Cytotoxicity and growth inhibition by 5-fluorouracil in methotrexate-resistant dihydrofolate reductase gene-amplified KB cells in the presence of 30 μM thymidine correlates with incorporation of this fluorinated pyrimidine into RNA. Growth of these cells over several generations in the presence of inhibitory concentrations of 5-fluorouracil does not depress the steady state levels of either 18 or 28 S RNA but actually causes an increase. Similarly the rates of RNA and protein synthesis in 5-fluorouracil-treated cells are not decreased. The level of dihydrofolate reductase RNA from 5-fluorouracil-treated cells in a dose-dependent manner correlated with 5-fluorouracil incorporation into RNA. The qualitative size distribution of the dihydrofolate reductase RNA species is unaffected when examined by the Northern blotting technique indicating an RNA processing lesion is not induced by 5-fluorouracil incorporation into RNA. As the dose of dihydrofolate reductase RNA increases, there is no change in the level of dihydrofolate reductase specific activity, but the level of enzyme activity per cell increases. The relevance of these phenomena to the mechanism of 5-fluorouracil effect on RNA and relevance to combination chemotherapy with methotrexate are discussed.

5-Fluorouracil is a pyrimidine analog which is used alone or in combination for the treatment of several solid human tumors (1-4). Many laboratories have studied FUra’s mechanisms of cytotoxicity (5). It is clear that in many systems, metabolism to FdUMP results in blockage of DNA synthesis through inhibition of thymidylate synthetase (5-7). Under certain conditions, FdUTP is formed and can be incorporated into DNA, an effect which is blocked by low concentrations of dThd (8).

In many in vitro and in vivo systems, intracellular conversion of FUra to 5-fluorouracil ribonucleoside triphosphate results in incorporation of FUra residues into RNA. This incorporation has been correlated with cytotoxicity in tissue culture (9-11) and gastrointestinal mucosa (12). The effects of FUra incorporation into RNA on the different classes of RNA vary and their relationships to cytotoxicity are controversial. In the case of rRNA, inhibition of 45 S rRNA processing to mature 18 and 28 S RNA occurs with the administration of high concentrations of FUra (9, 13, 14) or by FUra in the presence of the metabolic modulator inosine (15). In the case of Escherichia coli tRNA, substitution of 90 to 95% of uridine and uridine-derived nucleosides with FUra leads to the substantial inhibition of aminoacylation of several tRNA species (16). In contrast, no effects were observed in tRNA where only 50% replacement had taken place (17). Incorporation of FUra residues into mRNA can cause miscoding during translation in E. coli (18) and mammalian cell lines in vitro (19). The effect of FUra incorporation on mammalian mRNA is not well defined, in part due to the heterodisperse nature of poly(A) RNA. Translation of mammalian poly(A) RNA using an in vitro translation system demonstrated an enhanced degree of protein synthesis and the appearance of some high molecular weight protein species when poly(A) RNA isolated from the regenerating liver of FUra-treated rats was used as a messenger source (20), a result which can be interpreted as being due to FUra incorporation into RNA or the result of different mRNA synthesis. The interpretation of these results is important to the understanding of the RNA-mediated effects of FUra and its metabolites. However, interpretation is limited by the lack of a single mRNA species to study as a model for the effect of FUra on this class of RNA.

This study was initiated to examine the effects of FUra on RNA and a particular mRNA class under conditions of pharmacologic relevance. A clonally selected stable gene-amplified human cell line (KB/TD) which overproduces dihydrofolate reductase and its mRNA has been employed to monitor FUra effects on a single class of mRNA (21-23). This report demonstrates a previously unobserved phenomenon, i.e. an increase in a particular mRNA species as a result of cell growth in the presence of FUra. In addition, this report demonstrates that cell kill by low levels of FUra during long term exposure correlates with its incorporation into RNA in the absence of a depression of the levels of 18 and 28 S RNA and protein synthesis. The relevance of these observations to FUra’s mechanism of action, clinical significance, and previous observations is discussed.

**MATERIALS AND METHODS**

*Chemicals—DNase, RNase A, dThd, NADPH, methotrexate, and FUra were obtained from Sigma. FUra was obtained from Calbiochem-Behring; all other nucleosides and nucleotides were obtained from P-L Biochemicals. Poly(U) Sephadex, CsCl (optical grade), urea and ADNA were obtained from Bethesda Research Laboratories. Nitrocellulose (BA 85) was obtained from Schleicher & Schuell. H₃PteGlu was synthesized and purified by the method of Blakely (24). HindIII and DNA polymerase I were obtained from BioTec. [6-3H]FUra (18 μCi/mol) was obtained from Moravek Biochemicals Inc. [8-3H]Guo, [U-14C]Urd (529 μCi/mmol), and [α-32P]dCTP (800 Ci/mmol) were purchased from Amersham Corp. RPMI 1640, dialyzed horse serum, and pancreatin were obtained from Grand Island Biologicals. Garamycin was purchased from Scher-
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Icing Corp. Tissue culture flasks were obtained from Corning and Linbro dishes from Flow Laboratories, Inc.

Cell Culture—KB and the methotrexate-resistant dihydrofolate reductase gene-amplified KB7D subline were maintained in RPMI 1640 medium supplemented with 5% dialyzed horse serum, 40 μg/ml of kanamycin, and 50 μg/ml of dThd at 37 °C and 5% CO2 atmosphere. KB7D cells were carried in the above medium with an additional 75 μM methotrexate. Cells were placed in the absence of methotrexate for experiments unless otherwise indicated. KB and KB7D were passaged twice weekly by detachment with pancreatin and a subsequent 4-fold dilution in fresh medium. The doubling time for KB cells is 24 h and 30 h for KB7D.

For the estimation of growth inhibitory effects of drugs, cells were plated in duplicate vessels at 6.67 × 104 cells/cm2 in complete medium (plus or minus dThd as indicated in the text) and allowed to attach at 37 °C overnight. After 24 h drug was added to the indicated concentration. Cells were allowed to grow for 6 days (an average of six cell doublings for KB cells and four to five for KB7D) at which time they were harvested and counted to determine the extent of cell growth. For some experiments cytotoxicity by cloning efficiency was estimated by plating 100, 200, and 300 cells in 2 ml of fresh medium (minus drug) in duplicate 4.5-cm wells. Cells were allowed to grow 14 to 21 days at which time colonies were stained with 2% (w/v) methylene blue in methanol. Colonies containing at least 50 cells were deemed viable, cloning efficiency was 50 to 70% for KB and 20 to 30% for KB7D cells. All tissue culture experiments were repeated at least twice with similar results.

Preparation and Gel Analysis of RNA—Total cellular RNA was prepared essentially by the urea extraction and CsCl discontinuous gradient centrifugation method of Ross (25) with minor modifications. RNAs were washed twice with 3.0 M sodium acetate, pH 4.5 (4 °C), twice with 70% ethanol, 100 mM NaCl, and finally once with 100% ethanol. The RNA was then desiccated, dissolved in sterile distilled water, and stored at −70 °C. Yields were typically about 15 mg per 109 cells, using 50 μg of RNA per 3 × 105 cells to determine amounts. RNA was analyzed by denaturation with deionized glyoxal and electrophoresis on 0.5-cm thick 1.4% agarose gels submerged in 10 mM sodium phosphate buffer, pH 7.0, with buffer recirculation as described (26). Typically 20 μg of total cellular RNA was used per lane. Gels were stained for 45 min with 10 μg/ml of acridine orange and photographed using a long wave UV transilluminator and Kodak XRP-1 film to the washed filters at −70 °C with Cronex Quanta 11 intensifying screens.

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Preparation-Dihydrofolate reductase mRNA was prepared using the acid-insoluble fraction. As Fig. 3 indicates, growth inhibition and cytotoxicity by FUra in KB and KB7D cells was determined at 110,000 g for 30 min to generate a cleared supernatant. KB and KB7D cells were inoculated into duplicate wells of a Linbro dish (see under "Materials and Methods") and allowed to attach overnight. On the following day (day 1), FUra was added to the concentrations indicated (Fig. 2). Dishes were harvested daily on days 3 to 6 (KB) or 4 to 7 (KB7D) and the cells were counted. The results presented in Fig. 2 demonstrate the similarity of the ID50 of FUra in KB and KB7D cells. Cell viability, determined by the cloning efficiency of drug-treated cells yields similar results to the growth inhibition experiments (see Fig. 3). Fig. 2 also demonstrates that in KB and KB7D cells sensitivity to FUra remains unchanged during cell growth from mid- to late log (i.e. 3.9 to 5.5 doublings for KB and 3.8 to 5.3 doublings for KB7D). To determine whether growth inhibition and cytotoxicity by FUra was associated with its incorporation into RNA, cells were grown in the presence of [3H]FUra. After 6 days of growth in medium containing various concentrations of [3H]FUra, cells were harvested and counted. A fraction of the cells was plated to determine viability, and the remainder was extracted to determine the incorporation of [3H]FUra into the acid-insoluble fraction (i.e. RNA) (5). Net counts per min incorporated per cell were linear over the range studied (data not shown).

To determine whether there was any effect of FUra on the steady state level of dihydrofolate reductase RNA in cells grown in the presence of FUra, KB7D cells were seeded into flasks and incubated for 6 days in the presence of various concentrations of FUra. At this time the cells were harvested, counted, and RNA was prepared. The RNA was dot blotted on nitrocellulose and hybridized against 32P-labeled nick translation product of dihydrofolate reductase cDNA.

Species of RNA-containing regions of dihydrofolate reductase mRNA were identified by a modification of the Northern blotting procedure (27, 28).

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Quantitation of Dihydrofolate Reductase RNA—Dihydrofolate reductase RNA was quantitated by a modification of the dot blot method of Kafatos et al. (32). RNA was dissolved in 3 M NaCl, 0.15 M sodium citrate, pH 7.0. The RNA was spotted on a sheet of nitrocellulose paper with the same buffer using a Brandel (Rockville, Md.) research Laboratories Hybri-dot manifold to apply the RNA in 3-mm2 circles. In some experiments, 1 to 10 μg of RNA were added to determine if the presence of a carrier was required to obtain linear results with the amount of RNA applied. Additional RNA had no effect on the outcome of experiments. After applying the RNA samples (amounts as indicated in the figures), the nitrocellulose was baked for 2 h at 70 °C, prehybridized and hybridized as described above. The resulting autoradiogram was scanned at 600 nM using a Gilford 2600 microprocessor-controlled spectrophotometer with a densitometer scanner-integrator attachment. The area under the absorbance curves was integrated and plotted as a function of micrograms of RNA applied. Results used for quantitation derived from areas of the curve which were linear with amounts of added RNA. The slope of these curves was used to estimate relative amounts of dihydrofolate reductase RNA.

Enzyme Assay and Preparation—Dihydrofolate reductase was assayed spectrophotometrically by the method of Osborne and Huennekens (53). Reaction mixtures contained in a final volume of 1 ml: 20 μM H3PteGlu, 100 μM NADPH, 150 mM KCl, 100 mM Tris-HCl, pH 7.5, and KB7D. Reactions were preincubated for 2 min at 37 °C prior to initiation by the addition of H3PteGlu. All activities were linear with time and enzyme concentration. One unit of enzyme activity is defined as the amount of enzyme necessary to form 1 μmol of product per min. Cells were extracted for dihydrofolate reductase as described previously (34) except the cell sonicate was centrifuged at 110,000 × g at 4 °C for 30 min to generate a cleared supernatant.

RESULTS

Our purpose was to utilize a cell line which overproduces a particular mRNA species to study the effects of FUra on RNA and mRNA. To this end we determined some growth inhibition parameters of FUra under conditions where most of FUra's action could be attributable to incorporation into RNA. The ID50 of FUra for KB and KB7D cells was determined after different times of continuous exposure. This method was chosen to determine a concentration range where FUra produces inhibition and an RNA complement which has a steady state percentage substitution of its pyrimidine components as FUra derivatives. Determinations of growth inhibitory effects were carried out in the presence of 30 μM dThd to circumvent possible inhibition of thymidylate synthetase by FdUMP biosynthesis as a mechanism of cell kill. Preliminary experiments indicated that 30 μM dThd promoted growth inhibition by FUra in both cell lines but did not inhibit growth itself (Fig. 1).

KB and KB7D cells were inoculated into duplicate wells of a Linbro dish (see under "Materials and Methods") and allowed to attach overnight. On the following day (day 1), FUra was added to the concentrations indicated (Fig. 2). Dishes were harvested daily on days 3 to 6 (KB) or 4 to 7 (KB7D) and the cells were counted. The results presented in Fig. 2 demonstrate the similarity of the ID50 of FUra in KB and KB7D cells. Cell viability, determined by the cloning efficiency of drug-treated cells yields similar results to the growth inhibition experiments (see Fig. 3). Fig. 2 also demonstrates that in KB and KB7D cells sensitivity to FUra remains unchanged during cell growth from mid- to late log (i.e. 3.9 to 5.5 doublings for KB and 3.8 to 5.3 doublings for KB7D). To determine whether growth inhibition and cytotoxicity by FUra was associated with its incorporation into RNA, cells were grown in the presence of [3H]FUra. After 6 days of growth in medium containing various concentrations of [3H]FUra, cells were harvested and counted. A fraction of the cells was plated to determine viability, and the remainder was extracted to determine the incorporation of [3H]FUra into the acid-insoluble fraction (i.e. RNA) (5). Net counts per min incorporated per cell were linear over the range studied (data not shown).

To determine whether there was any effect of FUra on the steady state level of dihydrofolate reductase RNA in cells grown in the presence of FUra, KB7D cells were seeded into flasks and incubated for 6 days in the presence of various concentrations of FUra. At this time the cells were harvested, counted, and RNA was prepared. The RNA was dot blotted on nitrocellulose and hybridized against 32P-labeled nick translation product of dihydrofolate reductase cDNA.
5-Fluorouracil Modulates Dihydrofolate Reductase RNA Levels

Fig. 1. Effect of dThd on growth inhibition of KB and KB7D cells by FUra. Cells were plated as described on day 0 in 4.5-cm² wells of a Linbro dish in duplicate for each concentration of drug (see under "Materials and Methods"). On day 1 dThd was added to the concentrations indicated with 0.5 µM FUra (C, □) or alone (■). The cells were harvested and counted on day 7: □, KB cells; O, ■, KB7D cells.

Fig. 2. Inhibition parameters of FUra for KB (A) and KB7D (B) cells. Cells were plated as described (see under "Materials and Methods") into duplicate wells (4.5 cm²) of a Linbro dish with 30 µM dThd on day 0. On day 1 FUra was added to the concentrations indicated. The cells were harvested and counted for duplicate wells on days 3 (□), 4 (×), 5 (○), 6 (△), or 7 (□). One hundred percent control cell growth refers to the cell number for the respective cell line without FUra. Symbols represent the average of duplicate determinations.

5-fluorouracil (FUra) translated pDHFR26. The filter was washed and subjected to autoradiography. The results are presented in Fig. 4. The differences in dot intensity corresponding to relative amount of dihydrofolate reductase RNA were quantitated by integrating the area under a densitometric tracing of the autoradiogram and plotting the area as a function of micrograms of RNA. Slopes were taken from the linear portion of these curves, and the ratio of slopes from the drug versus control cells is taken as an estimate of the relative concentration of dihydrofolate reductase. As Fig. 5 indicates, the level of dihydrofolate reductase RNA appears to correlate inversely with inhibition of cell growth (up to 50% growth inhibition) reaching a peak change of approximately 3-fold.

It has been demonstrated by many researchers that FUra can inhibit maturation of 45S precursor rRNA to the mature 18 and 28S forms. It was anticipated that this phenomenon could be responsible for the observed increase in dihydrofolate reductase RNA. As Fig. 6 demonstrates, the 18 and 28S RNA species are as prominent and integral in the drug-treated cells as in control cells. The maximum mass contribution of the initial cell population RNA to the

Fig. 3. Correlation of growth inhibition and cloning efficiency with incorporation of FUra into RNA. Fourteen 25-cm² flasks were inoculated on day 0 with KB7D cells in medium containing 30 µM dThd as described (see under "Materials and Methods"). On day 1 [3H]FUra (4.0 µCi/µmol) was added to the concentrations indicated and the cells were allowed to grow until day 7. On day 7 the cells were harvested, counted, and plated to determine cloning efficiency (see under "Materials and Methods"). The remaining cells were pelleted by centrifugation and extracted with ice-cold 10% (w/v) trichloroacetic acid. The acid-insoluble precipitates were collected by filtration through nitrocellulose, washed twice with 5% (w/v) ice-cold trichloroacetic acid, twice with 70% (v/v) ethanol, dried, and counted in 10 ml of Aquasol. Symbols indicate the average of duplicate determinations. The range of determinations for net counts per min incorporated per flask of cells (C) are indicated by error bars. Control cell values were 2.5 × 10⁶ cells/flask and are equivalent to 100% cell growth. Symbols are: O, per cent control cell growth; ×, cloning efficiency.
5-Fluorouracil Modulates Dihydrofolate Reductase RNA Levels

Fig. 4. Dot blot of RNA for dihydrofolate reductase RNA as a function of FUra concentration. Autoradiogram of a RNA dot blot hybridized with [32P]pDHFR26 as described in the text (see under “Materials and Methods”). The amounts of RNA loaded per well and the concentration of FUra at which their respective cell sources were grown are indicated.

Fig. 5. Graphic representation of change in dihydrofolate reductase (DHFR) RNA levels with growth of KB7D cells in medium containing FUra. The data represented here are the averages of two experiments conducted approximately 5 months apart. The error bars are the range of the determinations of relative dihydrofolate reductase RNA levels (●) where identical concentrations were used for each experiment. The result of 5 μM FUra shown in Fig. 4 is not presented here since a repeat of this concentration was not performed. ---, represents an idealized curve tracing the change in dihydrofolate reductase RNA levels. ○, △, represent percent control cell growth and plating efficiency, respectively. Error bars for cell growth show the ranges of two determinations.

RNA extracted from cells grown for 6 days is about 3.6% for the control cells and about 19% for RNA from cells growth inhibited 50% (based upon 4.8 cell doublings for control and 2.4 for drug-treated cells). Therefore, the increase in dihydrofolate reductase RNA is not due to a proportional decrease in cellular rRNA. This estimate is based on the assumption that

the amount of RNA per cell has not decreased significantly in drug-treated cells. In fact, in all experiments where the RNA recovery per cell has been estimated, the average recovery of RNA from control cells is found to be less than that of drug-treated cells (see Table I), indicating that the estimate of original RNA as a percentage of the RNA shown in Fig. 6 is an upper estimate.

A gel similar to that portrayed in Fig. 6 was blotted to nitrocellulose as described (see under “Materials and Meth-
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Table II

| Condition | Control growth* | Protein | 5 min | 10 min | 20 min |
|-----------|-----------------|---------|-------|--------|--------|
|           | %               | dpm/cell|       |        |        |
| Control   | 100 (11.4)      | 0.33    | 0.43  | 0.61   |
| 0.65 μM FUra | 78 (8.9)     | 0.58    | 0.58  | 0.68   |
| 1.0 μM FUra | 55 (6.3)     | 0.57    | 0.68  | 0.87   |

"Numbers in parentheses represent -fold growth over 1.7 × 10^6 cells seeded per 25-cm² flask.

Fig. 7. Northern blot of RNA from methotrexate-resistant KB cells grown in the presence of FURA, analysis of dihydrofolate reductase RNA. RNA was prepared from cells grown in the presence of various concentrations of FURA as described in Fig. 6. 10 μg of each RNA were denatured and electrophoresed as described (see under "Materials and Methods"). The gel was blotted to nitrocellulose and hybridized with the [32P]pDHFR26. The autoradiogram is labeled as described for Fig. 6 except the species of dihydrofolate reductase RNA (kilobases) are indicated in the left margin and the positions of the λDNA markers on the right (kilobases).

As we were using total cellular RNA (minus tRNA) to estimate the relative amount of dihydrofolate reductase RNA in control and FURA-treated cells, it was necessary to obtain an estimate of the per cent of our RNA as poly(A) RNA. If the amount of poly(A) RNA from drug-treated cells was increasing, a general explanation for the increase in dihydrofolate reductase RNA would be evident. Cells were grown in the absence or presence of FURA (1.0 μM) for 6 days as described previously. Control cells were pulsed for 60 min with either [3H]Urd-labeled RNA from control cells was harvested and the disintegrations per min determined. The results are shown in Table I. Table I shows that the amount of RNA recovered from drug-treated cells is higher than controls. (Increases in RNA from drug-treated cells are found to be elevated routinely; increases range from 60 to several hundred per cent depending on the amount of drug used.) Poly(U) Sephadex chromatography was carried out by mixing [3H]Urd-labeled RNA from control and cultures treated with [3H]Guo-labeled RNA from control or FURA-treated cells to serve as an internal standard. As Table I indicates the per cent of RNA recovered as poly(A) RNA is not significantly different in control versus FURA-treated cells.

Since previous reports have implicated processing of 45 S precursor rRNA as the lesion associated with RNA-mediated
5-Fluorouracil Modulates Dihydrofolate Reductase RNA Levels

**Table III**

Effect of FUra on dihydrofolate reductase activity

| FUra | Growth | Enzyme activity | Units/µg (x 10^6) | Units/µg (x 10^6) | Units/cell equivalent (x 10^6) |
|------|--------|-----------------|-------------------|------------------|-------------------------------|
| µM   | %      |                 |                   |                  |                               |
| 0    | 100    | 3.1 ± 0.2       | 3.1 ± 0.2         | 2.5 ± 0.2        |
| 0.1  | 91     | 3.3 ± 0.5       | 3.2 ± 0.5         | 2.6 ± 0.6        |
| 0.3  | 67     | 3.5 ± 0.4       | 3.2 ± 0.4         | 2.8 ± 0.5        |
| 0.65 | 37     | 3.9 ± 0.1       | 3.2 ± 0.1         | 3.1 ± 0.1        |
| 1.0  | 22     | 4.2 ± 0.5       | 2.8 ± 0.3         | 3.4 ± 0.4        |
| 2.0  | 12     | 4.2 ± 0.4       | 2.3 ± 0.2         | 3.4 ± 0.3        |

* Assumes equivalent cell extraction of 1.25 x 10^6 cells/µl.

FUra toxicity, we decided to measure protein synthesis in drug-treated cells. This was particularly important since we had not observed a change in the distribution of 18 and 28 S RNA from steady state drug-treated cells and 2 g/l of radioencoded RNA from [³H]Guanine-pulsed drug-treated and control cells (Table I) showed the loss of 18 S RNA and increase in 45 S RNA pattern as others had observed (data not shown, see Refs. 5, 9, 13, and 15). We reasoned that if protein synthesis was not significantly impaired in FUra-treated cells, then the inhibition of 45-S RNA processing observed over the short term pulse of cells grown for several generations in FUra was probably not a mechanism of cell death. Table II shows an experiment which measures the amount of [³H]Leucine incorporated into control and drug-treated cells as a function of FUra concentration and time. The amount of [³H]Leucine incorporated into protein on a per cell basis increases in a dose-dependent fashion over the concentration range of FUra shown. Thus, protein synthesis is not inhibited in FUra-treated cells, but is actually increased.

The increase in dihydrofolate reductase RNA per µg of cellular RNA was an indication that the level of enzyme activity may be increased in FUra-treated cells. This was expected since protein synthesis in FUra-treated cells was not depressed. Cells grown for 6 days in the presence of various concentrations of FUra were extracted for dihydrofolate reductase enzyme activity determinations (see under “Materials and Methods”). A constant volume of extraction buffer to cell number was used so as to be able to relate activity to a cellular value. The results are presented in Table III. As Table III indicates, as cells are grown in the presence of increasing concentrations of FUra, the dihydrofolate reductase activity does not change significantly per µg of protein until 2.0 µM FUra where a significant decrease is observed. In contrast the enzyme activity increases significantly on a per cell basis reaching a maximum at 1.0 µM FUra with an increase over basal level of 36% (p < 0.05).

**DISCUSSION**

FUra has been demonstrated over the years to be a drug with multiple mechanisms of action (5). In mammalian tissue culture systems where growth inhibition and/or cytotoxicity correlate with the incorporation of FUra into RNA, the primary mechanism of action has been ascribed to the inhibition of 45 S precursor rRNA maturation (5, 9, 13). Other effects on RNA have been reported, such as effects on in vitro translation rates of mRNA with FUra substitution as well as a perturbation in the size distribution of the products synthesized using in vitro translation systems (20). However, no studies of FUra effect on a particular mRNA species related to function or cellular levels have been reported for a mammalian cell line. Additionally, most studies on the effects of FUra on RNA metabolism and function have approached this problem from the standpoint of short term drug exposure (i.e. less than one cell generation time). These studies usually require higher levels of drug than do experiments involving long term growth in the presence of drug to attain equivalent cell kill and growth inhibition. We have studied cells which have been treated with concentrations of FUra which inhibit cell growth over a long term period (i.e. greater than 2 generations) to achieve a steady state level of FUra substitution in RNA and to avoid possible epiphenomena which may be associated with high drug levels (e.g. possible inhibition of some RNA processing enzyme(s)). We have attempted to monitor a particular class of mRNA (i.e. dihydrofolate reductase RNA) which codes for the target enzyme of a drug (i.e. methotrexate) whose cytotoxic action is known to be affected by FUra. This study is the first to report an effect of FUra on the levels of a mRNA coding for a specific enzyme. Studies were carried out in the presence of 30 µM dThd (see Fig. 1) in an effort to eliminate the inhibition of thymidylate synthetase by FUra as a mechanism of growth inhibition and thus channel growth inhibitory effects to the incorporation of FUra into RNA.

The results presented here show that in the gene-amplified methotrexate-resistant cell line KB7D, growth inhibition and cytotoxicity correlate with the incorporation of FUra into RNA. This RNA-directed effect was attained by the growth of cells in medium containing low concentrations of FUra (0 to 2 µM) and 30 µM dThd to circumvent a block at thymidylic synthetase. The distribution and integrity of 18 and 28 S RNA from control and FUra-treated cells were found to be indistinguishable as determined by denaturing gel electrophoresis. Since these RNA preparations were from cells which had grown at least several-fold in the presence of drug (see Fig. 6 and “Results”), the contribution of the RNA from the seeded cell population is calculated to be 19% at most in cells growth inhibited 50%. Experiments presented here (Table I) which indicate the FUra-treated cells contain more RNA per cell than controls suggest that the original cellular RNA probably contributes much less than 19% (even if RNA turnover is not taken into account) to the RNA being analyzed. This result contradicts the conclusion that the RNA-mediated cytotoxic effect of FUra is due to an inhibition of the processing of 45 S precursor rRNA to 18 and 28 S RNA (9, 13). Other results from our laboratory confirm the observation that 45 S precursor rRNA maturation is affected but indicate the process is only delayed during continuous exposure to FUra. This conclusion is further substantiated by experiments which show that in cells grown in the presence of FUra, 45 S RNA processing was found to be inhibited during an hour pulse with [³H]Guanine, but protein synthesis actually increased in FUra-treated cells (see under “Results” and Table II).

Additionally, there was no change in the per cent of cellular RNA as poly(A) RNA (Table I). Cellular RNA was, therefore, used for dot blots (Fig. 4) and Northern blotting (Fig. 7) as an estimation of the amount of dihydrofolate reductase RNA. This allowed use of small amounts of cells for RNA analysis. These studies indicated that dihydrofolate reductase RNA increased per µg of RNA in FUra-treated cells (Figs. 4 and 5). Analysis of the dihydrofolate reductase RNA by denaturing gel electrophoresis, Northern blotting, and hybridization with a cloned [³P]cDNA probe indicates the major species of dihydrofolate reductase RNA appear unaltered in terms of molecular weight. Thus, FUra incorporation into RNA does not appear to have affected the processing of these
dihydrofolate reductase RNAs which have been shown by others to possess mRNA activity (35). However, there is a progressive increase in the autoradiographic signal from the lane containing RNA from the cells grown in the presence of increasing concentrations of FUra. This increase occurs mainly in the 3.5 kilobase species and is not observed for the 1.0 kilobase species until 2.0 µM FUra (Fig. 7). Since the percentage of cellular RNA as poly(A) RNA does not change in FUra-treated cells (Table I), this increase in dihydrofolate reductase RNA is not an artifact generated by a loss in rRNA content or an increase in the cellular level of poly(A) RNA. There are several explanations for this increase. It is possible that the increase in dihydrofolate reductase RNA reflects enhanced transcription of the dihydrofolate reductase gene or an enhanced distribution of cells in a phase of the cell cycle where dihydrofolate reductase RNA (and possibly other mRNAs) increases due to growth in the presence of FUra. FUra has been reported to act at G1 when its cytotoxicity is correlated with an RNA effect (36). Since in many cell systems dihydrofolate reductase is elevated in S phase, retardation of a cell in late G1 may cause an increase in the mRNA for this enzyme. Increases in the mRNA for dihydrofolate reductase just prior to S phase has been demonstrated previously (37) although these increases may be the result of nuclear RNA turnover and not transcriptional rates (38). This generalized cell cycle phenomenon (i.e. an unequal distribution of FUra-treated cells in a pre-S state) as an explanation for the observed increase in dihydrofolate reductase RNA per µg of RNA (Figs. 4 and 5) but without a substantial increase in the level of dihydrofolate reductase enzyme activity per µg of protein (Table III) is an attractive hypothesis currently under investigation in our laboratory.

The mechanism of cell kill related to incorporation of FUra into RNA is unclear. Cell kill and growth inhibition correlate with incorporation of FUra into RNA in the presence of dThd. However, protein synthesis and steady state levels of rRNA are not decreased even though the short term matura-
tion of 45 S RNA to 18 and 28 S RNA appears to be affected (see under “Results,” Tables I and II, and Fig. 6). We conclude from these observations that the inhibition of 45 S RNA processing is probably an epiphenomenon of the effect of FUra incorporation into RNA not responsible for cytotoxicity in this system (although this may be a contributing factor with high FUra doses). Our interpretation is that the incorporation of FUra into RNA which correlates with growth inhibition of these cells may be due to impairment of mRNA function or aberrant translation of mRNA in general to form miscoded translation products such that the cell must commit excessive resources to synthesize greater than normal amounts of RNA to achieve normal levels of enzyme-mediated cell functions. This is suggested by recent experiments in our laboratory which show an enhanced ability of total cellular RNA from FUra-treated cells to stimulate protein synthesis in an in vitro protein synthesis system.2 Alternative explanations such as an effect on some critical nuclear RNA species are not excluded. This hypothesis (i.e. impaired RNA function due to FUra incorporation) is also consistent with the disproportionate increase in dihydrofolate reductase RNA compared to the lack of change in dihydrofolate reductase enzyme activity and cannot be distinguished from a late G1/S block by FUra as an explanation by these studies.

The elevated levels of dihydrofolate reductase RNA and enzyme per cell may be a partial explanation for the antagonism observed between FUra and methotrexate during their combined use in certain schedules of tumor treatment. It has been demonstrated that in regimes where methotrexate precedes FUra, enhanced tumor kill is observed in vivo (39, 40). However, in vivo antagonism is observed when the two drugs are coadministered or when FUra precedes methotrexate (41–43). Some explanations revolve about the thymidylate synthetase site of FUra action (42) and do not account for RNA-related effects. In this report we present evidence that FUra can cause increases in the intracellular levels of dihydrofolate reductase RNA and enzyme activity. These increases may result in the enhanced resistance of FUra-treated cells to methotrexate. Although a maximal elevation of dihydrofolate reductase activity of 36% was observed, removal of FUra may result in an even greater increase if the elevated dihydrofolate reductase RNA contributes to enzyme levels due to a released block in cell cycle progression which allows translation of the accumulated message.

Further studies are necessary to evaluate the change in dihydrofolate reductase RNA as a possible mechanism of FUra antagonism to methotrexate. The functionality of the elevated dihydrofolate reductase RNA is also a question which needs to be answered. These areas are currently under investiga-
tion and it is hoped they will provide more insight as to the possible role of FUra incorporation into RNA as a mecha-

Acknowledgments—We wish to express our thanks to Dr. Yung-
Chi Cheng for his gift of KB and KB7D cells.

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J. Biol. Chem. 1983, 258:13299-13306.

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