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5-Fluorouracil Augmentation of Dihydrofolate Reductase Gene Transcripts Containing Intervening Sequences in Methotrexate-Resistant KB Cells

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SUMMARY

Several fragments of the human dihydrofolate reductase gene (tetrahydrofolate dehydrogenase, 5,6,7,8-tetrahydrofolate $NADP^+$ oxidoreductase, EC 1.5.1.3) were isolated from gene-amplified KB7B cells and characterized. Recombinant plasmids containing intron sequences were constructed. Probes prepared from these plasmids were tested for dihydrofolate reductase precursor mRNA specificity via solution hybridization studies and Northern blot analysis. One probe, p0.69EH, was shown to be specific for dihydrofolate reductase RNA by its greatly enhanced level of hybridization with total RNA from dihydrofolate reductase gene-amplified versus non-amplified cells. In addition, solution hybridization studies with various classes of RNA and Northern blot analysis revealed that p0.69EH hybridizes predominantly with polyadenylated, high molecular weight, nuclear RNA species. Subsequent solution hybridization studies revealed a disproportionate 5-fluorouracil-induced increase in dihydrofolate reductase intron-containing RNA over dihydrofolate reductase mRNA. These results suggest that 5-fluorouracil incorporation into RNA may inhibit the conversion of precursor mRNA to mature mRNA.

The base analog, FUra, is a chemotherapeutic agent used to treat a wide variety of human tumors (1). Three major mechanisms for its cytotoxicity have been proposed. The FUra metabolite FdUMP has been shown to be a tight binding inhibitor of thymidylate synthetase and, consequently, to inhibit DNA synthesis through reduced levels of thymidylate (2). FUra residues have recently been detected in DNA both *in vivo* and in tissue culture, and this incorporation has recently been correlated with increased levels of DNA strand breakage (3–5). Finally, FUra, via its conversion to FUTP, is incorporated into RNA (6). The incorporation of FUra into RNA has been correlated with its cytotoxic effects both in tissue culture and *in vivo* (7–10). In particular, it has been demonstrated in several cell lines that thymidine administration, which causes an increase in FUra incorporation into RNA while circumventing inhibition of thymidylate synthetase, can cause an increase in cell death (10–12). In addition, other compounds which increase the incorporation of FUra into RNA have been shown to enhance cytotoxicity (13, 14). However, the precise mechanism whereby FUra incorporation into RNA causes cell death in mammalian cells is unclear. Several studies have demonstrated that high concentrations of FUra inhibit the processing of 45 S rRNA to mature 18 S and 28 S rRNA and that this inhibition correlates with cytotoxicity (7, 15, 16). The effect of FUra on other types of RNA, such as mRNA and precursor mRNA, is not well defined. To understand better the effect of FUra on the metabolism of mRNA, studies in our laboratory, utilizing a single mRNA species as a model, were undertaken. Specifically, we have studied the effects of FUra on dihydrofolate reductase mRNA and dihydrofolate reductase enzyme activity in gene-amplified KB7B cells which overproduce this enzyme and its mRNA (10, 17, 18). These studies were carried out in the presence of 30 μM thymidine to bypass the thymidylate synthetase block by FdUMP and to channel FUra into RNA (10). Cells were exposed to FUra for an extended period of time (greater than 2 generations) to achieve a steady state level of FUra substitution into RNA (10). Under these conditions, incorporation of FUra into RNA was shown to correlate with growth inhibition and cytotoxicity (10). However, no decrease in the steady state levels of 18 S or 28 S rRNA was observed (10). Rather, Northern blot and dot blot analyses revealed a dose-dependent increase in dihydrofolate reductase mRNA species with increasing FUra concentrations (10). Additional studies provided evidence consistent with FUra-induced miscoding as a mechanism of RNA-mediated FUra cytotoxicity (17).

Here, we report studies, utilizing this system, which address

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ABBREVIATIONS: FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FUTP, 5-fluorouridine-5′-triphosphate; pre-mRNA, precursor mRNA; EDTA, ethylenediaminetetraacetic acid; kb, kilobase pairs; bp, base pairs.

the effect of FUra on the metabolism of dihydrofolate reductase pre-mRNA molecules. There is little information regarding FUra effects on mRNA precursors. In one study, Glazer and Peale (19) report that, of all classes of nuclear RNA, the amount of FUra incorporated per unit RNA is greatest in the polyadenylated, heterogeneous RNA fraction. Recent studies by Armstrong and Cadman (20) demonstrate an increase in nuclear dihydrofolate reductase exon-containing RNA, as opposed to cytoplasmic, in C3-L5178Y murine leukemia cells exposed to FUra for relatively short periods of time. We describe the construction and characterization of a probe specific for dihydrofolate reductase pre-mRNA molecules and, via solution hybridization assays, demonstrate a disproportionate increase in dihydrofolate reductase intron-containing RNA compared to dihydrofolate reductase mRNA in FUra-treated KB7B cells. The results of this report, therefore, provide evidence consistent with FUra inhibiton of pre-mRNA processing and suggest an additional mechanism whereby FUra incorporation into RNA may lead to growth inhibition and cell death.

Materials and Methods

Chemicals. Restriction endonucleases were obtained from Promega Biotec, New England Biolabs, or Bethesda Research Laboratories. Seakem Le Agarose and Sea Plaque Agarose were purchased from FMC Corporation. DEAE Sephacel, RNase A, S_1 nuclease, and formamide were obtained from Sigma. DNase-free RNase A was prepared as described by Maniatis *et al.* (21). Formamide was deionized by mixing for 16 hr at 4° with a mixed bed resin, AG 501-X8 (D) purchased from Bio-Rad Laboratories. NACS Prepac columns were obtained from Bethesda Research Laboratories, RNAsin from Promega Biotec, and Sephadex G-50 from Pharmacia. DNase I was purchased from Worthington Biochemicals and prepared free of RNase by passage through agarose 5′-(*p*-aminophenyl phosphoryl) uridine 2′(3′)-phosphate obtained from Miles-Yeda LTD as described (19). Vanadyl ribonucleoside complexes were prepared as described from vanadium (IV) sulfate oxide purchased from Alfa Products (21). [α-^{35}S]UTP (1283 Ci/mmol) and [α-S]-UTP were obtained from New England Nuclear. [α-^{35}S]dATP (1200 Ci/mmol), [α-^{32}P]dCTP (800 Ci/mmol), and Hybond N transfer membranes, were purchased from Amersham.

Cloning and characterization of dihydrofolate reductase gene fragments. Standard procedures were utilized to introduce restriction fragments of both KB7B genomic DNA and subsequently cloned fragments of the human dihydrofolate reductase gene into complementary restricted plasmid vectors. EcoRI-digested fragments of KB7B genomic DNA were size selected via electrophoresis through an 0.8% agarose gel, isolated by electroelution of excised gel slices, and purified by DEAE-Sephacel chromatography (21). Restriction fragments of cloned DNA were isolated by electrophoresis through low melting temperature agarose gels and purified by DEAE-Sephacel chromatography or passage through NACS Prepac columns as described by the manufacturer (21). Ligations and $CaCl_2$-mediated transformation into the *Escherichia coli* strains HB101 or MC1061 were performed as described (21). Desired recombinants were detected by the colony hybridization method of Grunstein and Hogness (22) using ^{35}S- or ^{32}P-nick-translated probes and/or via small scale plasmid preparation followed by restriction enzyme site analysis (21, 22). Large scale plasmid preparations were carried out essentially as described by Birnboim and Doly (23). All restriction enzyme digests were carried out as described except that Bg1 II digestions were performed in high salt (100 mM NaCl) buffer (21). Detailed restriction site mapping was accomplished by double restriction digests and partial restriction digests of end-labeled fragments (21). The position of dihydrofolate reductase coding sequences was determined by Southern blotting techniques and subsequent hybridization with the [α-^{35}S]dATP nick-translated Pst I insert of pHD84 (10, 24).

***In vitro* transcription of cloned DNA.** *In vitro* transcription was performed using the Riboprobe System marketed by Promega Biotec. The reaction was carried out as described by the manufacturer with minor modifications. Briefly, the linearized pSP6 template, at a final concentration of 20 μg/ml, was transcribed in a total reaction volume of 25 μl containing the following: 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each of ATP, CTP, and GTP, 16.8 μM [α-S]UTP, 3.2 μM [α-^{35}S]UTP (1289 Ci/mmol), 4 mM dithiothreitol, 37 units of RNAsin, and 5 units of SP6 RNA polymerase. The mixture was incubated at 40° for 45 min, after which an additional 5 units of polymerase were added and incubation was continued for an additional 45 min. RNase-free DNase was added to a final concentration of 20 μg/ml and the reaction was incubated for 10 min at 37° to hydrolyze the DNA template. The reaction was terminated by addition of 2 volumes of 20% (w/v) sodium dodecyl sulfate, 2 volumes of 200 mM vanadyl ribonucleoside complexes, and 50 μg of *E. coli* tRNA. RNA transcripts were purified by phenol/chloroform extraction followed by passage over Sephadex G-50 in 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 0.2% (w/v) sodium dodecyl sulfate. The proper size of the transcripts was confirmed by formaldehyde gel electrophoresis and autoradiography (data not shown) (21).

Cell culture. KB and KB7B, a methotrexate-resistant dihydrofolate reductase gene-amplified subline, were maintained as described previously (10, 17). Drug exposure was for 5 days as previously described (10, 17).

DNA and RNA preparation. KB7B genomic DNA was isolated by the method of Perucho *et al.* (25) with the following modification: RNA was removed by the addition of DNase-free RNase A to 100 μg/ml and incubation at 37° for 2 hr followed by repeated phenol/chloroform extraction and, finally, ethanol precipitation. KB and KB7B total cellular RNA was prepared by urea extraction and CsCl discontinuous gradient centrifugation as previously described (10). poly(A) RNA and non-poly(A) RNA were isolated from total cellular RNA by poly(U) Sephadex column chromatography as described (10, 17). Nuclear and cytoplasmic RNA were prepared as described with the following modification: isolated nuclei were resuspended in cell lysis buffer and recentrifuged twice through the discontinuous sucrose gradient in an effort to minimize the amount of contaminating cytoplasmic RNA (21). RNA was quantitated spectrophotometrically by the formula 1 A_{260} = 50 μg.

Solution hybridizations. Solution hybridizations were carried out essentially as described by Durnam and Palmiter (26) with minor modifications (26). Briefly, hybridization reactions contained 0.6 M NaCl, 10 mM Tris-HCl (pH 7.5), 4 mM EDTA, 19 mM β-mercaptoethanol, 40% (w/v) deionized formamide, 0–80 μg of RNA and approximately 20,000 dpm of ^{35}S-labeled anti-RNA transcripts (specific activity 0.5–2.2 × 10^8 dpm/μg) in a total volume of 30 μl. The reaction mixture was covered with paraffin oil (~100 μl), microfuged for 5 sec, and incubated at 55° for 18 hr. Preliminary studies revealed that the hybridization reactions proceeded to completion by 16 hr (data not shown). The samples were then treated with S_1 nuclease to remove any unhybridized probe. S_1 digestions were carried out by adding 1 ml of 0.3 M NaCl, 30 mM sodium acetate, pH 4.5, 3 mM $ZnSO_4$, 100 μg/ml of herring-sperm DNA and 20 units of S_1 nuclease, vortexing, and incubating at 55° for 1 hr. The reaction was terminated by addition of trichloroacetic acid to 5% (w/v) final concentration and cooled on ice for 5 min. The acid-precipitable RNA-RNA hybrids were collected on Scientific Products grade 131 filters, washed three times with 5% trichloroacetic acid containing 1% sodium pyrophosphate, once with 70% ethanol, and subsequently counted in 2 ml of scintillation fluid. Background ranged from 0.5 to 7.3% of the input dpm.

Northern blot analysis. poly(A) RNA was denatured with formaldehyde and separated by electrophoresis in 0.8% agarose gels containing 2.2 M formaldehyde (21). RNA was transferred to Hybond N membranes and covalently bound as described by the manufacturer. Prehybridization and hybridization were carried out as described previously (10). The probes used, pSP65-13 and p0.69EH, were nick-

translated with [α-^{32}P]dCTP (800 Ci/mmol) as previously described (10). Hybridized, nick-translated probe was removed from the Hybond N membrane by washing with 95% deionized formamide for 3 hr at 85°. Removal of the probe was monitored by autoradiography of the dehybridized membrane.

Results

EcoRI fragments of the human dihydrofolate reductase gene were isolated from KB7B genomic DNA. KB7B, a methotrexate-resistant subline, contains approximately a 10-fold greater number of dihydrofolate reductase genes than the parent cell line (KB) and therefore facilitated the isolation of dihydrofolate reductase gene fragments. In addition, the sizes of dihydrofolate reductase exon containing EcoRI fragments were determined by Southern blotting techniques, utilizing nick-translated [^{32}P] pHD84 as a probe (data not shown). pHD84 consists of a human dihydrofolate reductase cDNA containing all of the coding sequence and 85 bases of 3′ noncoding sequences, inserted at the Pst I site of pBR322 (24). Hence, total EcoRI-restricted DNA was enriched for dihydrofolate reductase sequences via size selection of electrophoretically separated fragments. Subsequent cloning into EcoRI-restricted pBR322 and screening with nick-translated Pst I insert of pHD84 led to the isolation of a 4-kb and a 1.65 kb dihydrofolate reductase gene fragment (Fig. 1). Southern blotting and detailed restriction mapping demonstrated that these two fragments consist of a contiguous stretch of the dihydrofolate reductase gene which encompasses exons 4 and 5, intron IV, and part of intron V (Fig. 1). The identity of the 1.65-kb fragment was further confirmed by dideoxy sequencing analysis of the coding region and a portion of the adjacent fourth intron (data not shown). Subfragments, consisting only of intervening sequences, were isolated for the purpose of constructing probes to study intron containing dihydrofolate reductase pre-mRNA (Fig. 1C). In particular, an 1100-bp SstI-EcoRI fragment from intron IV and a 690-bp Hind III-EcoRI fragment from intron V were isolated and cloned into the transcription vector pSP65, generating the plasmids p1.1ES and p0.69EH, respectively (Fig. 1). ^{35}S-labeled RNA transcripts, complementary to intron IV or intron V, were transcribed from p0.69EH and p1.1ES and tested for dihydrofolate reductase pre-mRNA specificity via solution hybridization studies. In addition, radiolabeled RNA transcripts complementary to dihydrofolate reductase exon sequences were transcribed from the transcription vector, pSP65-13. pSP65-13 contains the 0.69-kb Pst I insert of pHD84. These anti-exon transcripts were also used in solution hybridization studies to determine the levels of dihydrofolate reductase mRNA. Pre-mRNA molecules generally contribute only a very small amount to the total exon-containing RNA pool. This is evidenced by previous Northern blot analysis with radiolabeled dihydrofolate reductase cDNA in which the presence of dihydrofolate reductase pre-mRNA species is undetectable even though the dihydrofolate reductase mRNA species are readily apparent (10, 17). Thus, although pSP65-13 anti-exon transcripts will hybridize to both pre-mRNA and mature messages, the contribution by pre-mRNA molecules to the observed level of hybridization will be very small.

It was reasoned that a dihydrofolate reductase intron-specific probe should hybridize to a much greater extent with RNA isolated from KB7B cells as opposed to KB. Indeed, solution hybridization studies utilizing anti-dihydrofolate reductase coding sequence transcripts from pSP65-13 revealed that KB7B cells contain approximately 140–250 times more dihydrofolate reductase coding sequences per μg of total RNA than parental cells (data not shown). The extent of hybridization at various RNA concentrations with total RNA from KB and KB7B cells was determined for p0.69EH and p1.1ES ^{35}S-labeled transcripts (Fig. 2). As can be seen in Fig. 2A, there is no significant difference in the number of RNA hybrids formed between p1.1ES transcripts and total RNA from KB versus

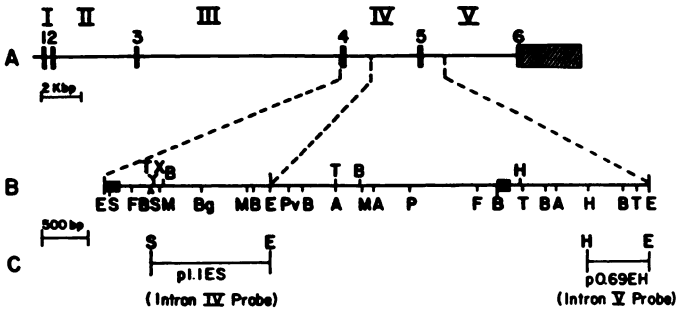

Fig. 1. Organization of the human dihydrofolate reductase gene and origin of intervening sequence probes. A. The organization of the human dihydrofolate reductase gene is drawn approximately to scale and adapted from Yang *et al.* (30). *Solid boxes* represent dihydrofolate reductase coding sequences, *line segments* labeled with *roman numerals* represent intervening sequences, and the *hatched box* represents 3′ untranslated sequences. B. Fine restriction site map of two cloned, contiguous EcoRI fragments. Restriction enzyme designations are as follows: *A*, Ava I; *B*, BstNI; *Bg*, Bg1 II; *E*, EcoRI; *F*, Fnu 4HI; *H*, Hind III; *M*, Msp I; *P*, Pst I; *Pv*, Pvu II; *S*, Sst I; *T*, Taq I; *X*, Xho I. *C*. Restriction enzyme fragments isolated and cloned into pSP65 transcription vector.

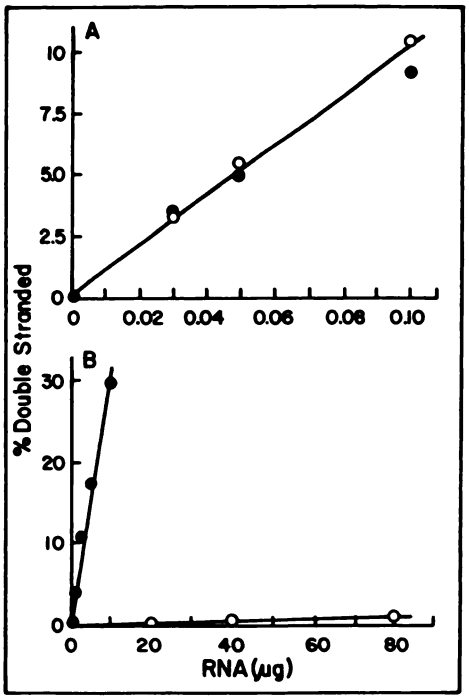

Fig. 2. Extent of solution hybridization of p1.1ES and p0.69EH ^{35}S-transcripts with KB vs. KB7B total RNA. A represents results obtained with p1.1ES ^{35}S-transcripts. B was generated using p0.69EH ^{35}S-transcripts. Solution hybridizations were performed as described in Materials and Methods. All data points represent the average of three determinations at a given RNA concentration. Standard deviation values did not exceed ±1.3% and, typically, were less than ±0.5%. ○, KB RNA, ● KB7B RNA.

KB7B cells. These results indicate that p1.1ES anti-intron IV transcripts are homologous to RNA sequence elements which are found to the same extent in both KB and KB7B cells and, therefore, are not specific for dihydrofolate reductase intron IV. Conversely, p0.69EH transcripts hybridize to a much greater extent with KB7B total RNA (Fig. 2B). Indeed, KB7B total RNA contains a 220-fold enrichment over the parental cell line of sequences complementary to p0.69EH transcripts. This level is comparable to the 140–250-fold increase of dihydrofolate reductase coding sequences in KB7B cells compared to the parental KB cell line and therefore suggests that p0.69EH transcripts are specific for dihydrofolate reductase RNA.

To further substantiate the intron-specific hybridizing character of p0.69EH transcripts, solution hybridization experiments were performed with various classes of KB7B RNA (Table 1). The extent of hybridization of pSP65-13 (i.e., exon specific) transcripts was determined as well. It is apparent from the data presented in Table 1 that RNA sequences complementary to pSP65-13 transcripts are much more abundant (36 times) than sequences which are complementary to p0.69EH transcripts. This is an expected result, as the number of mRNA molecules for a given RNA species is generally much larger than the number of precursor mRNA molecules in the steady state. It is also apparent that RNA complementary to p0.69EH transcripts predominantes in the nuclear RNA as opposed to the cytoplasmic RNA fraction. In particular, nuclear RNA contains a 100-fold enrichment over cytoplasmic RNA of sequences complementary to p0.69EH transcripts. In contrast, RNA complementary to pSP65-13 transcripts predominates in the cytoplasmic fraction. The product-precursor relationship of unprocessed, intron-containing pre-mRNAs with mRNA would account for a low level of hybridization of a coding sequence probe with nuclear RNA. The apparently high level of hybridization of anti-coding sequence transcripts with nuclear RNA can be explained by the lower percentage of rRNA sequences found in nuclear RNA as opposed to cytoplasmic RNA (36% versus 96% in mouse L cells) (27). This difference can account for a 16-fold increase in the level of hybridization per μg of RNA with nuclear versus cytoplasmic RNA fractions. In addition, the nuclear RNA fraction contains dihydrofolate reductase mRNA. This is apparent from Northern blot analysis which reveals a relatively high level of dihydrofolate reductase mRNA species in the nuclear RNA fraction (data not shown).

TABLE 1

Level of hybridization of p0.69EH and pSP65-13 ^{35}S-transcripts with various classes of RNA

RNA Class	dpm Hybridized/μg (±SD)[a]		Exon/intron ratio[b]
	p0.69 EH (intron)	pSP65-13 (exon)	
Total	$1.31 \pm 0.05 \times 10^3$	$4.67 \pm 0.05 \times 10^4$	36
Nuclear	$6.36 \pm 0.27 \times 10^3$	$2.74 \pm 0.04 \times 10^4$	4
Cytoplasmic	$6.40 \pm 0.60 \times 10$	$4.87 \pm 0.09 \times 10^4$	760
poly(A)	$1.78 \pm 0.18 \times 10^4$	$7.95 \pm 0.06 \times 10^5$	45
Non-poly(A)[c]	$2.42 \pm 0.04 \times 10^2$	$6.53 \pm 1.17 \times 10^3$	27

[a] RNAs were prepared and solution hybridizations were performed as described in Materials and Methods. Values were determined from triplicate determinations at three different RNA concentrations in the linear hybridization range. The dpm hybridized/μg of RNA values were normalized to account for differences in specific activity due to radioactive decay.

[b] p0.69 EH and pSP65-13 are the same length (690 bp) and, therefore, dpm hybridized are directly comparable.

[c] Non-poly(A) RNA is defined as that RNA which does not bind to a poly(U) Sephadex column after two individual applications under the conditions previously described (17).

The poly(A)-fractionated RNA contains greatly enhanced levels of RNA complementary to both probes. These results indicate that p0.69EH transcripts are hybridizing with polyadenylated dihydrofolate reductase pre-mRNA and not merely to excised intron V sequences. The large difference in dpm hybridized/μg of total, as opposed to non-poly(A) RNA, further indicates that p0.69EH transcripts form hybrids to a substantially greater extent with polyadenylated pre-mRNA than with non-polyadenylated excised intron. Thus, the results shown in Fig. 2 and Table 1 support the assertion that p0.69EH transcripts hybridize specifically with dihydrofolate reductase pre-mRNAs containing intron V sequences.

In order to determine whether p0.69EH is complementary to discrete, high molecular weight dihydrofolate reductase RNA species, Northern blot analysis of poly(A) RNA from KB and KB7B cells was performed. As shown in Fig. 3, lane *B*, ^{32}P-nick-translated p0.69EH hybridizes with two major RNA species, 4.7 and 8.5 kb in length. These species are larger than the 3.6- and 1.0-kb mRNA species which are typically observed with a dihydrofolate reductase cDNA probe (Fig. 3, lane *D*). The absence of any detectable hybridization with KB poly(A) RNA and the appearance of discrete high molecular weight bands indicate that p0.69EH is specific for dihydrofolate reductase intron-containing RNA. In order to establish that the 4.7- and 8.5-kb species are mRNA precursors, it is essential to demonstrate that these species contain dihydrofolate reductase exon sequences as well. Recent Northern blot analysis utilizing nuclear poly(A) RNA from KB7B cells has revealed that the 8.5-kb species does contain dihydrofolate reductase exon sequences.[2] However, hybridization of the exon probe with the 4.7-kb species was obscured by the signal of the 3.6-kb band. Hence, at this time, we cannot conclude definitively that the 4.7-kb species is a dihydrofolate reductase mRNA precursor. Nonetheless, these Northern blot analyses further support the conclusion that p0.69EH is specific for dihydrofolate reductase intron V-containing RNA.

The construction of a probe specific for dihydrofolate reductase pre-mRNAs was accomplished in order to study the effect of FUra on pre-mRNA metabolism. Recent studies in our laboratory demonstrated a dose-dependent increase in the steady state level of dihydrofolate reductase mRNA in FUra-treated cells (10, 17). We were interested in determining whether pre-mRNA levels are also affected by FUra incorporation into RNA. Solution hybridization studies utilizing total RNA isolated from KB7B cells exposed to 0–3 μM FUra for 5 days were performed with both anti-exon (pSP65-13) and anti-intron V (p0.69EH) transcripts. As shown in Fig. 4, there is a striking dose-dependent increase in the amount of RNA complementary to p0.69EH transcripts present in total RNA isolated from FUra-treated cells. Dihydrofolate reductase mRNA levels also increase, but to a lesser extent, and decline at the highest FUra concentration. One study revealed increases in dihydrofolate reductase mRNA of 2.4- and 1.8-fold at FUra concentrations of 2 and 3 μM, respectively, and increases in dihydrofolate reductase intron V-containing transcripts of 4.4- and 5.0-fold at these same concentrations (Fig. 4). Typically, the level of dihydrofolate reductase intron V-containing transcripts increased 1.7–2.7-fold over DHFR mRNA in the 2–3 μM FUra range. Hence, these results suggest that there is a dispro-

[2] C. L. Will and B. J. Dolnick, unpublished observation.

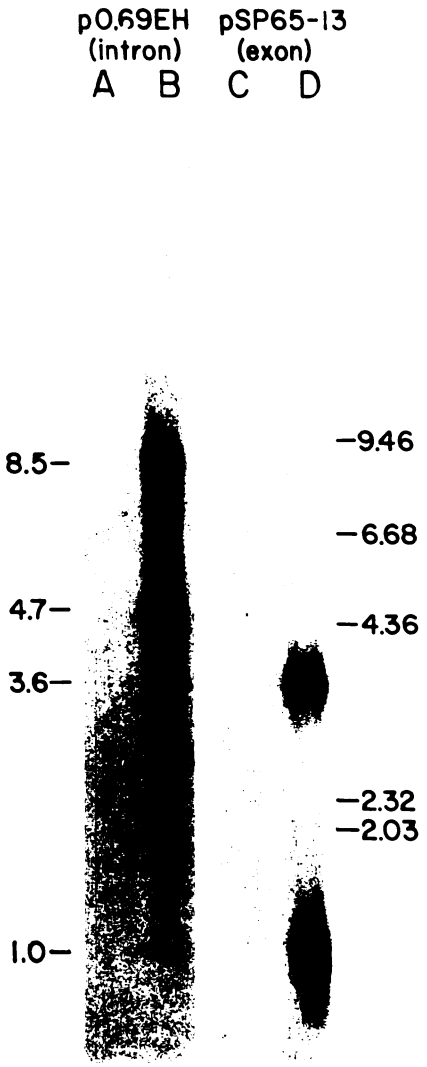

Fig. 3. Northern blot of poly(A) RNA isolated from KB and KB7B cells. poly(A) RNA was prepared from KB (lanes *A* and *C*) and KB7B (lanes *B* and *D*) cells as described in Materials and Methods. Twenty μg of each poly(A) RNA were denatured, electrophoresed, and transferred to Hybond N membrane as described in Materials and Methods. The membrane was hybridized first with ^{32}P-labeled p0.69EH (lanes *A* and *B*), dehybridized, and rehybridized with ^{32}P-labeled pSP65-13 (lanes *C* and *D*). Lanes *A* and *B* were exposed for 6.5 hr, whereas lanes *C* and *D* were exposed for only 0.5 hr. The species of dihydrofolate reductase mRNA and intron-containing RNA (kilobases) are indicated in the *left* margin and the positions of the Hind III cut λ DNA markers are on the *right* (kilobases).

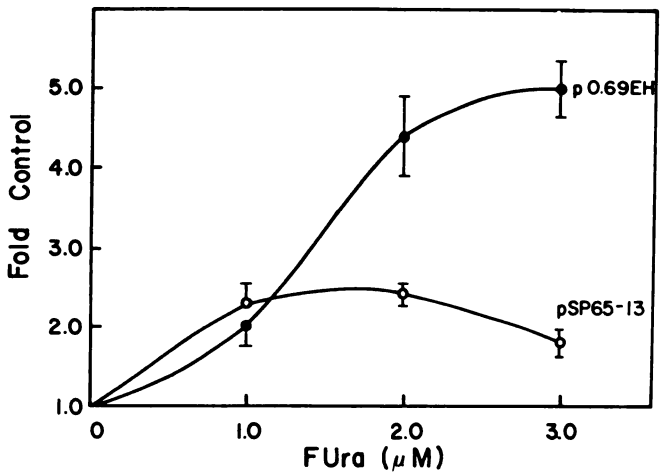

Fig. 4. The effect of FUra on the levels of KB7B RNA containing dihydrofolate reductase intron V sequences vs. dihydrofolate reductase exon sequences. Solution hybridization were performed on KB7B total RNA isolated from cells grown for 5 days in the presence of FUra at the indicated concentrations (see Ref. 10). The -fold increase over control was calculated from the averages of two or three determinations at three to four different RNA concentrations, within the linear range of hybridization, at each drug concentration. ○, pSP65-13 transcripts; ●, p0.69EH transcripts.

portionate FUra-induced increase in the steady state level of dihydrofolate reductase pre-mRNA over that of mature dihydrofolate reductase mRNA.

Discussion

Recent studies in our laboratory have provided evidence which suggests that one possible mechanism by which FUra incorporation into RNA leads to growth inhibition and cell death is through a decrease in the fidelity of translation resulting in increased levels of nonfunctional or functionally compromised protein (17). The experiments described here were undertaken to determine whether additional RNA-mediated mechanisms for FUra-induced cytotoxicity exist. In particular, we wished to determine what effect FUra had on the metabolism of mRNA precursors. Studies involving FUra effects on nuclear RNA have almost exclusively focused on rRNA precursors (7, 15, 16). Exposing murine leukemia cells to FUra for relatively short periods of time can result in an increase in dihydrofolate reductase exon sequences within the nucleus without a concurrent increase in the cytoplasm (20). These studies, however, do not distinguish between an increase in actual dihydrofolate reductase precursor mRNA and an increase in mature dihydrofolate reductase mRNA present within the nucleus. The results we present here demonstrate a novel effect of FUra on dihydrofolate reductase pre-mRNA molecules. Specifically, we observe a dose-dependent, disproportionate increase in intron-containing dihydrofolate reductase RNA over dihydrofolate reductase mRNA in FUra-treated KB7B cells. These results are consistent with the hypothesis that FUra incorporation into RNA may lead to inhibition of the conversion of pre-mRNA into mature messages. However, it should be noted that the results presented in Fig. 4 can also be explained by an increase in excised intron V sequences as opposed to an increase in actual pre-mRNA species. A more qualitative analysis of the effect of FUra on dihydrofolate reductase intron-containing RNA is currently under way in our laboratory and should allow us to distinguish between an increase in pre-mRNA versus excised intron molecules.

An increase in pre-mRNA levels can be achieved by an increase in the rate of transcription and/or a decrease in the rate of degradation or conversion to mRNA (i.e., increased half-life). An increase in the steady state levels of pre-mRNA should result in a proportional increase in mRNA levels for a given RNA species provided the rate of mRNA degradation does not increase and/or the rate of conversion of pre-mRNA molecules to mature messages does not decrease. Thus, one possible explanation for a disproportionate increase in the steady state levels of pre-mRNA versus mRNA is the inhibition of one of the steps required to convert a larger pre-mRNA molecule into a mature message. These steps include the repeated removal of intervening sequences and subsequent religation of exons. A

sufficient level of FUra incorporation into RNA may inhibit this process. It should be noted that FUra incorporation into the precursor RNA itself may not be the critical event for such inhibition. Rather, FUra substitution into other RNA species suspected to be involved in RNA processing, namely small nuclear RNAs, may give rise to processing inefficiency (28). The fact that small nuclear U-RNAs contain relatively high percentages of uracil (28) should make them particularly susceptible to structural and functional changes induced by FUra substitution. Indeed, Armstrong *et al.* (29) have demonstrated apparent structural changes in U4 and U6 snRNA, and an increase in the level of U1 small nuclear RNA, in 5-fluorouridine-treated S-180 cells. *In vitro* splicing systems could be used to determine whether FUra-substituted pre-mRNA alone is less efficiently processed. Such studies are currently under investigation in our laboratory.

These studies further confirm that FUra incorporation into RNA stimulates the production of dihydrofolate reductase mRNA in KB7B cells. Short-term labeling and pulse-chase experiments should reveal whether this increase is due to enhanced transcription of dihydrofolate reductase pre-mRNA or increased stability of the precursor and/or messenger RNAs. Possible reasons for an FUra-induced increase in dihydrofolate reductase mRNA levels were addressed previously (10, 17). One possible explanation was that FUra-induced miscoding and the subsequent production of structurally and/or functionally altered dihydrofolate reductase created a regulatory signal to produce more dihydrofolate reductase mRNA in order to compensate for reduced enzyme activity. It was further hypothesized that FUra-induced miscoding during general protein synthesis would, thus, create a metabolic stress on cells which could eventually lead to metabolic exhaustion and cell death. Data presented here extend this contention; that is, inhibition of pre-mRNA processing could contribute to growth inhibition and cell death by creating additional metabolic demands on the cell.

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