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Genetics

# Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity

(mutagenesis/methotrexate/cancer chemotherapy/tetrahydrofolate dehydrogenase)

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**ABSTRACT** **Mutants of Chinese hamster ovary cells lacking dihydrofolate reductase (tetrahydrofolate dehydrogenase, 7,8-dihydrofolate:NADP$^+$ oxidoreductase; EC 1.5.1.3) activity were isolated after mutagenesis and exposure to high-specific-activity [$^3$H]deoxyuridine as a selective agent. Fully deficient mutants could not be isolated starting with wild-type cells, but could readily be selected from a putative heterozygote that contains half of the wild-type level of dihydrofolate reductase activity. The heterozygote itself was selected from wild-type cells by using [$^3$H]deoxyuridine together with methotrexate to reduce intracellular dihydrofolate reductase activity. Fully deficient mutants require glycine, a purine, and thymidine for growth; this phenotype is recessive to wild type in cell hybrids. Revertants have been isolated, one of which produces a heat-labile dihydrofolate reductase activity. These mutants may be useful for metabolic studies relating to cancer chemotherapy and for fine-structure genetic mapping of mutations by using available molecular probes for this gene.**

The genetic locus (*dhfr*) specifying the enzyme dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase, 7,8-dihydrofolate:NADP$^+$ oxidoreductase; EC 1.5.1.3) is of considerable interest to mammalian cell geneticists for several reasons. This enzyme is responsible for the formation of intracellular tetrahydrofolic acid, a cofactor that is required for one-carbon transfers in various biosynthetic reactions (1, 2). The central role of DHFR in the synthesis of nucleic acid precursors, together with its great sensitivity to tetrahydrofolate analogs such as methotrexate (MTX, amethopterin), has made this enzyme a target of wide use in cancer chemotherapy (3). The metabolic consequences of this sensitivity have also been exploited for the nutritional manipulation of cultured mammalian cells in somatic cell genetics (e.g., hypoxanthine/amethopterin/thymidine medium; see refs. 4 and 5).

Mutational studies of the *dhfr* locus have until now been confined to the phenotype of cellular resistance to the growth-inhibitory effects of MTX and related inhibitors of DHFR. MTX-resistant clones isolated from several different rodent cell lines usually exhibit one of the following three phenotypes: (*i*) decreased permeability to MTX (6); (*ii*) DHFR that is intrinsically less sensitive to MTX (6, 7); or (*iii*) overproduction of DHFR activity (6, 8, 9). The last class appears to be the most common, and the overproduction has been shown to result from increased synthesis of wild-type enzyme (10, 11) due to *dhfr* gene amplification (12).

The abundance of DHFR mRNA in MTX-resistant mouse cell mutants has made it possible to prepare purified cDNA for this gene and to clone this sequence in a bacterial host (13). This clone represents a molecular probe that could be used to analyze mutational alterations in the chromosomal genes for DHFR.

For these reasons, we undertook the development of a selective system for the isolation of mutant mammalian cells carrying lesions at the *dhfr* locus such that functional DHFR is no longer produced. This was accomplished in a Chinese hamster ovary (CHO) cell line with the use of [6-$^3$H]deoxyuridine ([$^3$H]dUrd) as the principal selective agent. The successful isolation of DHFR-deficient mutants required the creation of a putative heterozygote as an intermediate. A preliminary account of this work has been presented (14).

## MATERIALS AND METHODS

**Culture Conditions.** All cells were derivatives of the CHO-K1 line (15) and were propagated in F12 medium (ref. 16; GIBCO) modified as indicated. The medium was supplemented with 10% (vol/vol) fetal calf serum (GIBCO) for general growth or with 10% extensively dialyzed (17) fetal calf serum whenever cell nutrition was being manipulated. Cells were grown at 37°C in an atmosphere of 5% carbon dioxide.

**Selection of DHFR-Deficient Mutants.** Mutagenesis with ethyl methanesulfonate (EtMes) and selection of 6-thioguanine-resistant mutants have been described (18). Mutagenesis with $\gamma$ rays was carried out by immersing vials containing cell suspensions in a water tank containing a cobalt-60 source for varying time intervals. The dose used for the isolation of mutants described in Table 2 was 690 rads (1 rad = $1.00 \times 10^{-2}$ J/kg), which reduced viability to 9%.

A partially DHFR-deficient, presumptive heterozygote clone was selected by a stepwise enrichment procedure (see *Results*). After mutagenesis and a 7-day expression period, $5 \times 10^5$ CHO-K1 cells ($3.6 \times 10^3$ cells per cm$^2$) were incubated for 24 hr in F12 medium modified to contain 0.3 $\mu$M thymidine, 0.15 $\mu$M [$^3$H]dUrd (24 Ci/mmol, New England Nuclear; 1 Ci = $3.7 \times 10^{10}$ becquerels), and 0.1 $\mu$M MTX. After an additional 8 days in F12 medium containing 0.1 $\mu$M MTX, the surviving colonies were pooled and again treated with [$^3$H]dUrd and MTX as above. This procedure was repeated several times; each time at least several hundred colonies were pooled for the subsequent round.

For selection of fully deficient mutants, mutagenized cells were allowed 6–7 days of growth for phenotypic expression and then plated at $1.1 \times 10^3$ cells per cm$^2$ in F12 medium modified to contain 0.3 $\mu$M thymidine and 0.15 $\mu$M [$^3$H]dUrd (24 Ci/mmol). Reconstruction experiments showed a decreased recovery of mutant cells at higher cell densities, suggesting that crossfeeding of tritiated derivatives or of tetrahydrofolate itself was taking place. After 24 hr, the medium was changed to regular F12 (supplemented with whole rather than dialyzed fetal calf serum). After 6–8 days of further growth, surviving colonies were cloned and screened for their inability to grow in F12 medium lacking glycine, hypoxanthine, and thymidine.

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Abbreviations: CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; *dhfr*, dihydrofolate reductase locus; MTX, methotrexate; EtMes, ethyl methanesulfonate; $ED_{50}$, mean effective dose.

**DHFR Assays.** Catalytic activity was measured by the procedure of Frearson *et al.* (19) at room temperature in a total volume of 0.5 ml. Cell extracts were prepared by sonication (20) followed by dialysis against extraction buffer (19).

[$^3$H]MTX binding was measured basically as described by Johnson *et al.* (21). Cytoplasmic extracts were prepared by resuspending washed cells at $2 \times 10^8$/ml in buffer A (10 mM potassium phosphate/0.15 M KCl, pH 6) containing 0.5% Nonidet P-40 (Bethesda Research, Rockville, MD), incubating for 5 min at 0°C, then centrifuging for 30 min at 12,000 × *g*. To 0.2 ml of the supernatant solution was added 0.1 ml of a solution containing bovine serum albumin (1 mg/ml), 0.3 mM NADPH, and 40 nM [3′,5′,9(*n*)-$^3$H]MTX (7.5 Ci/mmol, Amersham). After 5 min at room temperature, 0.6 ml of charcoal suspension [33 mg of acid-washed Norit per ml, 0.3 mg of Dextran T-2000 (Pharmacia) per ml, and 8 mg of bovine serum albumin per ml, at pH 6.2] was added, the mixture was centrifuged for 5 min at 350 × *g*, and 0.8 ml of the supernatant solution was added to another 0.6 ml of charcoal suspension. After a second centrifugation, the radioactivity of 1.2 ml of the supernatant, containing nonextractable [$^3$H]MTX bound to protein, was measured in 10 ml of Triton X-100-based scintillation fluid. Background values (no extract) ranged up to 500 cpm per original assay tube, which is equivalent to about 0.1 pmol of [$^3$H]MTX.

**Miscellaneous.** Protein was measured (22) after precipitation with 10% (wt/vol) trichloroacetic acid. The mutant clone OY21, resistant to 1 mM ouabain, was selected according to Baker *et al.* (23) after EtMes mutagenesis of clone YHD13 (24). Cell fusion with inactivated Sendai virus has been described (18). Biochemicals were purchased from Sigma unless otherwise noted.

## RESULTS

**Selection Method.** The selection against DHFR-positive cells was based on the role of this enzyme in the *de novo* biosynthesis of thymidylic acid. DHFR catalyzes the reduction of folic acid, supplied in the medium, to tetrahydrofolic acid. The latter is the active form of the cofactor that is used in several biosynthetic pathways involving one-carbon transfers. Mutants lacking DHFR activity would be unable to carry out the *de novo* synthesis of glycine, purine nucleotides, and thymidylate. Such mutants should be viable, however, as long as salvageable sources of these end products are supplied in the medium; that is, DHFR-deficient mutants should simply be triple auxotrophs for glycine, hypoxanthine (a purine source), and thymidine.

The selective agent chosen was tritiated deoxyuridine ([$^3$H]dUrd) of high specific activity (24 Ci/mmol). Like tritiated thymidine, [$^3$H]dUrd should be toxic to wild-type cells by virtue of its incorporation into DNA and subsequent radioactive decay. In order to be incorporated into DNA, deoxyuridine must first be converted to thymidylate. The four reactions necessary for this conversion are listed in Table 1. A cell lacking any one of these enzymatic steps should be resistant to the toxic effects of [$^3$H]dUrd. The growth phenotype would be different in each case, however. As mentioned above, a DHFR-deficient mutant would be a triple auxotroph. Mutants partially deficient in step 2 have been isolated (25, 26) and shown to require only glycine for growth. Step 3 mutants should have no growth requirement, but should be resistant to BrdUrd (27). Mutants lacking step 4 have not been demonstrated in mammalian cells; they should be thymidine auxotrophs. Thus, all four potential classes of [$^3$H]dUrd-resistant mutants should be easily distinguishable by their growth characteristics alone.

Table 1. Reactions converting deoxyuridine (dUrd) to thymidylate (TMP)

| |
|---|
| 1. Folic acid → tetrahydrofolic acid ($FH_4$) |
| 2. Serine + $FH_4$ → glycine + methylene-$FH_4$ |
| 3. dUrd + ATP → dUMP + ADP |
| 4. dUMP + methylene-$FH_4$ → TMP + $FH_2$ |

Enzymes involved are: 1, dihydrofolate reductase; 2, serine hydroxymethyltransferase; 3, thymidine kinase; and 4, thymidylate synthetase.

The effectiveness of [$^3$H]dUrd as a killing agent is shown in Fig. 1. In this experiment wild-type CHO-K1 cells were exposed to increasing amounts of [$^3$H]dUrd and tested for their ability to subsequently form colonies. A minimal amount of thymidine (0.3 μM) was also included in the medium, just enough to allow full-size colony formation when *de novo* TMP synthesis is blocked. Although thymidine, as expected, does compromise the effectiveness of killing by [$^3$H]dUrd (data not shown), its inclusion is necessary because DHFR-deficient mutants will require exogenous thymidine. As can be seen in Fig. 1, [$^3$H]dUrd is an effective killing agent. In subsequent experiments (not shown) it was found that after a 24-hr exposure to 0.15 μM [$^3$H]dUrd, survival is typically 0.005–0.01%. Frozen storage of cells to permit additional radioactive decay was not necessary to achieve this level of killing. To test the idea that DHFR-deficient mutants would be relatively resistant to killing by [$^3$H]dUrd, we exposed wild-type cells to [$^3$H]dUrd in the presence of 2 μM MTX, a tight-binding inhibitor of DHFR (2). Under these conditions, cellular DHFR activity is completely suppressed and wild-type cells are converted into phenocopies of DHFR-negative mutants. As can be seen in Fig. 1, the inclusion of MTX effectively spares wild-type cells from killing by [$^3$H]dUrd.

**Attempts at One-Step Selection.** In our initial experiments, we attempted to isolate DHFR-deficient mutants starting directly with the wild-type CHO-K1 clone. If, by chance, the *dhfr* locus were functionally haploid (X-linked or already heterozygous) in CHO cells, then an EtMes-induced mutation frequency on the order of $10^{-4}$ might be expected, by analogy with our experience with two other single-allele systems (18, 20).

In two experiments, CHO-K1 cells were mutagenized with EtMes, allowed an expression period of 6–7 days, and then treated with [$^3$H]dUrd as described for the selection of fully deficient mutants. Samples of mutagenized populations were also challenged with 6-thioguanine. Resistance to this purine analog is usually caused by mutation in the single functional gene for hypoxanthine phosphoribosyltransferase. Mutants

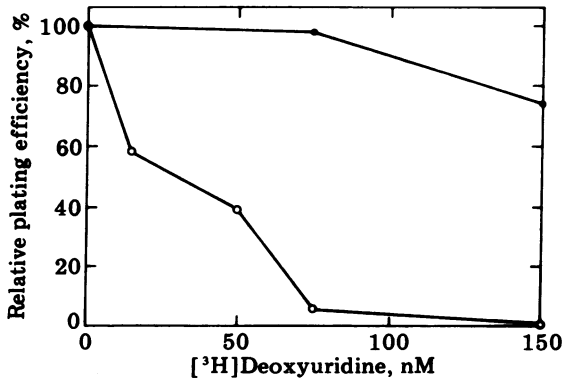


FIG. 1. Killing of cells by [$^3$H]dUrd and its reversal by MTX. Cells were plated in duplicate in F12 medium containing 0.3 μM thymidine. [$^3$H]dUrd (26 Ci/mol) was added as indicated. ○, No other additions; ●, plus 2 μM MTX. Colonies were stained and counted after 7 days of growth. The absolute plating efficiency with no additions was 52%.

resistant to 6-thioguanine were present at a frequency of $2 \times 10^{-4}$, representing an induction of over 100-fold and attesting to the efficacy of the mutagenic treatment. The frequency of colonies surviving the [$^3$H]dUrd treatment was similar in the two experiments, the average being $1 \times 10^{-4}$. However, the great majority of tested colonies did not exhibit the triple auxotrophy (27, 28) expected of DHFR-deficient mutants and were considered wild-type cells that had statistically survived the [$^3$H]dUrd treatment. Four clones did exhibit the triple auxotrophy (i.e., required glycine, hypoxanthine, and thymidine), but when cell-free extracts were prepared and assayed, they proved to have wild-type levels of DHFR activity. Mutants with this phenotype have been isolated in CHO cells (25, 29) and in some cases have been shown to lack folylpolyglutamate synthetase activity (30). This enzyme adds up to five glutamic acid residues to tetrahydrofolic acid, a modification that is thought to play a role in retaining the cofactor within cells (29). Consistent with the thymidine requirement and [$^3$H]dUrd resistance, one mutant of this type tested incorporated only one-third as much radioactive [$^3$H]dUrd into trichloroacetic acid-insoluble material as compared with wild type (data not shown).

No DHFR-deficient mutants were found by direct [$^3$H]dUrd selection on mutagenized CHO-K1 cells. Based on the number of cells challenged ($1.6 \times 10^6$) and the fraction of surviving colonies tested (31/160), an induced mutation frequency of less than $3 \times 10^{-6}$ can be calculated. The absence of DHFR$^-$ mutants is consistent with the idea that two hits are necessary to produce a complete deficiency of DHFR activity. For instance, in our previous studies with CHO cells, EtMes induced mutants at the functionally haploid locus for hypoxanthine phosphoribosyltransferase at a frequency of $2 \times 10^{-4}$ (18), whereas for mutants at the presumably diploid locus for adenine phosphoribosyltransferase, the frequency was only about $10^{-7}$ (20). The cost of [$^3$H]dUrd and the low density at which cells must be plated for recovery of [$^3$H]dUrd-resistant mutants precluded the direct screening of $10^8$ cells for such double mutants.

**Selection of Cells Heterozygous for *dhfr*.** If the *dhfr* locus is present in a diploid state in CHO cells and if each allele is approximately as mutable with EtMes as are the phosphoribosyltransferase genes mentioned above, then mutants affected at one allele only should be present in the mutagenized population at a frequency of about $10^{-4}$. These heterozygotes should contain only half the amount of DHFR activity of wild-type cells if gene dosage relationships obtain. By addition of a judicious amount of MTX to the medium, these partially deficient heterozygotes would become fully deficient and thus resistant to killing by [$^3$H]dUrd. Wild-type cells, on the other hand, would still have considerable DHFR activity remaining under these conditions (ideally 50%), enough to allow them to convert [$^3$H]dUrd to [$^3$H]TMP and still be killed. The amount of MTX in the medium that would inactivate 50% of a wild-type level of cellular DHFR was not known. Therefore, an amount of the inhibitor was added that increased survival in the presence of [$^3$H]dUrd from the usual $5 \times 10^{-5}$ to approximately $10^{-3}$. After an initial exposure of EtMes-mutagenized CHO-K1 cells to this combination of MTX and [$^3$H]dUrd, the survivors were pooled and expanded. This procedure was repeated three times. The survival after each of the rounds was 0.07%, 0.11%, 16%, and 2.5%. After the fourth round, 11 surviving colonies were picked and initially screened for quantitative sensitivity to MTX; we reasoned that a cell with only 50% wild-type enzyme level should be slightly more sensitive to inhibition of growth by the drug. Five of the clones did show a 2-fold increase in MTX sensitivity, the mean effective dose ($ED_{50}$) for colony formation decreasing from 10 nM to 5 nM (data not shown). Because of the mass culture enrichment used, the clones isolated were not necessarily independent, and only one was chosen for further analysis. As expected, this clone (UKB25) is relatively resistant to the selection regimen used (13% survival).

The presence of DHFR in extracts from wild-type and mutant cells was quantitated in two ways: spectrophotometric assay of catalytic activity and [$^3$H]MTX binding. Both methods indicated that UKB25 contains half the specific activity of wild-type (homozygous positive, $d^+/d^+$) CHO-K1 cells, as predicted for a cell heterozygous ($d^+/d^-$) at the *dhfr* locus (Table 2, lines 1 and 2). A more critical test is the ability of heterozygous cells to give rise to completely deficient (homozygous negative, $d^-/d^-$) mutants at high frequency.

**Selection of Completely Deficient Mutants from the Presumptive Heterozygote.** UKB25 cells were again mutagenized with EtMes and then challenged with [$^3$H]dUrd, this time with no MTX present. Surviving colonies appeared at a frequency of $2.5 \times 10^{-4}$. About half of these survivors appeared to be statistical in that they did not require glycine, hypoxanthine, or thymidine. Those colonies that did exhibit the triple auxotrophy were recloned and assayed for DHFR by either the catalytic or MTX-binding assay or both. In 19 cases examined (four experiments), all of the triple auxotrophs isolated from UBK25 proved to be deficient in DHFR (Table 2). Eighteen of the mutants contained no detectable DHFR, whereas one clone (DUK22) did exhibit a low level of residual activity, approximately 2% of wild type. The lack of DHFR activity in mutant cells was not due to the presence of a diffusible inhibitor; no decrease in activity was observed when wild-type extract was mixed with an excess of mutant extracts (DUK22 and DUK51). As indicated in Table 2, DHFR-deficient mutants have been isolated after mutagenesis with $\gamma$ irradiation as well as with EtMes. In both cases, DHFR-deficient mutants appeared at a frequency of approximately $10^{-4}$. Only one spontaneous DHFR$^-$ mutant has been isolated among $6 \times 10^5$ UKB25 cells screened.

**Reversion.** All of the DHFR-negative mutants isolated have maintained their characteristic growth phenotype of an inability to grow in medium lacking glycine, hypoxanthine, and thymidine after two subclonings and extensive cultivation in nonselective medium. The only indication of possible instability was found in clone DUK22, the mutant that contains a small amount of residual DHFR activity. After several hundred generations in nonselective medium, these cells are able to grow

Table 2. DHFR levels in mutant clones

| Clone | Mutagen | Presumed genotype | Relative enzymatic activity | Relative [$^3$H]MTX binding |
|---|---|---|---|---|
| CHO-K1 | — | $d^+/d^+$ | 1.00 ± 0.05 | 1.00 ± 0.08 |
| UKB25 | EtMes | $d^+/d^-$ | 0.50 ± 0.08 | 0.53 ± 0.02 |
| DUK22 | EtMes | $d^-/d^-$ | 0.02 | 0.02 |
| DUK51 | EtMes | $d^-/d^-$ | <0.02 | <0.005 |
| DUK-D1 | EtMes | $d^-/d^-$ | ND | <0.005 |
| DUK-S1 | — | $d^-/d^-$ | ND | <0.003 |
| 15 clones | $\gamma$ rays | $d^-/d^-$ | ND | All <0.005 |
| DUK51-R1 | EtMes | $d^+R/d^-$ | ND | 0.52 |
| DUK51-R2 | EtMes | $d^+R/d^-$ | ND | 0.49 |
| DUK22-R1 | EtMes | $d^+R/d^-$ | 0.18 | 0.05 |
| DUK22-R2 | EtMes | $d^+R/d^-$ | 0.07 | 0.13 |

All values are normalized to enzyme levels in CHO-K1, which are 3.2 nmol/min per mg of protein for catalytic activity and 6.0 pmol/mg of protein for [$^3$H]MTX binding. Standard errors are included for an experiment comparing CHO-K1 and UKB25 in which three independent cultures of each were assayed on the same day. The suffix, R, denotes a revertant. ND, not determined.

in the absence of hypoxanthine, although they still require glycine and thymidine. This phenomenon may be due to amplification of a gene with a leaky mutation.

Although these mutants are basically stable, revertants can be isolated after further mutagenesis. In theory, revertants should be selectable in a medium lacking glycine, hypoxanthine, and thymidine, but preliminary experiments showed that omission of the purine alone was sufficient to effectively kill mutant cells and yielded revertants more consistently. Two EtMes-induced mutants (DUK22 and DUK51) were mutagenized again with EtMes, allowed a 3-day expression period in nonselective medium, and plated in F12 medium lacking hypoxanthine. Revertant colonies appeared at a frequency of $10^{-6}$. In two independent revertants of clone DUK51, DHFR activity has returned to the parental (UKB25) level (Table 2), and these revertants are capable of growth in the absence of glycine, hypoxanthine, and thymidine. Revertants of clone DUK22, on the other hand, still require glycine and thymidine and contain a lesser amount of DHFR activity (Table 2).

The nature of the DHFR activity in two revertants was compared with that of wild-type cells with respect to two properties. DUK22-R1 DHFR activity was slightly but reproducibly less sensitive to MTX inhibition in cell-free extracts ($ED_{50}$ = 2.5 nM, compared with 1.2 nM for CHO-K1). A more dramatic difference was found by measuring the heat lability of enzyme activity. DUK22-R1 DHFR activity is extremely unstable at temperatures that hardly affect the wild-type enzyme, whether measured by catalytic activity (Fig. 2*A*) or [$^3$H]MTX binding (Fig. 2*B*). This revertant thus produces an altered enzyme, perhaps due to a second site mutation in the *dhfr* structural gene that compensates for the initial lesion. The DHFR activity in the second revertant tested, DUK51-R1, was indistinguishable from wild type by these two criteria.

**Cell Hybridization Experiments.** To test for the dominant or recessive character of the enzyme-deficient phenotype, we fused two mutants (DUK22 and DUK51) with cells that are wild type with respect to *dhfr*. The latter clone (OY21) carries a dominant ouabain resistance marker and a recessive hypoxanthine phosphoribosyltransferase deficiency. After promotion of cell fusion with inactivated Sendai virus, hybrids were selected in medium containing 1 mM ouabain, 0.02 mM azaserine (to inhibit *de novo* purine synthesis), and hypoxanthine. Hybrids derived from both mutants proved to be independent of glycine, hypoxanthine, and thymidine, indicating that the DHFR deficiencies in DUK22 and DUK51 are recessive to the wild-type allele. As part of the same experiment, DUK22 and DUK51 were fused with each other and then subjected to selection in medium lacking glycine, hypoxanthine, and thymidine. No hybrids were found among $10^6$ parental cells subjected to fusion. Because the hybrid frequency in parallel dishes fusing the mutants to the wild-type cells was $2 \times 10^{-4}$, the lack of hybrids when mutants were fused with each other can be taken as evidence for a lack of complementation between the independent mutations in these two clones. These hybridization data are what would be expected if these clones represent structural gene mutants for *dhfr*.

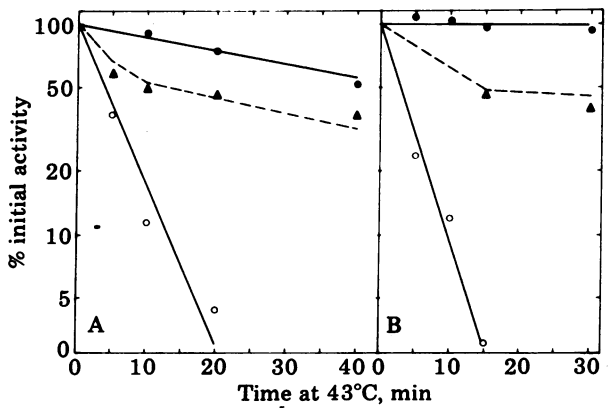


FIG. 2. Heat inactivation of DHFR activity from wild-type and revertant cell lines. Extracts were prepared as usual except for the inclusion of 10 $\mu$M NADPH, for measuring either catalytic activity (*A*) or [$^3$H]MTX-binding activity (*B*). Samples were heated at 43°C for the indicated times and then transferred to a 0°C bath until the conclusion of the heating treatment, when all samples were assayed together. ●, Wild-type CHO-K1 cells; ○, revertant DUK22-R1; ▲, mixture of approximately equal activities of each; – –, theoretical activity of the mixture calculated from the algebraic sum. The initial activities for the wild-type, revertant, and mixture, respectively, were 0.8, 0.7, and 0.7 nmol/min for the catalytic assay and 1.1, 0.17, and 0.40 pmol for the [$^3$H]MTX-binding assay.

## DISCUSSION

The [$^3$H]dUrd-resistant variants of CHO cells described here exhibit many of the characteristics expected of true *dhfr* structural gene mutants: (*i*) they lack DHFR catalytic activity and the ability to tightly bind a substrate analog ([$^3$H]MTX); (*ii*) they are rare in untreated populations ($10^{-6}$) but can be induced 100-fold by the known mutagens EtMes and $\gamma$ rays; (*iii*) they are stable, but can be induced to revert; (*iv*) their mutations are recessive to wild type in cell hybrids; and (*v*) one revertant produces an altered DHFR activity, consistent with the idea of two amino acid substitutions present in the revertant enzyme.

The fact that CHO-K1 cells did not directly give rise to a mutant lacking DHFR activity suggests that there are at least two wild-type alleles for *dhfr* in these pseudodiploid cells. It is probable that K1 cells are diploid at this locus because, in the partially deficient, putative heterozygous derivative (UKB25), DHFR activity has been reduced by a factor of 2. Karyotypic data support this conclusion: the homogeneously staining chromosomal region that contains the amplified sequences of the *dhfr* gene in a MTX-resistant CHO cell mutant is located on the long arm of chromosome 2 (31). Giemsa banding indicates that CHO cells contain both homologues of this arm (28, 31, 32). The *dhfr* locus thus falls into the predominant category of diploid genes (20, 33) and is not among the class of haploid (or functionally haploid) autosomal genes found in CHO cells (34, 35).

Completely deficient mutants could be generated starting with the partially deficient heterozygote by a second mutation in the one remaining wild-type *dhfr* allele. However, an alternative mechanism is the production of a homozygous negative genotype by mitotic recombination or gene conversion. Although we have shown (24) that mitotic recombination does not occur with high frequency between two X-linked markers in these cells, those negative results may not apply to all loci (36, 37). The fact that second mutations can occur in this system is shown by the isolation of mutant clone DUK22 because it exhibits a distinctive phenotype with regard to residual enzyme activity and reversion. A more detailed survey of independent mutants will be necessary to decide whether or not second mutation is the predominant mechanism involved.

A survey of mutant genotypes has been, in fact, one of the principal objectives in the development of this selective system. In a variety of cell lines, DHFR mRNA is overproduced due to gene amplification at this locus (reviewed in ref. 38). In mouse cells, this has permitted the cloning in *Escherichia coli* of DNA sequences complementary to DHFR message. The cloned mouse sequence contains sufficient homology so as to hybridize with Chinese hamster *dhfr* sequences (31). The

availability of molecular probes such as this should permit fine-structure mapping by DNA sequence analysis of mutations resulting in DHFR deficiency. Such an analysis should help in determining what aspects of gene structure are necessary for gene expression and in defining the molecular consequences of spontaneous and induced mutation at the level of DNA. In addition, structural gene mutants that alter the catalytic properties of DHFR may be useful in pointing out structure–function relationships in this small (22,000 daltons) single polypeptide enzyme (2).

The primary metabolic effect of DHFR deficiency in these cells is a triple auxotrophy (for glycine, a purine, and thymidine). More complex aspects of folate metabolism may now be investigated by using these mutants. These include the role of DHFR in the transport of folate compounds and in the interconversion of folate metabolites and antimetabolites. Previous studies of this type often have been complicated by the interaction of these compounds with DHFR (39).

Proteins other than DHFR that are capable of binding MTX and other folates with high affinity have been reported in some tissues (39). The fact that DHFR catalytic activity and [$^3$H]-MTX-binding ability are always lost simultaneously in these experiments indicates that DHFR is the only cytoplasmic protein in CHO cells capable of binding MTX with high affinity.

MTX was used in this work to partially titrate cellular DHFR activity and so magnify the effects of gene dosage. This method should be generally applicable to other diploid loci where a tight-binding inhibitor of the gene product is available and where selective pressure for a negative phenotype can be applied. Moreover, this principle might also be used with cells of even higher ploidy or when genes are present in multiple copies in order to select for the stepwise elimination of functional genes.

The fact that cells with a higher level of DHFR can be selectively killed with [$^3$H]dUrd illustrates an approach that may be useful in conjunction with the use of MTX in cancer chemotherapy. In many cases, MTX treatment must be discontinued because of the development of MTX resistance in the tumor cell population (3). We have recently shown that MTX-resistant cells (containing high levels of DHFR activity) can be killed by a combination of [$^3$H]dUrd and a high dose of MTX; this treatment does not greatly affect wild-type cells. The use of this or an analogous regimen for resistant tumors may decrease the number of drug-resistant cells and allow resumption of MTX chemotherapy.

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