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Thymidylate Synthase Overproduction and Gene Amplification in Fluorodeoxyuridine-Resistant Human Cells

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SUMMARY

Cytotoxicity to 5-fluoro-2′-deoxyuridine (FdUrd) derives from its conversion to 5-fluorodeoxyuridine-5′-monophosphate, which binds to and inhibits thymidylate synthase (TS) in the presence of the cofactor, 5,10-methylenetetrahydrofolate. We have selected FdUrd-resistant variants of the human cell line HEp-2 following adaptation to stepwise increases in drug concentration. In the initial selection, maximal drug resistance was associated with a 26-fold increase in the cellular level of TS. Greater TS overproduction (80-fold) was obtained by selection for FdUrd resistance in the presence of 10 μM folinic acid and 100 μM deoxyinosine. The latter agents were included to expand the folate pool to ensure adequate levels of cofactor during the selection process. Using cDNA plasmid pMTS-4, which is complementary to mouse TS mRNA, we show that TS overproduction in the HEp-2 variants is accompanied by a 100-fold increase in TS mRNA and a 100-fold amplification of the TS structural gene. Thus, TS overproduction and gene amplification is a mechanism of resistance to FdUrd in human cells.

INTRODUCTION

Fluoropyrimidine antimetabolites, particularly FUra[1] and FdUrd, are cytotoxic in diverse biological systems and have been extensively used in the clinical treatment of carcinomas of the ovary, breast, and gastrointestinal tract (1). One primary mode of action of these drugs is through their conversion to FdUMP, which is a tight-binding inhibitor of TS (EC 2.1.1.45) in the presence of the cofactor, 5,10-MTHF) (2). Since TS provides the sole *de novo* source of thymidylate, which is crucial for DNA biosynthesis, the enzyme has been a target of cancer chemotherapy and the subject of extensive investigation.

Resistance to antimetabolites is a major barrier to their long term use in the control of neoplastic disease. For example, only 15–20% of patients bearing tumors of the colon and rectum experience an objective response to FUra therapy (3, 4); the responding patients eventually develop resistant tumors that are refractory to further treatment. Clearly, understanding the biochemical and molecular mechanisms of antimetabolite resistance both in model systems and in the clinic will be critical in assessing and improving chemotherapeutic protocols.

Studies on mammalian cells that have been selected for resistance to cytotoxic drugs in culture have provided insight into the variety of genetic alterations leading to a drug-resistant phenotype. Altered drug transport (5) and metabolism (6, 7), decreased affinity between target enzyme and drug (8, 9), and overproduction of critical gene products (10–15) have all been identified as mechanisms leading to drug resistance. The latter mechanism has received considerable attention, for it has been shown that resistance to a number of drugs results from increased levels of target enzymes; the high enzyme levels are generated by specific mRNA overproduction which, in most cases, is a consequence of amplification of the appropriate structural gene (reviewed in Ref. 12). This mechanism may play a role in the development of drug-resistant tumors during clinical therapy (16, 17).

Fluoropyrimidine-resistant mutants have been isolated in several laboratories and have enabled identification of several mechanisms associated with the resistance. These include altered metabolism (6, 7), decreased binding of FdUMP to TS (8), and increased levels of TS (10, 11). Recently it was shown that the overproduction of TS in FdUrd-resistant mouse fibroblasts is accompanied by TS gene amplification (11a). In this paper we have selected FdUrd-resistant populations of the human cell line HEp-2, and we show that the resistance is generated by TS overproduction resulting from TS gene

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[1] Abbreviations used are: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluorodeoxyuridine-5′-monophosphate; TS, thymidylate synthase; CF, folinic acid; dIno, 2′-deoxyinosine; 5,10-MTHF, N^5N^{10}-methylenetetrahydrofolate.

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amplification. This is the first example of TS overproduction and gene amplification in human cells.

MATERIALS AND METHODS

Materials. [6-^{3}H]FdUMP (20 Ci/mmol) was purchased from Moravek Biochemicals (City of Industry, CA). FdUrd, deoxyinosine, folic acid, CF, ammonium bicarbonate, and acid-washed activated charcoal were purchased from Sigma Chemical Company (St. Louis, MO). Horse serum and powdered RPMI 1640 medium were obtained from Grand Island Biological Company (Grand Island, NY). Dextran T-70 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). (±)-5,10-MTHF was prepared from folic acid and purified by DEAE-cellulose chromatography by the method of Zakrzewski and Sansone (18).

Cells and culture conditions. Human laryngeal carcinoma HEp-2 cells were maintained as monolayers in RPMI 1640 medium containing 5% horse serum. In this medium, FdUrd-resistant cells were selected by adaptation to drug concentrations of 0.03, 0.1, 0.3, and 1 μM. At each step a vigorously growing monolayer was obtained prior to addition of a higher concentration of drug; usually 2–4 weeks were required between steps. In medium containing dIno and CF (see text), cells were successively adapted to FdUrd concentrations of 1, 3, 6, 12, 24, 50, 100, 200, and 500 nM. It should be emphasized that the sublines analyzed in the present studies have not been cloned. Low cloning efficiencies have thus far precluded isolation of single colonies of HEp-2 or its drug-resistant derivatives.

TS level determination. Following removal of growth medium, cells in confluent monolayers were rinsed three times with cold PBS (10 mM sodium phosphate, 153 mM sodium chloride, pH 7.5). The cells were collected as described previously and frozen at −70° (2). The methods of Spears *et al.* (19) were utilized to prepare cell extracts and to dissociate intracellular FdUMP from TS in the extracts. After dissociation, the extracts were dialyzed against the dissociation buffer at 4° for 16 hr. TS levels were determined by titrating the enzyme with [6-^{3}H]FdUMP in the presence of excess 5,10-MTHF, as described previously (20). Samples were counted in 10 ml of Liquiscint-2 scintillation solution (National Diagnostics, Inc., Somerville, NJ) using a liquid scintillation spectrometer.

Isolation and analysis of nucleic acids. Cells in confluent monolayers were washed three times with cold PBS and collected as described previously (21). DNA was isolated from the cells by the method of Pellicer *et al.* (22). RNA was isolated by the method of Cox (23) as described by Labarca and Paigen (24).

In vitro translation (25) was in the presence of L-[^{35}S]methionine using nuclease-treated lysate from rabbit reticulocytes (Bethesda Research Laboratories, Rockville, MD). Following incubation, the mixture of labeled polypeptides (10 μl) was fractionated on two-dimensional polyacrylamide gels (26) and observed by fluorography (27). Antibody-reactive polypeptides were isolated using Pansorbin A (Calbiochem-Behring Corp., San Diego, CA) following incubation of 5 μl of translation mixture with rabbit antibody to human TS, as described elsewhere (28). The rabbit antibody was made in response to a partially purified preparation of TS and was supplied by Dr. Y.-C. Cheng.

For Northern blotting (29), 15 μg of total RNA were denatured, fractionated on formaldehyde-containing 1.5% agarose gels, blotted onto nitrocellulose, and hybridized to nick-translated ^{32}P-labeled pMTS-4, which contains cDNA complementary to mouse TS RNA (30). Dot blot analysis was performed according to the method of Thomas (31): dilutions were made in water.

Southern analyses (32) were done by digesting 10- to 15-μg samples of genomic DNA with the appropriate restriction nucleases, fractionating the DNA fragments on 0.8% agarose gels and transferring them to nitrocellulose, and hybridizing to the ^{32}P-labeled probe. DNA transfer was quantitative as assessed by ethidium bromide staining of gels following the transfer.

RESULTS

Selection of FdUrd-resistant HEp-2 cells. Initially, selection for FdUrd-resistant HEp-2 cells was performed in medium (see Materials and Methods) in which the ID_{50} for FdUrd is 40 nM (33). Cells were adapted in a stepwise fashion to FdUrd concentrations ranging from 0.03 to 1 μM; the final cell population, termed HEp-2/1, grew very slowly and proved somewhat difficult to maintain. We were unable to obtain viable cells in 3 μM FdUrd even after prolonged incubation with this concentration of drug. TS levels were assayed by [^{3}H]FdUMP binding (20). In this method, the labeled nucleotide binds covalently to TS in the presence of folate cofactor, thereby enabling titration of enzyme concentration. HEp-2/1 extracts were assayed for TS and values were compared to those in parental HEp-2 extracts. As shown in Table 1, HEp-2/1 contained 26-fold higher FdUMP-binding activity relative to HEp-2. Since the [^{3}H]FdUMP binds TS covalently, the labeled TS complex can be observed after gel electrophoresis in the presence of sodium dodecyl sulfate. The results showed that all of the bound [^{3}H]FdUMP in the assay was associated with a single species of molecular weight 37,000 (data not shown), which is identical to the known molecular weight of purified human TS (34); thus, increased [^{3}H]FdUMP binding activity in HEp-2/1 extracts is due to TS and not to some other FdUMP-binding species.

In considering the reasons for our inability to select cells resistant to FdUrd levels greater than 1 μM, we took note of the following two observations. First, the levels of intracellular folate(s) which stimulate(s) ternary complex formation between FdUMP and TS is in 25-fold molar excess relative to TS in HEp-2 cells (35); in HEp-2/1, therefore, TS and these folates are approximately stoichiometric. Since the folate cofactor 5,10-MTHF is required for the catalytic activity of TS, increases in TS

TABLE 1

TS levels in FdUrd-resistant HEp-2 cells

Cells were adapted to and maintained in the indicated concentrations of FdUrd and other additions. Cells were collected, extracts were prepared, and TS was assayed as described in Materials and Methods. Values for TS concentration are the mean ± standard error, with the number of independent measurements indicated in parentheses.

Additions during selection	Concentration of FdUrd	TS concentration	Fold change
	μM	*pmol TS/mg protein*	
None	0	3.1 (1)	1.0
	0.03		
	0.1		
	0.3		
	1.0	79.2 (1)	26
Deoxyinosine (100 μM) and folinic acid (10 μM)	0	3.9 ± 0.42 (16)	1.0
	0.001	4.1 ± 0.50 (2)	1.1
	0.003	4.4 ± 0.80 (2)	1.1
	0.006	44.4 ± 5.0 (4)	11
	0.012	58.2 ± 3.4 (4)	16
	0.024	62.0 (1)	16
	0.050	78.0 ± 13 (4)	20
	0.100	108 ± 10 (4)	28
	0.200	166 ± 13 (2)	43
	0.500	297 ± 19 (3)	76

production above 25-fold would result in cofactor deficiency and would leave an enzymatically inactive enzyme, which would be of no survival value to the cell. Second, we observed that addition of CF to the culture medium in the continued presence of FdUrd rapidly killed HEp-2/1 cells. CF is a reduced folate which has been shown to increase the intracellular level of folate(s) by 4-fold in HEp-2 cells (35). Several studies have shown that CF renders cells more sensitive to FUra and FdUrd; this is a consequence of effects of higher folate levels on the equilibrium between free and FdUMP-bound TS (2, 20, 36) and indicates that folate pools are important determinants of the selection process.

As a consequence of folate pool expansion, we expected to achieve higher TS levels in FdUrd-resistant cells selected in the presence of CF. In addition, we chose to include dIno. dIno is metabolized to deoxyribose-1-phosphate and hypoxanthine. The former, by shifting the equilibrium of thymidine phosphorylase toward nucleoside synthesis, prevents any accumulation of FUra from FdUrd; in effect, the dIno maintains high FdUrd levels in the cells which, in turn, increases FdUMP levels to maximize cytotoxicity. Moreover, hypoxanthine, being a source of purines, decreases folate utilization in the *de novo* purine biosynthetic pathway to further increase cofactor availability to TS.

Using medium supplemented with CF and dIno, we adapted cells to stepwise increases in the level of FdUrd. Since FdUrd toxicity is augmented in this medium (see above), we carried out the selection in lower drug concentrations, which ranged from 1 nM to 500 nM (see Materials and Methods for details). Extracts from cells withdrawn at various steps during selection were assayed and found to have increasing concentrations of TS (Table 1); a maximal increase of about 80-fold was found in cells from the HEp-2/500 population, which was resistant to 500 nM FdUrd. We were subsequently able to select cells that were resistant to 1 μM FdUrd and that contained a 200-fold increase in the TS level. These cells grew very poorly, probably as a result of cofactor deficiency as described above. We therefore chose to use HEp-2/500 as the population displaying maximum TS overproduction.

Previous workers have isolated cells that are FdUrd resistant as a consequence of a deficiency in thymidine kinase, the enzyme required for the conversion of FdUrd to FdUMP (6, 7). We have assayed thymidine kinase in several extracts of FdUrd-resistant HEp-2 cells and find equal activities in all (data not shown).

Stability of the high TS phenotype in the absence of selection was tested by culturing the HEp-2/500 cells in FdUrd-free medium. As shown in Fig. 1, after about 2 weeks in nonselective medium, TS levels began to decrease with a half-time of 18 days; this phenotypic reversion continued for at least 2 months. Thus, the high TS phenotype is unstable when cells are grown in the absence of inhibitor.

Electrophoretic comparison of TS in HEp-2 and HEp-2/500. To compare the TS produced by normal and FdUrd-resistant cells, we subjected [^{3}H]FdUMP-TS complexes to gel electrophoresis in the presence of sodium dodecyl sulfate. All of the radioactivity migrated as a single species of molecular weight 37,000 (data not shown). Comparisons at higher resolution were made by two-dimensional acrylamide gel electrophoresis. In both HEp-2 and HEp-2/500, [^{3}H]FdUMP-TS complexes migrated as a series of 37,000-dalton proteins showing charge heterogeneity in the pI range of about 6.0–6.3 (shown for HEp-2/500 in Fig. 2). The reason for the TS charge heterogeneity is not known. Since TS produced by *in vitro* mRNA translation is nonheterogeneous (see below), the TS charge forms must be generated post-translationally.

Overproduction of TS mRNA in HEp-2/500. We used

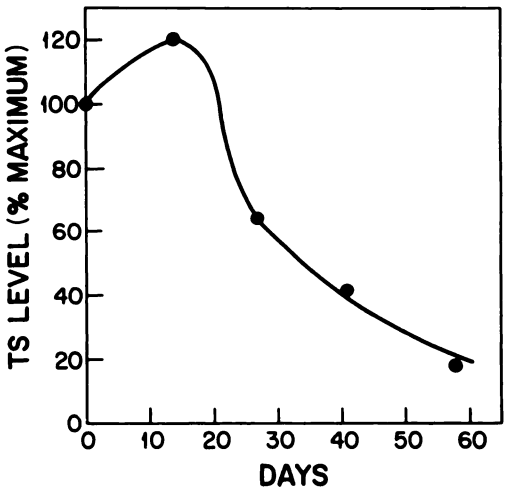

FIG. 1. *Stability of TS phenotype in HEp-2/500 cells*
HEp-2/500 cells were grown in FdUrd-free medium containing dIno and CF; at various times, extracts were prepared and assayed for TS. The TS level (percentage of that in HEp-2/500) is plotted as a function of time in drug-free medium.

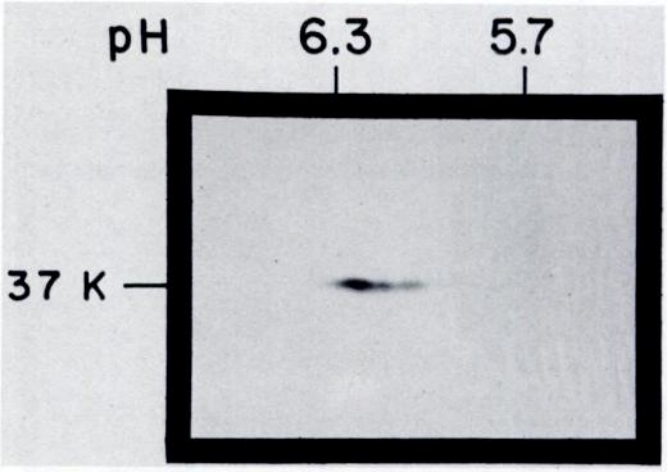

FIG. 2. *Two-dimensional gel electrophoresis of the [^{3}H]FdUMP-TS complex from HEp-2 cells*
Extracts of HEp-2 and its FdUrd-resistant derivatives were incubated in the presence of [^{3}H]FdUMP under conditions promoting binding to TS. Aliquots were subected to two-dimensional gel electrophoresis and the [^{3}H]-FdUMP-TS complex was observed by fluorography. The figure shows the region of the gel containing the label; there was no other radioactivity on the gel. Only the results for HEp-2/500 cells are shown; the same pattern was observed for parental HEp-2 cells. The molecular weight of the complex is indicated in kilodaltons.

cell-free mRNA translation to assess whether TS mRNA was present in an increased concentration in FdUrd-resistant HEp-2/500 cells. Total RNA from HEp-2 or HEp-2/500 cells was translated *in vitro* in the presence of [^{35}S]methionine; the labeled products were fractionated on two-dimensional polyacrylamide gels and detected by autoradiography. As seen in Fig. 3, among the translation products of HEp-2/500 mRNA was a polypeptide with molecular weight 37,000 and pI of about 6.5; this polypeptide was not detected among the translation products of parental HEp-2 mRNA. No other differences in translation products of the two RNAs were apparent. This new polypeptide, presumed to be TS, reacted with antibody generated against partially purified human TS (provided by Dr. Y.-C. Cheng; data not shown). These results suggest that HEp-2/500 contains increased levels of translatable TS mRNA.

More detailed analysis of TS mRNA was accomplished with a mouse TS cDNA-containing plasmid, pMTS-4, that was recently isolated from a cDNA bank prepared from TS-overproducing mouse fibroblasts (30). Total RNAs from cells at several stages of drug selection were fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pMTS-4 DNA (Fig. 4). There were no discernible changes in total

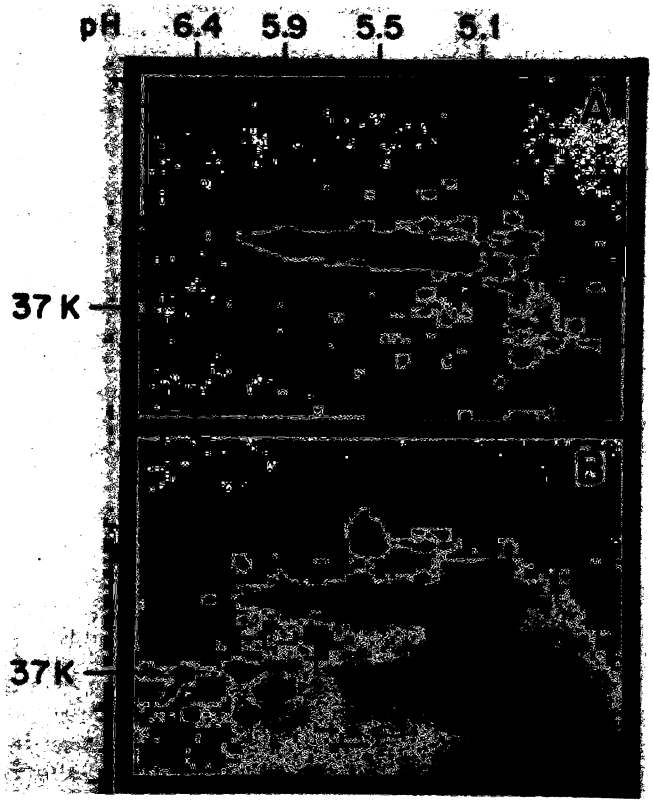

FIG. 3. *Two-dimensional gel electrophoresis of in vitro translation products of RNA from HEp-2 cells*

Total RNAs from HEp-2 (A) and HEp-2/500 (B) cells were translated in the presence of [^{35}S]methionine using a cell-free system derived from rabbit reticulocytes; the products were fractionated by two-dimensional gel electrophoresis. Radioactivity was detected by fluorography. The *arrow* indicates the single polypeptide that was present in products of HEp-2/500 RNA but undetectable in products of parental HEp-2 RNA.

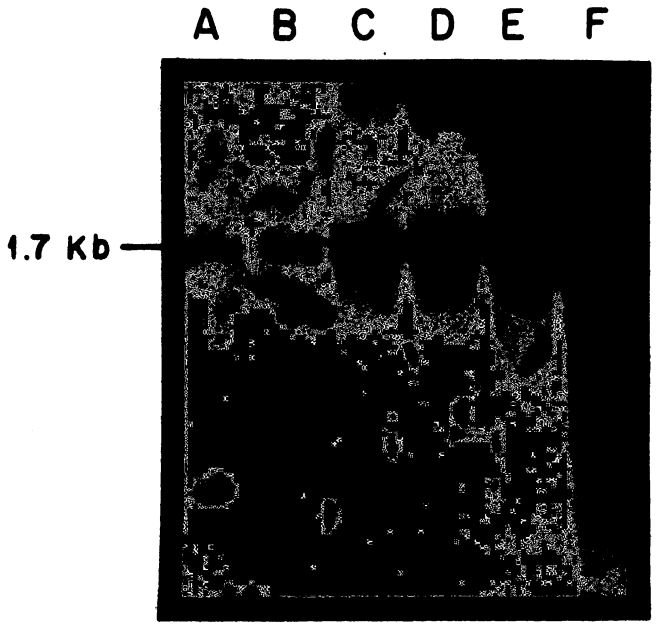

FIG. 4. *Northern blot analysis of TS mRNA in HEp-2 and its drug-resistant derivatives*

Total cellular RNAs were fractionated on formaldehyde-containing 1.5% agarose gels, blotted onto nitrocellulose, and hybridized to ^{32}P-labeled pMTS-4 DNA, which contains cDNA corresponding to mouse TS mRNA (13). RNAs were from parental HEp-2 cells (lane A), and from cells resistant to FdUrd concentrations of 0.001 μM (lane B), 0.024 μM (lane C), 0.05 μM (lane D), 0.10 μM (lane E), and 0.50 μM (lane F). Ribosomal RNAs were used as size markers.

RNA among the various cell populations, as determined by ethidium bromide staining following electrophoresis. The concentration of the major hybridizing species, which was 1.7 kb in length, increased significantly as the cells were adapted to higher concentrations of drug and generally reflected TS enzyme levels in the respective cells. No new RNA species or changes in RNA size appeared following drug selection. Dot blot analysis indicated a greater than 100-fold difference in TS mRNA level between HEp-2 and HEp-2/500 (Fig. 5). Thus, TS protein overproduction in FdUrd-resistant cells appears to be generated predominantly, if not exclusively, by an increase in TS mRNA concentration.

Amplification of the TS structural gene in HEp-2/500. To assess the role of structural gene rearrangement and/or amplification in TS mRNA overproduction, we have compared the TS genes in HEp-2 and its drug-resistant sublines by Southern blotting of genomic DNA. The DNAs were digested with restriction endonucleases *HindIII* or *EcoRI*, fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pMTS-4 DNA. As shown in Fig. 6, several DNA fragments hybridized to the mouse TS cDNA probe; all but two of these (see below) underwent amplification during acquisition of FdUrd resistance. A 100-fold dilution of HEp-2/500 DNA was required to obtain a hybridization signal similar to that from HEp-2 DNA (Fig. 7), indicating a 100-fold amplification. Copy numbers for the genes encoding ornithine decarboxylase, dihydrofolate reductase, and phosphoglycerate kinase

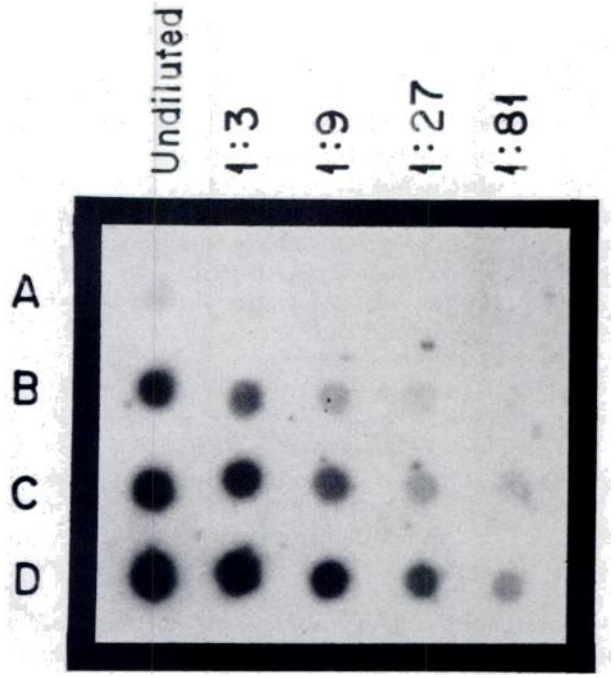

FIG. 5. *Quantitation of TS mRNA in HEp-2 cells and its drug-resistant derivatives*

Various dilutions of total RNA from HEp-2 cells (row A) and from cells resistant to FdUrd concentrations of 0.05 μM (row B), 0.10 μM (row C), and 0.50 μM (row D) were spotted onto nitrocellulose in 2-μl aliquots and hybridized to ^{32}P-labeled pMTS-4 DNA. Each row contains a 3-fold dilution series; undiluted RNAs were at a concentration of 1 mg/ml.

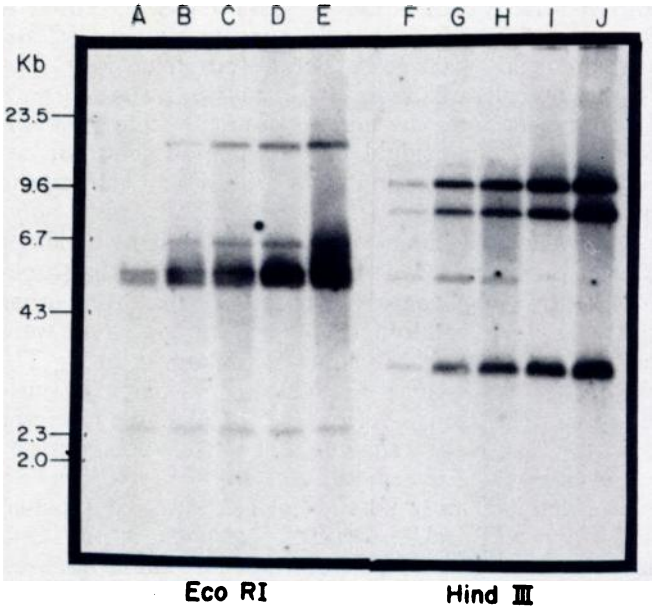

FIG. 6. *Southern blot analysis of TS genes in HEp-2 and its drug-resistant derivatives*

DNAs (20 μg) were digested with *EcoRI* (lanes A–E) or *HindIII* (lanes F–J); products were fractionated in 0.8% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pMTS-4 DNA. DNAs were from parental HEp-2 cells (lanes A and F) and from cells resistant to FdUrd concentrations of 0.006 μM (lanes B and G), 0.05 μM (lanes C and H), 0.10 μM (lanes D and I), and 0.50 μM (lanes E and J). Markers are *HindIII*-generated fragments of phage lambda DNA.

were not different between HEp-2 and HEp-2/500, as measured by Southern blot hybridization to appropriate cDNA probes (data not shown).

A 12-kb *HindIII* fragment and a 2.3-kb *EcoRI* fragment were present at similar concentrations in DNA from all cells. This suggests the existence of a TS gene (or pseudogene) that is not linked to the domain of amplification. It has recently been reported that four dihydrofolate reductase pseudogenes, none of which undergoes amplification during methotrexate selection, are present within the human genome (37).

A 5.8-kb *HindIII* fragment disappeared during drug selection (Fig. 6). It is unlikely that this rearrangement is a prerequisite for the amplification, since the fragment disappeared after considerable amplification had already occurred; more likely, it represents a polymorphic gene that was lost during drug selection (see Discussion).

HEp-2/500 cells grown in the absence of FdUrd lose the TS mRNA overproduction and gene amplification phenotypes (data not shown), in parallel with the loss of the TS enzyme levels (Fig. 1). Thus, the amplified TS genes probably exist as extrachromosomal elements (12); karyotype analysis is necessary to validate this conclusion.

These results show that increases in TS mRNA levels in FdUrd-resistant HEp-2 cells are paralleled by amplification of the TS structural gene. The correlation between mRNA concentrations and gene copy number exists for cells analyzed at several steps during the selection procedure.

DISCUSSION

Current knowledge of fluoropyrimidine metabolism and action in mammalian cells suggests that a variety of alterations could lead to a drug-resistant phenotype. Indeed, FdUrd resistance has been found to be associated with reduced levels of thymidine kinase (7), with impaired interaction between TS and FdUMP (8) and with increased TS concentrations (10, 11). Furthermore, biochemical studies indicate that other parameters, such as cellular levels of deoxyuridylate or folate cofactors, are potential determinants of FdUrd sensitivity (20).

In the present study we have found that exposure of human cells to progressive increases in the concentration of FdUrd results in a variant population with a high level of TS. Selection in FdUrd alone resulted in cells with a 26-fold higher concentration of TS (Table 1); greater TS overproduction could not be obtained. By supplementing the medium with CF and dIno, cells with as much as an 80-fold increase in TS were selected. This difference in maximal overproduction is probably a consequence of folate pool expansion elicited by one or both of these agents. In the supplemented medium, higher concentrations of reduced folates are available for TS and other indispensable folate-requiring enzymes, so that the cofactor is not limiting during selection for enzyme overproduction. CF is a precursor of 5,10-MTHF, which is required for TS catalytic activity. dIno is rapidly converted to hypoxanthine which, via the salvage pathway, is converted to IMP, a central metabolite in purine nucleotide biosynthesis; thus, dIno spares folates from

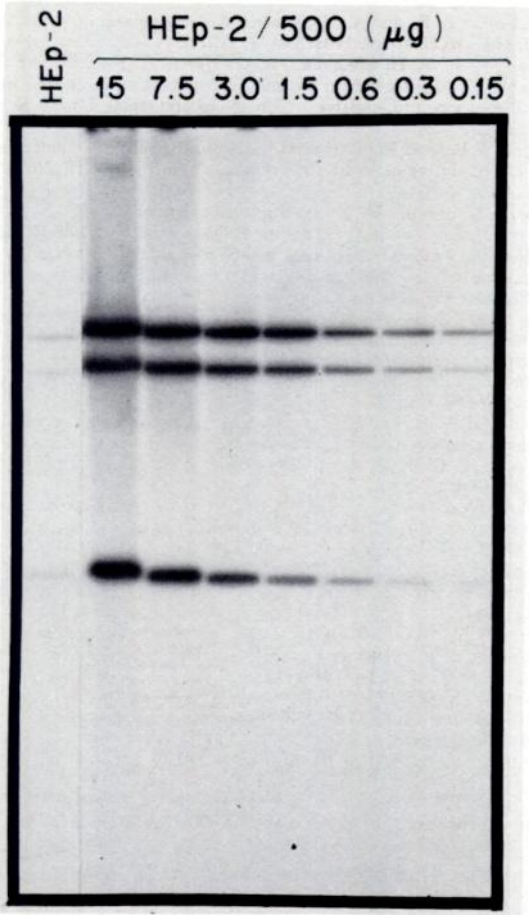

FIG. 7. *Quantitation of TS DNA in HEp-2 cells*

Parental HEp-2 DNA and various quantities of HEp-2/500 DNA were digested with *HindIII*, and TS DNA fragments were visualized following fractionation on agarose gels and Southern blot hybridization to pMTS-4. The HEp-2 lane contains 15 μg of DNA while the HEp-2/500 lane contains 0.15–15 μg of DNA, as indicated on the figure. The 5.8-kb *HindIII* fragment did not show up in the HEp-2 DNA lane due to the shorter autoradiographic exposure in this experiment relative to that in Fig. 6.

the *de novo* purine pathway. It might be expected that cofactor can become limiting during moderate TS overproduction, since the levels of intracellular folates which stimulate ternary complex formation are in 25-fold molar excess relative to TS in HEp-2 cells (35). Thus, cellular cofactor levels, as well as other physiological parameters, may be important determinants of drug resistance via enzyme overproduction. Indeed, excessive levels of particular enzymes could have severe metabolic and/or physiological consequences to a cell. Recent studies with *Leishmania tropica* indicate that two separate genetic events occur during acquisition of methotrexate resistance: one is overproduction of the bifunctional TS-dihydrofolate reductase and the other is overproduction of an unidentified factor which may facilitate survival of the cells during high enzyme overproduction (38). Secondary genetic events could, therefore, be critical elements in acquisition of drug resistance, i.e., a major limitation to enzyme overproduction may be requirements for genetic changes that mediate adaptation to the primary event conferring resistance.

The high enzyme level in FdUrd-resistant cells is generated by increased production of TS mRNA which, in turn, results from amplification of the TS structural gene. Enzyme levels in drug-resistant cells increased by as much as 80-fold, whereas the mRNA concentration and gene copy number increased by 100-fold or more; enzyme measurements may be underestimated slightly as a consequence of incomplete dissociation of endogenous FdUMP-bound enzyme in extracts from cells grown in high FdUrd concentrations. Clearly, the HEp-2/500 cells will be an important resource for the isolation of relatively large amounts of highly purified human TS.

Although no evidence for structural changes in the TS polypeptide (Fig. 2) or mRNA (Fig. 4) in FdUrd-resistant cells was obtained, we did observe minor changes in the pattern of TS DNA sequences on Southern blots. One fragment did not amplify during drug adaptation (Fig. 6). This observation, which has also been made during studies of TS gene amplification in FdUrd-resistant mouse fibroblasts (11a) may indicate the existence of a TS gene or pseudogene that is not linked to the region of amplification. It is of interest that the 2.3-kb *EcoRI* fragment maps to a human chromosome that is different from those of other fragments.[2] Human dihydrofolate reductase pseudogenes that are not amplified in methotrexate-resistant cells have been described (37).

Another change in the TS DNA patterns involved a loss of one restriction fragment (Fig. 6). Lewis *et al.* (39) reported that one of two polymorphic dihydrofolate reductase alleles undergoes amplification during selection for methotrexate resistance in Chinese hamster lung fibroblasts; the other allele remains unamplified, resulting in a change in the Southern blot pattern. It is unlikely that this explains the loss of the 5.8-kb *HindIII* fragment in HEp-2/500 DNA, since we readily see unamplified DNA fragments in the blots (e.g., note the 2.3 kb *EcoRI* and 12-kb *HindIII* fragments in Fig. 6). Rather, it is likely that, among cells within the HEp-2 population, there is TS DNA polymorphism that is lost during drug selection. Subcloning of the parental HEp-2 population should verify the presence of such TS gene heterogeneity.

Since TS is expressed ubiquitously and is regulated during the cell cycle (40), comparative studies of the gene in a variety of organisms will be informative from an evolutionary standpoint. Of interest will be the search for putative regulatory sequences that are conserved among the TS genes from various eucaryotic genomes. In addition, future studies will include examination of the potential role of TS gene structure and expression in clinical fluoropyrimidine resistance that is observed during cancer chemotherapy (3, 4). Although extensive information on molecular and biochemical mechanisms of drug resistance in model cell culture systems is now available, almost nothing is known about mechanisms that operate in human tumors under clinical conditions. Recent experiments do suggest a role for gene amplification in generating dihydrofolate reductase overproduction in leukemia cells from patients who have acquired methotrexate resistance during the course of therapy (16, 17). Further studies must focus on the role of gene

[2] R. Nussbaum, personal communication.

amplification, as well as enzyme alterations and other mechanisms, in determining clinical response to a variety of antineoplastic drugs. These analyses will be greatly facilitated by the availability of cloned human TS DNA sequences.

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