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Regulation of Dihydrofolate Reductase in Human Breast Cancer Cells and in Mutant Hamster Cells Transfected with a Human Dihydrofolate Reductase Minigene

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SUMMARY

The regulation of dihydrofolate reductase (DHFR) gene expression was studied in gene-amplified, estrogen-responsive human breast cancer cells (MTX^R MCF-7). Previous studies have shown that estrogen increases, whereas tamoxifen decreases the rate of DHFR enzyme synthesis resulting in corresponding changes in the level of this enzyme. DHFR levels also increase following incubation with methotrexate (MTX), an effect which is dependent on both the concentration of extracellular drug and the duration of exposure and which occurs at concentrations that are insufficient to inhibit cell growth. MTX, like estrogen and tamoxifen, has no apparent effect on the rate of DHFR enzyme degradation. The increase in DHFR in response to MTX is additive with that of estrogen and is not prevented by tamoxifen. Whereas hormone-mediated changes in DHFR are associated with changes in the level of DHFR mRNA, there is no apparent change in DHFR mRNA concentrations in cells exposed to MTX. The regulation of DHFR enzyme levels was also studied in gene-deleted Chinese hamster ovary cells which were transfected with a functional human DHFR minigene constructed from human DHFR genomic and cDNA sequences. Incubation with MTX increases DHFR levels in Chinese hamster ovary cells transfected with the human DHFR minigene but has no effect in cells transfected with a DHFR minigene which uses a viral promotor and polyadenylation signal. Thus, the human DHFR minigene contains sequences other than the protein coding region which effect the regulation of this gene by MTX.

The folic acid analogue, MTX is a potent inhibitor of the enzyme DHFR (EC 1.5.1.3). Since this is the only enzyme capable of regenerating the intracellular pool of reduced folate cofactors, inhibition of DHFR results in the diminished formation of thymidylate, purine nucleotides, and certain amino acids. Although MTX is an effective antineoplastic agent with activity in a wide variety of tumors including breast cancer (1), its clinical usefulness is limited by the development of drug resistance. Several mechanisms associated with reduced sensitivity to MTX have been identified: 1) diminished drug uptake (2–5), 2) induction of an altered DHFR enzyme with a reduced affinity for MTX (6–9), 3) decreased levels of thymidylate synthase (10–13), 4) diminished conversion of MTX to its polyglutamate derivatives (14, 15), and 5) increased levels of its target enzyme, DHFR (16–27). This latter class of cells is of particular interest because of the finding originally reported by Alt *et al.* (21) that DHFR gene sequences are amplified in MTX-resistant cell lines that overproduce DHFR enzyme.

The availability of MTX-resistant cells containing increased levels of DHFR enzyme and amplified DHFR genes has facilitated studies on the regulation of DHFR gene expression. These studies have demonstrated that, although this enzyme provides an essential cell housekeeping function and must be present in all dividing cells, the level of DHFR enzyme is regulated in cells under a variety of conditions (28–44), including: 1) viral infection, 2) serum deprivation, 3) amino acid deprivation, 4) contact inhibition, 5) changes in cyclic AMP levels, and 6) incubation with MTX. Since DHFR enzyme synthesis is cell cycle regulated (45, 46), some of the above conditions may effect DHFR levels by altering the rate of cell growth. However, the regulation of DHFR gene expression is apparently complex and may involve transcriptional (39, 44) as well as post-transcriptional (36, 41, 42) control mechanisms.

Previous studies from our laboratory have examined the regulation of DHFR levels in an estrogen-responsive human breast cancer cell line (MTX^R MCF-7) containing amplified DHFR genes (24, 37). These studies have demonstrated that estradiol induces an increase in the rate of DHFR enzyme synthesis in these MTX^R MCF- 7 cells, resulting in a 1.5–3.0-fold increase in enzyme level. Conversely, treatment of these

ABBREVIATIONS: MTX, methotrexate; DHFR, dihydrofolate reductase; IMEM, improved minimal essential medium; FCS, fetal calf serum; GHT, medium supplemented with glycine, hypoxanthine, and thymidine; CHO, Chinese hamster ovary cells; kb, kilobase.

cells with the antiestrogen tamoxifen lowers the intracellular level of DHFR enzyme, an effect which is mediated through a decrease in the rate of DHFR enzyme synthesis.

In the present study, we have examined the effect of MTX, a known modulator of DHFR levels in some cell lines (29, 38), on the regulation of DHFR in MTX^R MCF-7 cells, as well as the effects of estradiol or tamoxifen on the modulation of DHFR by MTX. Furthermore, in an attempt to define the mechanisms involved in the induction of DHFR by MTX, we have studied the regulation of this enzyme in gene-deleted CHO cells (47) in which DHFR activity was restored following transfection with a human DHFR minigene (48).

Experimental Procedures

Materials. MTX was obtained from the Drug Development Branch, National Cancer Institute (Bethesda, MD). [^{3}H]MTX (20 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). [^{35}S] Methionine was obtained from New England Nuclear (Boston, MA). Dihydrofolate, 2-mercaptoethanol, and 17β-estradiol were obtained from Sigma Chemical Co. (St. Louis, MO). Tamoxifen (1-(4-β-dimethylaminoethoxy-phenyl)-1,2-diphenylbut-1-ene) was provided by ICI Ltd. (Wilmington, DE). MTX and [^{3}H]MTX were further purified by DEAE-cellulose chromatography as described previously (24).

Cells and tissue culture. Conditions for the growth of MTX^R MCF-7 cells in IMEM (National Institutes of Health Media Unit, Bethesda, MD) containing 5% FCS were described earlier (24). Mutant CHO cells (DG 21), which lack DHFR enzyme activity, were generously provided by Dr. L. Chasin and were grown in F12 media (National Institutes of Health Media Unit) containing 5% FCS (47). For studies involving hormonal manipulations, MTX^R MCF-7 cells were twice passaged in medium (IMEM) containing 5% charcoal-stripped calf serum (49). MTX^R MCF-7 cells were then plated in six-well Linbro dishes (100,000 cells/well) or in T75 flasks in IMEM containing 5% charcoal-stripped calf serum. When the cells were 50% confluent, the medium was changed to serum-free IMEM in the presence or absence of either 10 nM estradiol or 2 μM tamoxifen. All incubations in the presence of MTX were performed in IMEM containing 5% FCS supplemented with 10 mg/ml of glycine, 13.6 mg/ml hypoxanthine, and 3.88 mg/ml thymidine (GHT) in order to prevent any MTX toxicity.

DHFR activity. DHFR activity was determined by spectrophotometric assay or by a [^{3}H]MTX binding assay (24, 50). In order to assay DHFR in cells which had been previously incubated with MTX, the intracellular enzyme-bound drug was first removed. This was accomplished by taking advantage of the fact that the binding of MTX to DHFR is markedly reduced under alkaline conditions (38). MTX-treated cells were washed with ice-cold phosphate-buffered saline at the end of the incubation period and harvested by gentle scraping. Following centrifugation at 600 × g for 15 min, the cell pellet was resuspended in 1 ml of 0.05 M Tris-HCl, pH 8.5, containing 45 μM dihydrofolate and 4 mM 2-mercaptoethanol, and sonicated for 10 sec in a Branson sonicator (Danbury, CT) using a microtip at a setting of 4 for 10 sec. Cell debris was removed by centrifugation at 13,000 × g for 30 min at 4° and the resulting supernatant was dialyzed extensively against 0.05 M Tris-HCl, pH 8.5, containing 45 μM dihydrofolate and 4 mM 2-mercaptoethanol across a membrane with a molecular weight exclusion limit of 12,000. The removal of MTX bound to DHFR during the alkaline dialysis was followed by adding [^{3}H]MTX to a control sample. Dialysis was continued until less than 5% of the total radioactivity present at the start of the dialysis remained in the control sample. Under these conditions there was no loss of control DHFR enzyme activity during the dialysis. Units of enzyme activity are expressed as μmol of NADPH consumed/hr or pmol of [^{3}H]MTX bound/mg of protein. Protein concentrations were determined using the Bradford method (Bio-RAD Laboratories, Richmond, CA) (51).

Bound and free intracellular MTX levels. Drug bound to DHFR was separated from free drug in the cytosol by DEAE-Sephadex chromatography as previously described (14).

DHFR enzyme degradation. The rate of DHFR degradation was determined in MTX-treated and control cells as described previously (24). Cells were incubated in methionine-free IMEM containing [^{35}S] methionine (100 μCi/ml) for 24 hr. The medium was then removed, and the cells were washed three times with IMEM and then incubated in the same medium containing unlabeled methionine (15 mg/ml) and 5% FCS in the presence or absence of 200 μM MTX. The amount of radioactivity remaining in DHFR was determined by affinity chromatography (24, 37), following exhaustive alkaline dialysis to remove MTX bound to DHFR. As described above, [^{3}H]MTX was added to control cytosol and then dialyzed in parallel in order to monitor the removal of MTX from enzyme. The dialysis was continued until >95% of the radioactive MTX was removed. The cell cytosol was then dialyzed against 0.05 M Tris-HCl, pH 7.0, prior to affinity chromatography.

DHFR mRNA studies. RNA was isolated from cells using guanidine-isothiocyanate (Fluka, Hauppage, NJ) and CsCl centrifugation (52, 53). DHFR mRNA concentration was analyzed using dot-blot hybridization in which varying amounts of total cellular RNA were bound to nitrocellulose filters (54, 55) and then hybridized to a ^{32}P-labeled probe containing human cDNA sequences (pHD84) (25) provided by G. Attardi. Hybridization and washing conditions were essentially the same as those described by Bresser *et al.* (56). Following the hybridization, the filters were cut and the radioactivity bound to each sample of RNA was analyzed.

DHFR minigene studies. A human DHFR minigene was constructed from cloned human DHFR genomic (48) and cDNA sequences (25) and subcloned into pUC8 plasmid vector (Pharmacia Inc., Piscataway, NJ) using standard techniques. The human DHFR minigene contains all of the DHFR protein coding sequences, plus the entire 3′ nontranslated region of the gene, as well as 1.25 kb of 5′ flanking sequences, 2.5 kb of 3′ flanking sequences, and intron 1 (48). The functional activity of the human DHFR minigene was examined following transfection of minigene DNA into mutant CHO cells (DG 21) which lack DHFR enzyme activity (47) using calcium phosphate-mediated DNA transfer of cloned plasmid DNA (57, 58). Stable transfectants containing DHFR activity were isolated following culture under selective conditions in IMEM containing 5% dialyzed FCS (no hypoxanthine, thymidine, and glycine). DG 21 cells were also transfected with another functional minigene construct, pMTVdhfr, which was generously provided by G. Ringold (59). pMTVdhfr contains mouse DHFR cDNA sequences, a viral promotor (MMTV), and an intervening sequence and polyadenylation site derived from SV40. Analyses of DHFR enzyme, mRNA, and minigene DNA in stably transfected cells were described previously (48).

Results

MTX induction of DHFR in MTX^R MCF-7 cells. The effect of MTX on the regulation of its target enzyme in human breast cancer cells is shown in Fig. 1. In this experiment cells were incubated in the presence of 200 μM MTX in medium containing GHT, and DHFR activity was measured following alkaline dialysis to remove bound MTX as described in Experimental Procedures. As shown in Fig. 1, the specific activity of DHFR in these cells increased linearly with time beginning within 2 hr after the addition of MTX. Following 24 hr of drug exposure the level of DHFR increased over 2-fold above the control value (24.4 versus 11.8 units/mg), and by 44 hr there was a greater than 3-fold increase in the level of DHFR. This does not simply reflect an increase in the number of cells with time but, instead, respresents an increase in the actual specific activity of this enzyme relative to other cellular proteins during incubations in the presence of MTX. The level of enzyme does

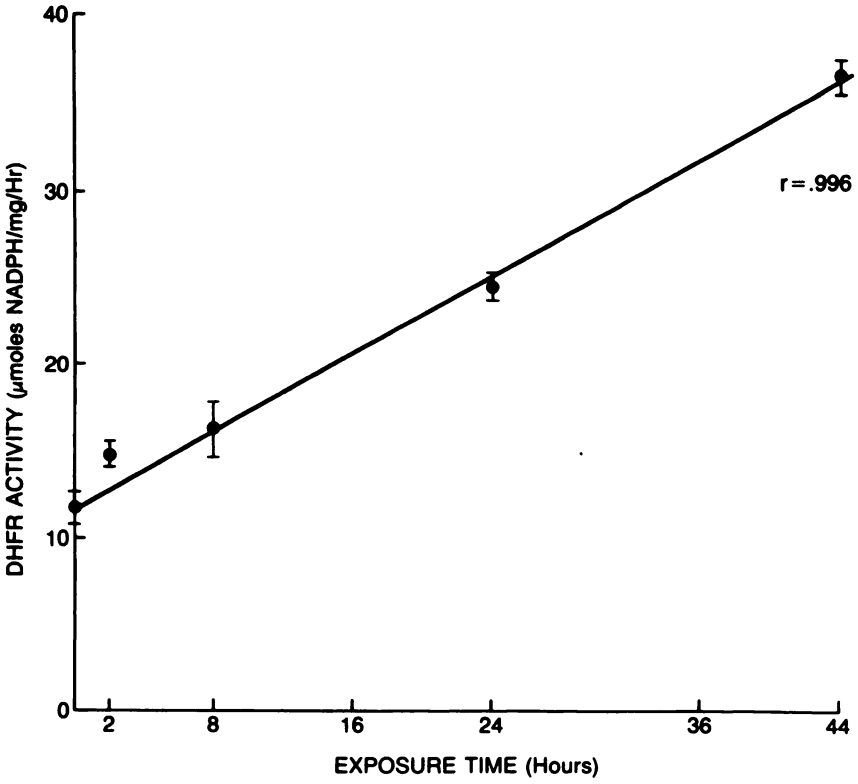

Fig. 1. Time-dependent effect of MTX on DHFR levels. Cells were incubated at 37° in the presence or absence of 200 μM MTX in IMEM containing 5% FCS and GHT. At various times, cells were harvested and the level of DHFR was measured following extensive alkaline dialysis to remove bound MTX as described in Experimental Procedures. Results represent the mean ± standard deviation of triplicate determinations.

not change significantly in untreated cells during log-phase growth.

As shown in Table 1, the effect of MTX on the level of DHFR enzyme is dose dependent and occurs at extracellular drug concentrations as low as 0.2 μM. Since the IC_{50} of this DHFR gene-amplified cell line for MTX is in excess of 100 μM (24), the increase in DHFR occurs at extracellular MTX concentrations which have no effect on the growth rate of these cells. Moreover, during these incubations the media were supplemented with hypxanthine, thymidine, and glycine in order to avoid the growth-inhibitory effect of MTX. At each increase in the extracellular concentration of MTX there was a corresponding increase in the level of DHFR. Incubation in the presence of 200 μM MTX for 24 hr resulted in approximately a 4-fold increase in the specific activity of DHFR (32.97 versus 8.29 units/mg). Thus, the increase in DHFR enzyme in MCF-7 cells exposed to MTX is both time and dose dependent.

Bound and free drug levels in MTX-treated cells. Previous studies have demonstrated that MTX cytotoxicity is dependent upon the complete saturation of DHFR-binding sites by MTX and the accumulation of excess free intracellular drug (60, 61). Since the transport of MTX into cells is relatively slow, the increase in DHFR by MTX could effectively limit the ability to achieve intracellular drug levels which are in excess of the binding sites on DHFR. In order to examine the effect of MTX on the ability to achieve enzyme-saturating levels of drug intracellularly, cells were incubated in the presence of 200 μM [^{3}H]MTX and the intracellular drug was separated into its enzyme-bound and free forms as described previously (14). Following 2 hr exposure to 200 μM MTX, only 4.2% of the total intracellular MTX was present in the form of free drug and the rest was enzyme bound (Fig. 2). After 24 hr of drug exposure the total intracellular drug concentration increased approximately 3-fold (655 nmol/g). However, this increase was accounted for almost entirely by an increase in the amount of enzyme-bound drug, as only 2.7% of the total intracellular MTX present at this time was present as free drug. When the cells were incubated in MTX for 48 hr the intracellular MTX level increased still further to 862.9 nmol/g. At this time the proportion of the drug present as free drug increased to 11.3% of the total intracellular drug.

TABLE 1

Dose-dependent increase in DHFR activity in MTX^R MCF-7 cells during 24-hr incubations with MTX

MTX^R MCF-7 cells were plated in six-well Linbro dishes as described in Experimental Procedures. When the cells had reached 50% confluency, various concentrations of MTX were added. After 24 hr of incubation at 37°, the cells were harvested, cytosol was prepared and dialyzed to remove enzyme-bound drug, and DHFR was assayed as described in Experimental Procedures. The results represent the mean ± standard deviation of triplicate samples.

MTX	DHFR activity
μM	units/mg
None	8.29 ± 0.76
0.2	10.75 ± 0.56
2	12.82 ± 0.26
20	20.75 ± 1.87
200	32.97 ± 1.03

Influence of hormonal manipulation on MTX induction of DHFR. Although these cells are resistant to MTX (24), they have retained the same apparent sensitivity to hormones that has been observed in the MCF-7 human breast cancer cell line from which they were derived. Previous studies have shown that incubation of this drug-resistant subline with estradiol results in an increase in the rate of DHFR enzyme synthesis and a 1.5–3-fold increase in the specific activity of DHFR approximately 24 hr after the addition of hormone (24). Converseley, treatment of these cells with the antiestrogen

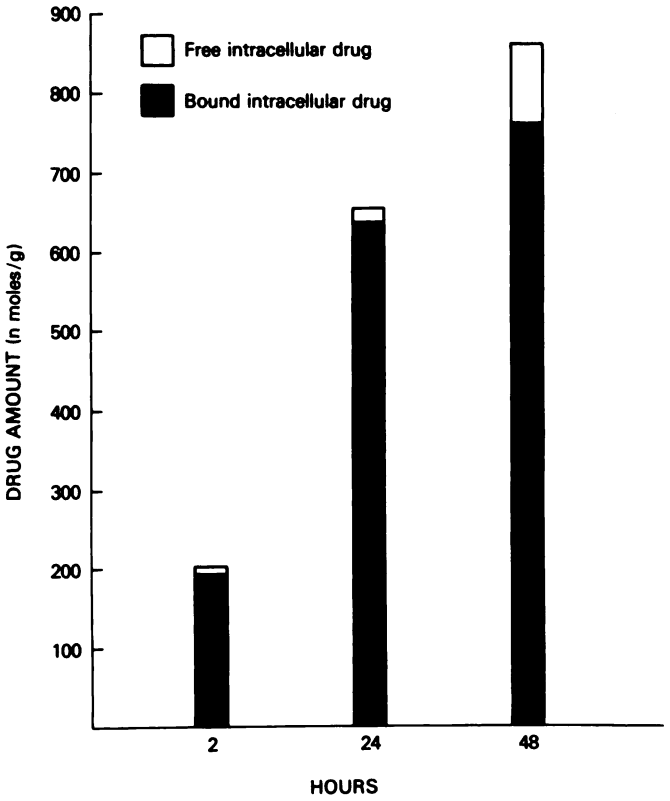

Fig. 2. Intracellular bound and free MTX levels. Cells were treated with 200 μM [^{3}H]MTX for various periods of time at 37°. At the appropriate time, cells were washed three times with ice-cold phosphate-buffered saline and harvested by scraping. The total amount of intracellular [^{3}H] MTX, the amount of enzyme-bound, and the amount of free drug were determined as described in Experimental Procedures.

TABLE 2

Influence of hormone pretreatment on MTX induction of DHFR

Cells were plated in six-well Linbro dishes in IMEM containing 5% charcoal-stripped serum. At 50% confluency, the medium was replaced with serum-free IMEM and the cells were incubated at 37° for 24 hr in the presence or absence of either 10 nM estradiol or 2 μM tamoxifen. At the end of the preincubation period, MTX (200 μM) was added to half of the cells and the incubation continued for an additional 8 hr. DHFR activity was determined in cell cytosol as described in Experimental Procedures. Results represent the mean ± standard deviation of triplicate samples.

24-hr Pretreatment	MTX	DHFR activity (U/mg)
	μM	units/mg
None	None	9.89 ± 0.49
None	200	14.21 ± 0.67
Estradiol	None	17.10 ± 1.17
Estradiol	200	29.96 ± 3.38
Tamoxifen	None	4.96 ± 0.56
Tamoxifen	200	6.85 ± 0.45

tamoxifen results in a decrease in the rate of DHFR enzyme synthesis and reduced cellular levels of this enzyme (37).

The effects of estrogen or tamoxifen on the increase in DHFR produced in response to MTX DHFR were examined by pretreating cells for 24 hr in the presence or absence of either 10 nM estradiol or 2 μM tamoxifen followed by exposure to 200 μM MTX for an additional 8 hr. As shown in Table 2, exposure of these cells to MTX alone results in a 1.5-fold increase in DHFR levels (9.89–14.21 units/mg), whereas treatment with estradiol alone resulted in almost a 2-fold increase in enzyme activity (9.9 versus 17.1 units/mg). Exposure of estrogen-treated cells to MTX for 8 hr resulted in an additional 1.6-fold increase in DHFR activity, so that the total increase in DHFR was more than 3-fold higher than in untreated cells (29.66 versus 9.89 units/mg). In contrast, treatment of MCF-7 cells with tamoxifen for 24 hr resulted in approximately a 50% decrease in the intracellular level of DHFR (9.89 versus 4.96 units/mg). Although subsequent exposure of tamoxifen-treated cells to MTX resulted in a 38% increase in DHFR levels above that present in cells treated with tamoxifen alone (4.96 versus 6.85 units/mg), the final enzyme level was still lower than that present in control cells (6.85 versus 9.89 units/mg). Thus, the increase in DHFR by MTX is apparently additive with that of estrogen and is not prevented by tamoxifen.

DHFR enzyme stability. The stability of DHFR enzyme was compared in MTX-treated and untreated cells. Following incubation of cells in the presence of [^{35}S]methionine for 24 hr at 37°, the radiolabel was removed from the medium and the cells were incubated in the presence or absence of MTX. At various periods of time the amount of radioactivity remaining in DHFR was measured by affinity chromatography (24). As shown in Fig. 3, under control conditions (no MTX), less than 50% of the radioactively labeled DHFR enzyme is lost during the subsequent 24-hr period of incubation. This is consistent with previous studies which demonstrated that the rate of enzyme degradation in these cells is slow (24). Furthermore, exposure to MTX during the incubation period had no apparent effect on the rate of DHFR degradation.

DHFR mRNA levels in MTX^R MCF-7 cells. We also examined the effects of hormonal manipulations and MTX exposure on the level of DHFR mRNA in these cells. Following the incubation of these cells in the presence or absence of either 10 nM estradiol or 2 μM tamoxifen for 24 hr, the relative concentration of DHFR mRNA was determined by dot blot hybridization. As shown in Fig. 4, the relative concentration of DHFR mRNA is approximately 30% higher in estradiol-treated cells relative to that present in control cells. Moreover, in cells treated with tamoxifen, the concentration of DHFR mRNA is reduced approximately 40% relative to the concentration in untreated cells. Overall there is a greater than 2-fold difference in the concentration of DHFR mRNA in tamoxifen-treated cells compared to cells exposed to estradiol. The results were essentially identical when poly A$^+$ RNA from control and hormone-treated cells were compared by dot blot analysis. Thus, the hormone-induced changes in DHFR enzyme are, at

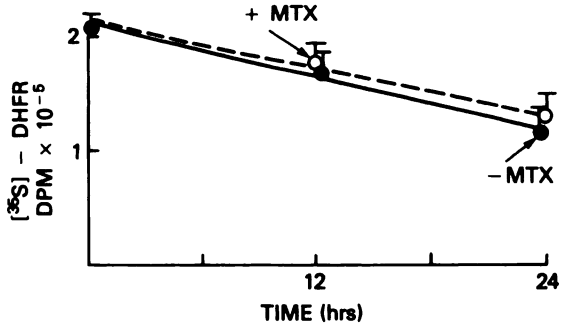

Fig. 3. DHFR stability in MTX-treated cells. Cells were incubated in [^{35}S] methionine (100 μCi/ml) for 24 hr. The medium was then removed and the cells were incubated in the presence of excess unlabeled methionine in the presence or absence of 200 μM MTX. The amount of radioactivity remaining in DHFR was determined as described in Experimental Procedures. Each *point* represents the mean ± standard deviation of triplicate samples.

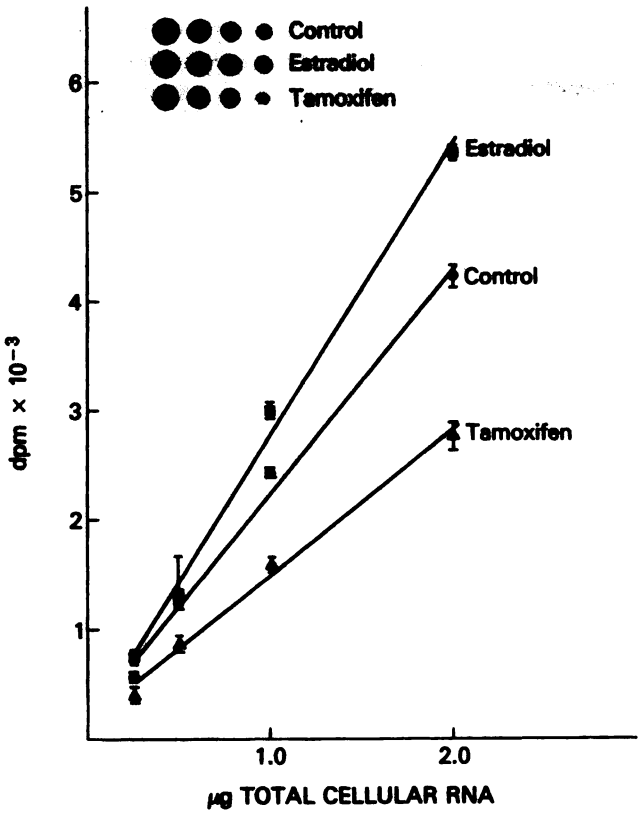

Fig. 4. DHFR mRNA levels in hormone-treated cells. Cells were grown in medium containing 5% charcoal-stripped calf serum for 2 weeks. When 50% confluent, cells were incubated in serum-free media containing either 10 nM estradiol, 2 μM tamoxifen, or the equivalent amount of carrier (ethanol), and RNA was isolated 24 hr later. Nitrocellulose filters containing varying amounts of total cellular RNA were hybridized with radiolabeled probe containing human DHFR cDNA sequences as described in the Experimental Procedures. The results are presented as the amount of radioactivity bound per μg of RNA. Each *point* represents the mean of duplicate filters.

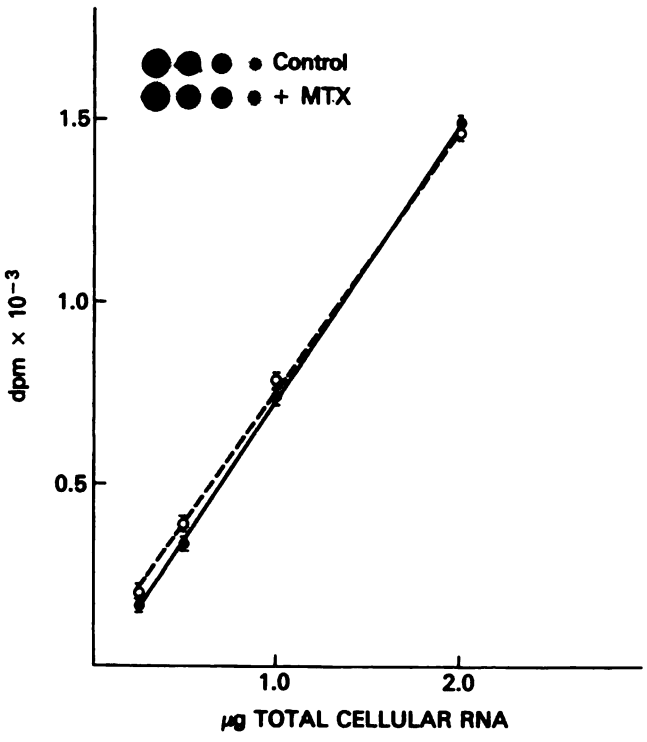

Fig. 5. DHFR mRNA levels in MTX-treated cells. Cells were incubated for 40 hr in IMEM containing 5% FCS and GHT in the presence or absence of 200 μM MTX. Cellular RNA was isolated and the amount of DHFR mRNA was determined as described in the legend of Fig. 4. Each *point* represents the mean of duplicate samples.

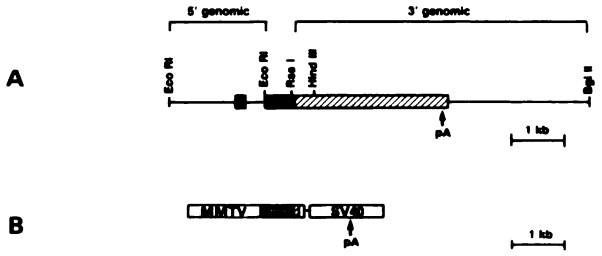

Fig. 6. DHFR minigenes. Depicted are schematic representations of (A) human DHFR minigene (Hdhfr) (48) and (B) pMTVdhfr (59). Coding sequences are represented by *black boxes*, 3′ untranslated regions by *hatched* or *clear boxes*, and flanking regions by *thin lines*. The portions of the 5′ and 3′ genomic DNA of the human DHFR gene which were used in the construction of the human minigene are outlined above the diagram. The locations of intervening sequences and the splice sites are also denoted.

least in part, mediated through changes in the level of DHFR mRNA. Whether these changes involve transcriptional or post-transcriptional control mechanisms is not known at present.

The analysis of DHFR mRNA in cells incubated with 200 μM MTX for 40 hr is shown in Fig. 5. Although in this experiment the level of DHFR enzyme increased more than 3-fold relative to that present in control (untreated) cells (395 versus 126 pmol/mg), there was no apparent change in the concentration of DHFR mRNA in the drug-treated cells compared to the control cells. In three other experiments in which cells were exposed to even 1000 μM MTX for 40 hr in the presence of GHT, the mean concentration of DHFR mRNA in the drug-treated cells was only slightly increased relative to that present in untreated cells (1.2 ± 0.22). Thus, in contrast to the hormone-induced changes in DHFR, the induction of enzyme which results during incubation in the presence of MTX is not associated with any significant change in the overall level of DHFR mRNA.

Regulation of DHFR in minigene-transfected CHO cells. We have isolated human DHFR genomic DNA sequences from these MTX^R MCF-7 cells and formed a functional human DHFR minigene using both genomic and cDNA sequences (see Fig. 6) (48) which is capable of restoring DHFR enzyme activity in gene-deleted CHO cells (47) following transfection. In other studies we have shown that this minigene is regulated in a fashion similar to that of the endogenous gene, as the level of DHFR in minigene-transfected CHO cells is inducible under two conditions which regulate DHFR levels in cells: release from serum starvation (48) as well as during release from amino acid deprivation.[1]

We have used this cellular expression system to further evaluate the mechanisms involved in the regulation of DHFR levels following exposure to MTX. Minigene-transfected CHO cells were incubated in the presence or absence of MTX for 24 hr and the level of DHFR was analyzed. In three independent

[1] M. E. Goldsmith and K. H. Cowan, unpublished observations.

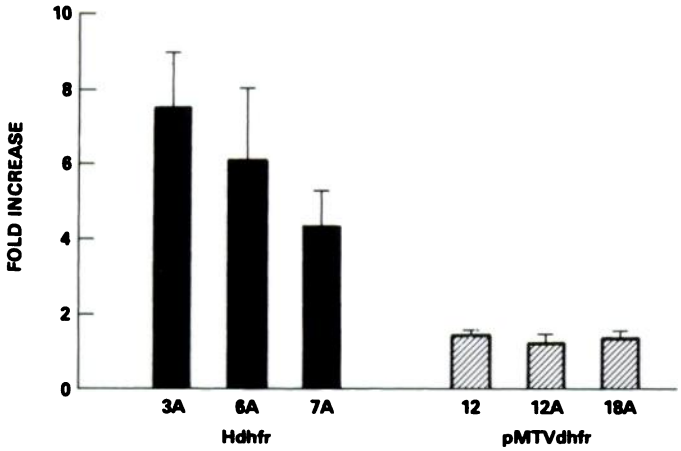

Fig. 7. DHFR induction by MTX in minigene-transfected CHO cells. Individual clones of DG 21 CHO cells which had been stably transfected with either the human DHFR minigene (Hdhfr) or pMTVdhfr (59) were incubated in IMEM containing 5% FCS and 2 mg/liter of proline and GHT. Following 24 hr incubation in the presence or absence of 2 μM MTX, cells were harvested and the level of DHFR was measured as described in Experimental Procedures. The *numbers* beneath the bars represent the individual clone of DG 21 cells transfected with the different minigenes. The *A* after the clone number indicates that the clones had been subsequently selected in medium containing gradually increasing amounts of MTX (to 2μM) in order to increase the gene copy number and increase the basal level of DHFR enzyme. Each clone was analyzed in four or five separate experiments exept pMTVdhfr 12 (n = 3) and 12A (n = 2). Each sample was analyzed in duplicate. The *bars* represent the -fold increase in DHFR in cells treated with MTX + standard deviation.

clones of CHO cells stably transfected with the human DHFR minigene there was a marked increase in DHFR levels (4.3–7.5-fold) following exposure to MTX (Fig. 7). For comparison, we also examined the effects of MTX on DHFR levels in mutant CHO cells which had been stably transfected with another DHFR minigene, pMTVDHFR (59). This latter construct (see Fig. 6) was formed using mouse DHFR cDNA sequences, a viral promotor (MMTV), and an intervening sequence and a polyadenylation site from SV40 virus. In contrast to the marked increase in DHFR enzyme observed in CHO cells transfected with the human DHFR minigene, there is little change in the enzyme in cells transfected with pMTVdhfr (1.2–1.4-fold increase). Thus, differences exist between CHO cells transfected with the human DHFR minigene and cells transfected with pMTVdhfr with respect to the regulation of DHFR following exposure to MTX.

Discussion

As discussed earlier, numerous conditions have been identified which result in the intracellular modulation of DHFR levels (28–47). However, the mechanisms involved in the regulation of DHFR gene expression are incompletely defined. In this report we have demonstrated that the hormone-mediated changes in DHFR in this estrogen-sensitive breast cancer cell line are regulated at least in part at the level of DHFR mRNA. Since these hormonal manipulations alter the rate of growth of this breast cancer cell line (estrogen increasing and tamoxifen decreasing), the accompanying changes in DHFR following hormonal manipulations most likely reflect the cell cycle regulation of DHFR gene expression (49, 50). Since previous studies have indicated that the regulation of DHFR may involve transcriptional (39, 44) as well as post-transcriptional (36, 41, 42) control mechanisms, it will be of interest to determine the level at which the hormone-mediated changes in DHFR are regulated in MCF-7 cells.

Studies presented in this report show that treatment of MCF-7 cells with MTX increases the level of DHFR enzyme. Although previous studies have suggested that the increase in DHFR by MTX may be due to enzyme stabilization (62), we find no evidence that MTX alters the rate of DHFR enzyme degradation in MCF-7 cells. The studies presented in this report used an affinity column technique to quantitate the rate of prelabeled DHFR enzyme degradation. If MTX remained bound to DHFR enzyme, the enzyme would not bind to the affinity matrix. This would result in underestimating the effect of the drug on the stability of the enzyme. For this reason, control samples were always incubated with radiolabeled MTX and dialyzed in parallel under alkaline conditions. The dialysis was continued until >95% of the radioactive MTX was removed from the control samples. Similar conclusions were reached by *Domin et al.* (26) in studies on KB cells using immunoprecipitation with anti-DHFR antibodies to measure the stability of DHFR enzyme during incubations with MTX. Thus, using two independent methods, no effect on enzyme stability by MTX could be found to account for the increase in enzyme levels produced during incubation with this drug.

Although both estrogen and MTX increase DHFR levels in MCF-7 cells, there are important differences between the effects of these two modulators of DHFR. First, whereas the induction of DHFR by MTX is rapid (within 2 hr) and linear over a prolonged period of time (>40 hr), the hormone-induced changes are somewhat delayed, with no changes observed during the first 6 hr of incubation and the maximal effect on DHFR induction occurring approximately 24 hr after the addition of estrogen (24). Second, whereas treatment with estrogen or tamoxifen alters the growth rate of these cells, the induction of DHFR by MTX is observed at concentrations of drug which are insufficient to saturate the enzyme and which have no effect on the growth rate of this cell line. Moreover, it should be noted that all of the experiments involving MTX incubations were performed in medium which was supplemented with hypoxanthine, thymidine, and glycine at concentrations that completely eliminate MTX toxicity in this cell line. Thus, the induction of DHFR by MTX is not related to the growth-dependent regulation of this enzyme.

In addition, marked differences exist between the effects of hormones and MTX on the level of DHFR mRNA. Whereas incubation of MCF-7 cells with estradiol results in a similar increase in both DHFR mRNA and DHFR enzyme, the induction of DHFR enzyme by MTX is associated with little or no change in the relative concentration of DHFR mRNA in these cells. This suggests that the drug-induced change in DHFR enzyme involves other mechanisms, perhaps at the level of translation. Bastow *et al.* (63) reported increases in DHFR enzyme as well as mRNA in KB cells treated with MTX. However, even in their study, the increase in mRNA was less than that of DHFR enzyme, suggesting that multiple levels of regulation exist.

We have also examined the induction of DHFR in mutant CHO cells that have been transfected with two different DHFR minigene constructs. In each of the clones of CHO cells transfected with the human DHFR minigene, there was a marked induction of enzyme levels following the addition of MTX.

Although the magnitude of the response varied somewhat among the different clones, the level of induction is similar to that observed in MCF-7 cells. In contrast, there is little change in DHFR levels in CHO cells that were transfected with another DHFR minigene (pMTVdhfr). Both the human DHFR minigene (Hdhfr) and pMTVdhfr are capable of converting the mutant CHO cells to a DHFR$^+$ phenotype. However, whereas the minigene Hdhfr is formed entirely from human DHFR DNA sequences (48), pMTVdhfr was formed using viral (MMTV and SV40) regulatory sequences (59). Although minor differences exist between the coding sequences of the human DHFR gene and the mouse cDNA sequences, these would not be expected to result in the observed differences in regulation. Thus, the human DHFR minigene construct apparently contains sequences that are not present in pMTVdhfr, which affect the regulation of gene expression during exposure to MTX. Analysis of various deletion mutants of the human DHFR minigene may provide additional insights into the actual mechanisms involved in the regulation of DHFR gene expression as well as the sequences responsible for these effects.

The studies presented in this report demonstrate that there are important pharmacological interactions between hormonal agents and cytotoxic drugs in estrogen-sensitive breast cancer cells which may effect drug sensitivity. Understanding the interactions of hormonal therapies and cytotoxic drugs at the molecular level may help to increase the effectiveness of these two treatment modalities when used in combination for the treatment of breast cancer.

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