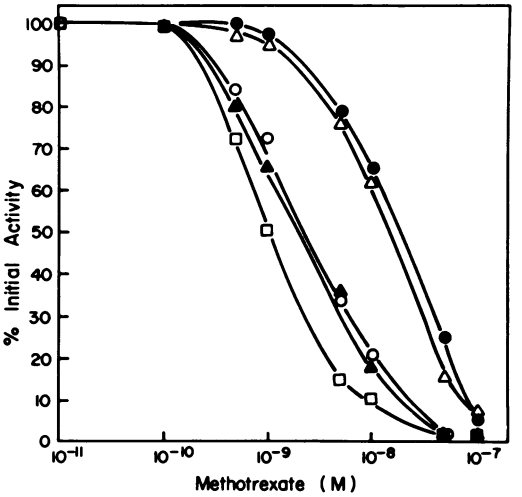

FIG. 3. Titration of DHFR in cell extracts. Cell extracts were prepared and the enzyme assay was carried out as described in the text. The residual activity in the presence of the drug is expressed as a percentage of the activity in the absence of the drug. Symbols: ○, Pro$^-$3; ●, Pro$^-$3 Mtx^{RIII} 1-2; △, Pro$^-$3 Mtx^{RVII} 1-1; ▲, Pro$^-$4 Mtx^{RV} 9-5; □, Pro$^-$4 Mtx^{RVI} 1-6.

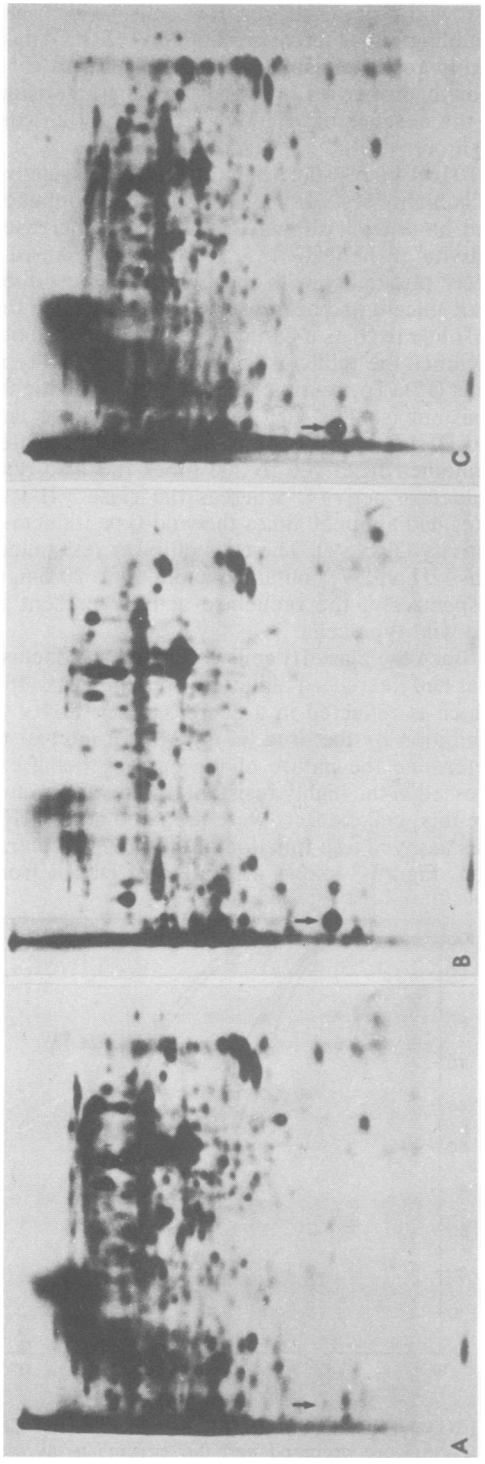

FIG. 4. Two-dimensional gel electrophoresis. Samples of ^{35}S-labeled cell extracts were dissociated and introduced onto the isoelectric focusing gels at the cathode as described in the text. Approximately 500,000 cpm (about 18 μg) of protein was added to each gel. Electrophoresis was conducted for 6,000 V-h. The isoelectric focusing gels were placed onto slab gels, and electrophoresis in the second dimension was carried out for 5 h at 25 mA. The gels were dried and exposed to X-ray films for 3 days as described in the text. The arrow, in each case, indicates the major DHFR component. Autoradiograms of (A) an extract from Pro$^-$3 cells; (B) an extract from Pro$^-$4 Mtx^{RVI} 1-6 cells; (C) an extract from Pro$^-$3 Mtx^{RVII} 1-1 cells.

Pro$^-$3, Pro$^-$3 Mtx^{RIII} 1-2, Pro$^-$3 Mtx^{RVII} 1-1, Pro$^-$4 Mtx^{RV} 9-5, and Pro$^-$4 Mtx^{RVI} 1-6 cells as a function of increasing drug concentrations. It is apparent that the class RIII and RVII isolates contain a reductase that is less sensitive to inhibition by Mtx than the enzyme present in the wild-type cells. The RV and RVI isolates, on the other hand, contain a reductase that is as sensitive to Mtx inhibition as the wild-type enzyme. The I_{50} values were determined from the curves in Fig. 3 to be 2×10^{-9}, 1.8×10^{-8}, 1.5×10^{-8}, 2×10^{-9}, and 1×10^{-9} M for the cell lines Pro$^-$3, Pro$^-$3 Mtx^{RIII} 1-2, Pro$^-$3 Mtx^{RVII} 1-1, Pro$^-$4 Mtx^{RV} 9-5, and Pro$^-$4 Mtx^{RVI} 1-6, respectively.

Two-dimensional gel electrophoresis. The preliminary observation that the Mtx^{RVI} or Mtx^{RVII} cells continually yielded more reductase protein after purification than an equivalent amount of wild-type cells (unpublished data) suggested that these highly resistant isolates may overproduce the reductase protein. This can be observed qualitatively by separation of ^{35}S-labeled soluble proteins by two-dimensional gel electrophoresis. Figure 4 shows an autoradiogram of labeled soluble proteins from Pro$^-$3, Pro$^-$4 Mtx^{RVI} 1-6, and Pro$^-$3 Mtx^{RVII} 1-1 cells. Since it has been previously shown that wild-type cells contain two reductase proteins, the arrow in each case represents the major reductase component representing about 90% of the total reductase protein (18). Previous results with purified reductase and cell extracts have also established that this spot corresponds to the reductase protein (18).

Since an equivalent number of counts were added to each gel, in wild-type cells, the reductase protein represents a small percentage of the total soluble protein, whereas in both the RVI and RVII cells this protein represents a major part of the total protein.

DHFR synthesis in sensitive and resistant cells. For a more quantitative estimate of the amount of reductase protein synthesized in the various resistant isolates, immunoprecipitation of ^{35}S-pulse-labeled proteins was carried out. All cell lines tested incorporated a similar amount of label into total soluble protein (Table 2). The isolates, however, which had an increased reductase activity, synthesized this protein at an

TABLE 2. Synthesis of DHFR[a]

Cell line	cpm incorporated into total soluble protein/mg of protein	cpm incorporated into reductase/mg of protein	Ratio, reductase incorporation/ total protein incorporation	Reductase synthesized in resistant cells/ reductase synthesized in wild-type cells (ratio of values from col 4)
Pro$^-$ 3	1.7×10^7	2.9×10^3	0.00017	1.0
Pro$^-$ 3 Mtx^{RI} 3-3	1.5×10^7	2.5×10^3	0.00016	0.95
Pro$^-$ 3 Mtx^{RIII} 1-2	1.5×10^7	1.2×10^4	0.00080	4.7
Pro$^-$ 3 Mtx^{RV} 9-5	1.3×10^7	1.9×10^4	0.0015	8.8
Pro$^-$ 3 Mtx^{RVI} 1-6	1.2×10^7	1.9×10^5	0.016	94
Pro$^-$ 4 Mtx^{RVII} 1-1	1.7×10^7	7.2×10^4	0.004	23.5

[a] Determined by [^{35}S]methionine incorporation and immunoprecipitation as described in the text.

elevated rate compared with the wild-type (Pro$^-$3) or class I (Pro$^-$3 Mtx^{RI} 3-3) resistant isolates. The Pro$^-$4 Mtx^{RVI} 1-6, Pro$^-$3 Mtx^{RVII} 1-1, Pro$^-$3 Mtx^{RIII} 1-2, and Pro$^-$4 Mtx^{RV} 9-5 isolates synthesized the reductase protein at 95, 25, 5, and 9 times, respectively, the rate in the wild-type cells.

Amplification of the reductase gene in resistant isolates. In other cell systems displaying high resistance to Mtx and overproduction of the reductase protein (3, 11, 26, 34, 35), a common mechanism appears to be an amplification of the gene coding for this function. The availability of a cloned cDNA complementary to murine reductase mRNA (10) and its partial homology with the reductase hamster DNA (2, 37) permitted the examination of whether the Mtx-resistant CHO isolates described here contained amplified sequences coding for the reductase protein.

This increase in the number of genes coding for DHFR was demonstrated by a restriction and Southern blot analysis (Fig. 5). In each case, the same general pattern after cutting with the endonuclease *Eco*RI was obtained; however, the intensity of the bands varied from cell line to cell line. (In this figure, some of the bands in the wild-type and RI lanes are not readily apparent. However, the bands are clearly visible on the original film.) Densitometric tracings of the bands indicated that there was an increase in DHFR gene copy number (Table 3).

Karyotype analysis. Karyotype analysis reveals that the class VI resistant cells contain several chromosomal alterations that are not present in the wild-type cells (Table 4). The most striking feature was the presence in the Pro$^-$4 Mtx^{RVI} 1-6 line of several chromosomes that have undergone increases in length (Fig. 6). Such new chromosomal regions were first described by Biedler and Spengler (7) and called homogeneously staining because of their lack of trypsin-Giemsa bands. In the Pro$^-$4 Mtx^{RVI} 1-6 cell line these HSR regions are associated with CHO chromosomes Z2 and Z5 in all of the cells examined. An HSR region was also frequently found on chromosome Z5a; however, after prolonged culture (18 months) approximately 10 to 40% of the cells retained this HSR chromosome. Another class VI line that was examined, Pro$^-$4

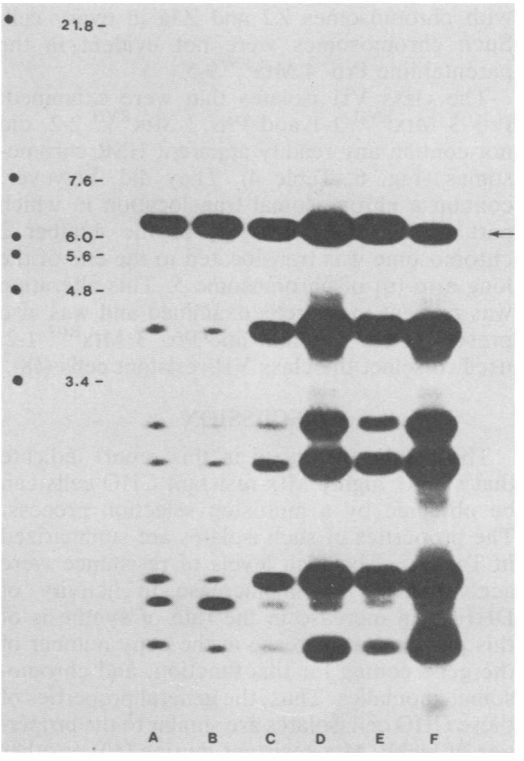

FIG. 5. Restriction analysis of DNA from various cell lines. A 10-μg portion of DNA isolated from (A) Pro$^-$3, (B) Pro$^-$3 Mtx^{RI} 3-3, (C) Pro$^-$3 Mtx^{RIII} 1-2, (D) Pro$^-$3 Mtx^{RVII} 1-2, (E) Pro$^-$4 Mtx^{RV} 9-5, and (F) Pro$^-$4 Mtx^{RVI} 1-6 cells was digested with *Eco*RI, electrophoresed in agarose, transferred to nitrocellulose paper, hybridized with 3×10^6 cpm of ^{32}P-labeled nick-translated cDNA, and exposed to X-ray film for 2 days. The numbers on the left side represent the kilobase fragments from an *Eco*RI digestion of λ DNA run in an adjacent lane. The arrow indicates the position of 50 pg of plasmid 26 cut with *Eco*RI applied to each lane as a control. The figure has been overexposed to bring out the bands in lanes A and B.

TABLE 3. Relative concentration of DHFR sequences in resistant cell lines

Source of DNA	Relative DHFR gene frequency from restriction blot analysis[a]
Pro$^-$ 3	1
Pro$^-$ 3 Mtx^{RI} 3-3	1
Pro$^-$ 3 Mtx^{RIII} 1-2	15
Pro$^-$ 4 Mtx^{RV} 9-5	40
Pro$^-$ 4 Mtx^{RVI} 1-6	150
Pro$^-$ 3 Mtx^{RVII} 1-1	75

[a] Determined by densiometric tracings of the 4-kilobase band in each cell line and normalized to the plasmid DNA band present in each lane.

Mtx^{RVI} 3-1, also displayed the HSR chromosomes (Table 4). In this case they are associated with chromosomes Z2 and Z5a in every cell. Such chromosomes were not evident in the parental line Pro$^-$4 Mtx^{RV} 9-5.

The class VII isolates that were examined, Pro$^-$3 Mtx^{RVII} 1-1 and Pro$^-$3 Mtx^{RVII} 2-2, did not contain any readily apparent HSR chromosomes (Fig. 6; Table 4). They did, however, contain a chromosomal translocation in which part of the short arm (p) of the number 2 chromosome was translocated to the end of the long arm (q) of chromosome 5. This alteration was present in all cells examined and was also present in the parental line Pro$^-$3 Mtx^{RIII} 1-2, used to select the class VII resistant cells (48).

DISCUSSION

The results described in this report indicate that stable, highly Mtx-resistant CHO cells can be obtained by a multistep selection process. The properties of such isolates are summarized in Table 5. The high levels of resistance were accompanied by an increase in activity of DHFR, an increase in the rate of synthesis of this protein, an increase in the copy number of the gene coding for this function, and chromosomal anomalies. Thus, the general properties of these CHO cell isolates are similar to the properties of highly Mtx-resistant murine (40) or other Chinese hamster systems (37). However, several features of the CHO system are of interest. Although single-step isolates were used, to obtain dramatic increases in DHFR levels, it was necessary to go through a gradual multistep selection similar to what was done in other systems. Cells overproducing either an apparent wild-type or altered enzyme can be obtained depending upon the nature of the cell line initially used to generate the highly resistant isolates, thus, indicating that, like the wild-type gene, the gene coding for this alteration can also be amplified. Data suggestive of gene amplification in the RIII mutant have been reported (17, 18, 20, 47).

As in other systems (2, 33, 40), the overproduction of the reductase protein appears to be specific since in general no other proteins appear to be present in elevated amounts over their level in the wild-type cells (see Fig. 4). However, in some cases, other changes have been observed. (See, for example, Fig. 4B; there appears to be an amplified spot in the lower right-hand corner). Such changes are not surprising based on the lengthy multistep selections involved in the isolation of these clones, and whether there are pleiotropic effects of amplification will require additional analyses.

There appears, however, to be some discrepancy between the activity and the synthesis of DHFR in the various cell lines. The starvation technique used to determine the synthesis rate may disrupt the steady-state equilibrium of cellular physiology and thus affect the rate of synthesis of the reductase protein (23). Perhaps the technique of radioaffinity labeling, as described by Hänggi and Littlefield (23), might be useful in determining enzyme synthesis rates under steady-state conditions.

We have previously shown that wild-type and class I Mtx-resistant CHO cells contain two forms of DHFR with differing pI's (18). The major component, representing 90% of the total reductase protein, had a pI of 8.0, whereas the minor component, representing 10% of the total reductase protein, had a pI between 7.2 and 7.6. In the highly resistant isolates both forms of the enzyme are overproduced. Preliminary experiments have indicated that the ratio of these two forms remains the same in the highly resistant

TABLE 4. Karyotype changes in resistant cells[a]

Cell line	Chromosome differences relative to wild type (Pro$^-$ 3)
Pro$^-$ 3 Mtx^{RIII} 1-2	t(2p$^-$; 5q$^+$)
Pro$^-$ 3 Mtx^{RVII} 1-1	t(2p$^-$; 5q$^+$)
Pro$^-$ 3 Mtx^{RVII} 2-2	t(2p$^-$; 5q$^+$)
Pro$^-$ 4 Mtx^{RV} 9-5	t(9q$^+$; Z2q$^-$)
Pro$^-$ 4 Mtx^{RVI} 1-6	−Z13, t(9q$^+$; Z2q$^-$), t(Xq$^-$; 7q$^+$), ISO(Z7q), t(7p; 5q$^+$) HSR Z2q$^-$, HSR 5q$^+$, HSR (Z5a) q
Pro$^-$ 4 Mtx^{RVI} 3-1	−Z13, t(9q$^+$; Z2q$^-$), t(Xq$^-$; 7q$^+$) HSR Z2q$^-$, HSR (Z5a)p

[a] t, Translocation; −, deletion; +, addition; ISO, isochromosome.

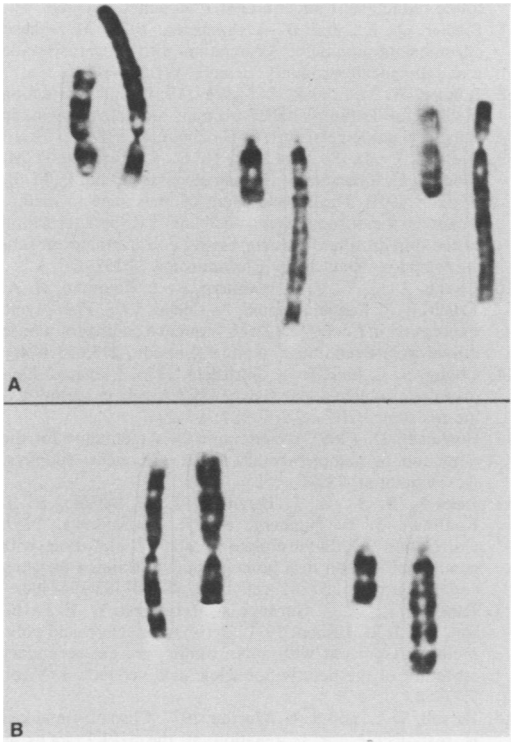

FIG. 6. Major chromosomal alterations present in the highly resistant isolates. (A) HSR chromosomes found in the Pro$^-$4 Mtx^{RVI} 1-6 cell line and their normal counterparts in wild-type cells. From left to right: normal Z2, HSR Z2, normal 5, HSR 5, normal Z5a, HSR Z5a. (B) 2p$^-$ and 5q$^+$ chromosomes of the Pro$^-$3 Mtx^{RVII} 1-1 cell line and their normal counterparts in wild-type cells. From left to right: normal 2, 2p$^-$, normal 5, 5q$^+$.

isolates as in the sensitive or class I resistant cells (Flintoff, unpublished data). At present, the nature of these two forms of the enzyme is not known. However, the availability of cells that overproduce both enzyme forms may provide a good source of material to determine the structural relationship between the forms.

The extent of amplification of the reductase genes in the CHO cell system has been determined by using a DNA complementary to the murine reductase cDNA (10) and the Southern blotting techniques. There is sufficient homology between the mouse and hamster genes that such a DNA can be used to estimate relative gene number (2, 37). With increasing levels of resistance to Mtx in the CHO cell system, there is a concurrent increase in the amount of reductase-specific DNA. Several mechanisms, including unequal exchanges between sister chromatids, insertion sequences, generation of extrachromosomal copies, or disproportionate replication, have been proposed to explain the amplification of specific genes (40), although to date it is not obvious what mechanism is operative in the various systems.

The restriction-blot analysis is of interest, suggesting that during the amplification of the DHFR gene no major rearrangements of the gene have occurred since the restriction pattern is similar in both wild-type and highly resistant isolates. It should be pointed out, however, that such conclusions are based on the information obtained with a murine reductase probe that is not completely homologous to the hamster gene (2, 37). Thus, such a probe might not identify all the DNA regions containing hamster DHFR sequences. Thus, the synthesis of a hamster reductase cDNA (29, 33) and subsequent use in an analysis of the DHFR gene in the various lines may be informative.

As previously described, high levels of Mtx resistance are often associated with chromosomal alterations (6, 9, 13, 25, 37). This is also true with one class (R^{VI}) of Mtx-resistant CHO cells. These cells contain HSR regions on several chromosomes. In all cases examined to date chromosome Z2 contains such a region, and this chromosome or chromosome 2 appears to have

TABLE 5. Summary of Mtx-resistant CHO lines

Class	Relative resistance to Mtx[a]	Relative DHFR activity[b]	Relative affinity of DHFR for Mtx[c]	Relative rate of DHFR synthesis[d]	Relative DHFR gene no.[e]	Presence of HSR chromosomes
WT	1	1	1	1	1	−
R^{I}	20	1	0.1	1	1	−
R^{III}	200	10	0.1	5	15	−
R^{V}	300	20	1	9	40	−
R^{VI}	20,000	150	1	95	150	+
R^{VII}	10,000	50	0.1	25	75	−

[a] Expressed as the ratio of the D_{10} value for the resistant lines to that for the wild-type line (1.2×10^{-8} M Mtx).
[b] Determined in crude cell extracts. The wild-type activity was 2.6 U/mg of protein.
[c] Expressed as the ratio of the I_{50} value for the resistant lines to that for the wild-type line.
[d] See Table 2.
[e] See Table 3.

the HSR regions in other highly Mtx-resistant Chinese hamster systems (6, 37). This is not surprising since it appears that the reductase gene is located on this chromosome (37, 48). It seems likely that the other chromosomes containing such HSR regions also contain sequences coding for the reductase function, although a direct determination of this has not been made. Preliminary experiments involving microcell-cell hybridization with the R^{VI} 1-6 line have indicated that for high levels of resistance all three HSR chromosomes are necessary (Flintoff, unpublished data).

High levels of resistance can, however, be obtained without any apparent HSR chromosomes, as illustrated by the R^{VII} isolates. In the cases examined to date, none have any readily identifiable HSR chromosomes nor are there very many chromosomal alterations. The reasons for this are not clear at present.

A further analysis of these highly resistant isolates by restriction endonuclease and blot analysis, using a hamster reductase-specific cDNA, will provide information on the organization of the reductase gene in sensitive and resistant cells and perhaps provide some insight into the mechanisms resulting in gene amplification and into the difference between cell lines with and without HSR chromosomes.

ACKNOWLEDGMENTS

We thank M. Coulter-Mackie, D. Fugita, G. Mackie, G. McFadden, V. Morris, R. Schimke, and R. Worton for supplying certain materials and helpful discussions.

This work was supported by a grant from the Medical Research Council of Canada to W.F.F. and a Public Health Service grant (HL-21831, National Institutes of Health) to W.S. W.F.F. is a Scholar of the Medical Research Council of Canada, and A.K.E. is a Government of Pakistan Overseas Scholar.

LITERATURE CITED

1. **Albrecht, A. M., J. L. Biedler, and D. J. Hutchison.** 1972. Two different species of dihydrofolate reductase in mammalian cells differentially resistant to amethopterin and methasquin. Cancer Res. **32**:1539–1546.
2. **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.
3. **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**:3063–3074.
4. **Berns, K. I., and C. A. Thomas.** 1965. Isolation of high molecular weight DNA from *Hemophilus influenzae*. J. Mol. Biol. **11**:476–490.
5. **Biedler, J. L., A. M. Albrecht, D. J. Hutchison, and B. A. Spengler.** 1972. Dose response, dihydrofolate reductase, and cytogenetics of amethopterin-resistant Chinese hamster cells *in vitro*. Cancer Res. **32**:152–161.
6. **Biedler, J. L., R. W. Melera, and B. A. Spengler.** 1980. Specifically altered metaphase chromosomes in antifolate-resistant Chinese hamster cells that overproduce dihydrofolate reductase. Cancer Genet. Cytogenet. **2**:47–60.
7. **Biedler, J. L., and B. A. Spengler.** 1976. Metaphase chromosome anomaly: Association with drug resistance and cell specific products. Science **191**:185–187.
8. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for Tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
9. **Bostock, C. J., E. M. Clark, N. G. L. Harding, P. M. Mounts, C. Tyler-Smith, U. van Heyningen, and P. M. B. Walker.** 1979. The development of resistance to methotrexate in a mouse melanoma cell line. 1. Characterization of the dihydrofolate reductases and chromosomes in sensitive and resistant cells. Chromosoma **74**:153–177.
10. **Chang, A. C. Y., J. H. Nunberg, R. J. Kaufman, H. A. Erlich, R. T. Schimke, and S. N. Cohen.** 1978. Phenotypic expression in *E. coli* of a DNA sequence coding for mouse dihydrofolate reductase. Nature (London) **275**:617–624.
11. **Chang, S. E., and J. W. Littlefield.** 1976. Elevated dihydrofolate reductase messenger RNA levels in methotrexate-resistant BHK cells. Cell **7**:391–396.
12. **Denhardt, D.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–652.
13. **Dolnick, B. J., R. J. Berenson, J. R. Bertino, R. J. Kaufman, J. H. Nunberg, and R. T. Schimke.** 1979. Correlation of dihydrofolate reductase elevation with gene amplification in a homogeneously staining chromosomal region in L5178Y cells. J. Cell Biol. **83**:394–402.
14. **Fanshier, L., A. C. Garapin, J. McDonnell, W. E. Levinson, and J. M. Bishop.** 1971. Deoxyribonucleic acid polymerase associated with avian tumor viruses: secondary structure of the deoxyribonucleic acid product. J. Virol. **7**:77–86.
15. **Farrell, S. A., and R. G. Worton.** 1977. Chromosomal loss is responsible for segregation at the HPRT locus in Chinese hamster cell hybrids. Somatic Cell Genet. **3**:539–551.
16. **Fisher, F. A.** 1962. Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolite in L5178Y leukemic cells. Biochem. Pharmacol. **11**:1233–1234.
17. **Flintoff, W. F., S. V. Davidson, and L. Siminovitch.** 1976. Isolation and partial characterization of three methotrexate-resistant phenotypes and Chinese hamster ovary cells. Somatic Cell Genet. **2**:245–261.
18. **Flintoff, W. F., and K. Essani.** 1980. Methotrexate-resistant Chinese hamster ovary cells contain a dihydrofolate reductase with an altered affinity for methotrexate. Biochemistry **19**:4321–4327.
19. **Flintoff, W. F., and M. Weber.** 1980. Selection of wild-type revertants from methotrexate-resistant cells containing an altered dihydrofolate reductase. Somatic Cell Genet. **6**:517–528.
20. **Gupta, R. S., W. F. Flintoff, and L. Siminovitch.** 1977. Purification and properties of dihydrofolate reductase from methotrexate-sensitive and methotrexate-resistant Chinese hamster ovary cells. Can. J. Biochem. **55**:445–452.
21. **Hakala, M. T., S. F. Zakresewski, and C. H. Nichol.** 1961. Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. J. Biol. Chem. **236**:952–958.
22. **Hänggi, U. J., and J. W. Littlefield.** 1974. Isolation and characterization of the multiple forms of dihydrofolate reductase from methotrexate resistant hamster cells. J. Biol. Chem. **249**:1390–1397.
23. **Hänggi, 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.
24. **Jackson, R. C., and D. Niethammer.** 1977. Acquired methotrexate resistance in lymphoblasts resulting from altered kinetic properties of dihydrofolate reductase. Eur. J Cancer **13**:567–575.
25. **Kaufman, R. J., P. C. Brown, and R. J. Schimke.** 1979. Amplified dihydrofolate reductase gene in unstably meth-

otrexate-resistant cells are associated with double minute chromosomes. Proc. Natl. Acad. Sci. U.S.A. 76:5669–5673.
26. **Kellems, R. E., F. W. Alt, and R. J. Schimke.** 1976. Regulation of folate reductase synthesis in sensitive and methotrexate-resistant sarcoma S-180 cells. *In vitro* translation and characterization of folate reductase mRNA. J. Biol. Chem. 251:6987–6993.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
28. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335–341.
29. **Lewis, J. A., D. T. Kurtz, and P. W. Melera.** 1981. Molecular cloning of Chinese hamster dihydrofolate reductase specific cDNA and the identification of multiple dihydrofolate reductase mRNAs in antifolate-resistant Chinese hamster lung fibroblasts. Nucleic Acids Res. 9:1311–1322.
30. **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.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
32. **McBurney, M. W., and G. F. Whitmore.** 1974. Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells. Cell 2:173–182.
33. **Melera, P. W., J. A. Lewis, J. L. Bredler, and C. Hession.** 1980. Antifolate-resistant Chinese hamster cells. Evidence for dihydrofolate reductase gene amplification among independently derived sublines overproducing different dihydrofolate reductases. J. Biol. Chem. 255:7024–7028.
34. **Melera, P. W., D. Wolgemuth, J. L. Biedler, and C. Hession.** 1980. Antifolate-resistant Chinese hamster cells: evidence from independently derived sublines for the overproduction of two dihydrofolate reductase encoded by different mRNAs. J. Biol. Chem. 225:319–322.
35. **Nakamura, H., and J. W. Littlefield.** 1972. Purification, properties, and synthesis of dihydrofolate reductase from wild-type and methotrexate-resistant hamster cells. J. Biol. Chem. 247:179–187.
36. **Nunberg, J. H., R. J. Kaufman, A. C. Y. Chang, S. N. Cohen, and R. T. Schimke.** 1980. Structure and genomic organization of the mouse dihydrofolate reductase gene. Cell 19:355–364.
37. **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. 11:5553–5556.
38. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of protein. J. Biol. Chem. 250:4007–4021.
39. **Ray, M., and T. Mohandas.** 1976. Proposed banding nomenclature for the Chinese hamster chromosomes (*Cricetulus griserus*). Cytogenet. Cell Genet. 16:83–91.
40. **Schimke, R. T., R. J. Kaufman, F. W. Alt, and R. F. Kellems.** 1978. Gene amplification and drug resistance in cultured murine cells. Science 202:1051–1055.
41. **Sirotnak, F. M., S. Kurita, and D. J. Hutchison.** 1968. On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia. Cancer Res. 28:75–80.
42. **Southern, E. M.** 1975. Detection of specific sequences among DNA separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
43. **Stanners, C. P., G. L. Elicieri, and H. Green.** 1971. Two types of ribosomes in mouse-hamster hybrid cells. Nature (London) New Biol. 230:52–54.
44. **Stavnezer, J., R. C. C. Huang, E. Stavnezer, and J. M. Bishop.** 1974. Isolation of messenger RNA for an immunoglobin kappa chain and enumeration of the genes for the constant region of kappa chain in the mouse. J. Mol. Biol. 88:43–63.
45. **Strohman, R. C., P. S. Moss, J. Micou-Eastwood, and P. Spector.** 1977. Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. Cell 10:265–273.
46. **Studier, T. W.** 1973. Analysis of bacteriophage T7 early RNA's and protein on slab gels. J. Mol. Biol. 79:237–248.
47. **Wigler, M., M. Perucho, D. Kurtz, S. Dana, A. Pellicer, R. Axel, and S. Silverstein.** 1980. Transformation of mammalian cells with an amplifiable dominant-acting gene. Proc. Natl. Acad. Sci. U.S.A. 77:3567–3570.
48. **Worton, R., C. Duff, and W. Flintoff.** 1981. Microcell-mediated co-transfer of genes specifying methotrexate resistance, emetine sensitivity, and chromate sensitivity with Chinese hamster chromosome 2. Mol. Cell. Biol. 1:330–335.
49. **Worton, R. G., C. C. Ho, and C. Duff.** 1977. Chromosome stability in CHO cells. Somatic Cell Genet. 3:24–45.