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5-Fluorouracil Alters Dihydrofolate Reductase Pre-mRNA Splicing as Determined by Quantitative Polymerase Chain Reaction

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SUMMARY

A quantitative polymerase chain reaction (PCR) assay has been developed to determine the absolute and relative amounts of each dihydrofolate reductase RNA species present at different stages of splicing (i.e., pre-mRNA, splicing intermediate, and mRNA). The ratios of each RNA species as measured by quantitative PCR have been confirmed by S1 nuclease mapping analysis. Quantitative PCR studies reveal a concentration-dependent decrease in the levels of dihydrofolate reductase mRNA and a splicing intermediate but little change in pre-mRNA levels after long term exposure of cells to 5-fluorouracil (FUra). The observed changes correlate with the extent of FUra incorporation into RNA and with cytotoxicity. These results, together with previous data from our laboratory, provide the first direct evidence that FUra incorporation into RNA can cause inhibition of pre-mRNA splicing *in vivo*. Inhibition of pre-mRNA splicing is thus a likely additional mechanism by which FUra incorporation into RNA may lead to growth inhibition and cell death.

The pyrimidine analog FUra has been extensively used to treat a variety of human solid tumors (1) since its introduction into clinical studies >30 years ago. However, its precise mechanism of action is still controversial. Three mechanisms for its cytotoxicity have been proposed (2). A FUra metabolite, 5-fluorodeoxyuridylate, has been shown to be a tight-binding inhibitor of thymidylate synthase. This enzyme catalyzes the only cellular reaction for the *de novo* synthesis of thymidylate (3). FUra residues have been detected in DNA both *in vivo* and in tissue culture. This incorporation has been related to increased DNA strand breakage (4, 5). Finally, FUra, via its conversion to 5-fluorouridine triphosphate, is incorporated into RNA, which can cause alterations in the structure and/or function of RNA (2, 6). The major site of FUra action may vary between cell types and may be dependent on the drug concentration and duration of exposure, as well as the presence of exogenous metabolites that can modify FUra action (1).

Incorporation of FUra into RNA is an important element of FUra cytotoxicity (2, 7-9), yet the precise mechanism of this RNA-directed cytotoxic activity is still not clear. The existence of many types of RNA suggests that there could be multiple RNA effects responsible for RNA-mediated cytotoxicity. The effects of FUra incorporation into various RNA species have been investigated in a number of studies. The first demonstration of a FUra effect on RNA in mammalian cells was the inhibition of 45 S rRNA precursor processing to mature 18 S and 28 S rRNA (10-12). Incorporation into *Escherichia coli* tRNAs has been shown to result in a decreased ability of certain tRNAs to be aminoacylated (13). Moreover, Armstrong *et al.* (14) demonstrated apparent conformational changes in U4 and U6 snRNA and an increase in the level of U1 snRNA in FUra-treated mouse S-180 tumor cells. Treatment of Hela cells with 5-fluorouridine resulted in decreased levels of U2 snRNA and the corresponding ribonucleoprotein particle, U2 snRNP (37).

To better understand the effects of FUra on mRNA metabolism, our laboratory has used DHFR gene-amplified human cell lines (i.e., KB7B and 1BT) to monitor FUra effects on a single class of mRNA. The results have shown that FUra incorporation into DHFR mRNA may cause translational miscoding, which could be a mechanism of RNA-mediated cytotoxicity (15). Other studies that focused on mRNA metabolism reported increased DHFR pre-mRNA levels, relative to mRNA levels (as determined by solution hybridization and nuclease mapping) (16-18), and a decreased rate of conversion of nuclear mRNA to cytoplasmic mRNA in cells exposed to FUra. These studies suggested the FUra may inhibit processing of pre-mRNA and/or affect the stability of nuclear DHFR mRNA (18).

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ABBREVIATIONS: FUra, 5-fluorouracil; MTX, methotrexate; DHFR, dihydrofolate reductase; PCR, polymerase chain reaction; snRNP, small nuclear ribonucleoprotein; snRNA, small nuclear RNA; bp, base pair(s).

To further define the effects of FUra on DHFR mRNA processing, we have developed a highly sensitive and quantitative PCR assay to monitor the level of each DHFR RNA species present throughout the splicing process. This assay uses a series of competitive templates and is based upon the principles described by others (19–21). This quantitative PCR assay has now allowed the measurement of levels of DHFR pre-mRNA and of a DHFR splicing intermediate RNA in a cell line that does not exhibit DHFR gene amplification and DHFR mRNA overexpression (i.e., KB cells). The similar ratios in the levels of DHFR RNA species representing different stages in the pre-mRNA splicing pathway in KB and 1BT cells indicate that the dynamics of pre-mRNA splicing for an overexpressed gene are similar to those for the same gene when not overexpressed. This assay has been used to examine the effect of cellular exposure to FUra on the splicing of DHFR pre-mRNA. The results reveal that there is a concentration-dependent decrease in the levels of both mRNA and a splicing intermediate but little change in pre-mRNA after long term exposure of cells to low concentrations of FUra. Changes in the levels of mRNA and the splicing intermediate correlate with the extent of FUra incorporation into RNA. The data indicate that FUra incorporation into RNA can cause inhibition of pre-mRNA splicing, which could contribute to the RNA-mediated cytotoxicity of FUra.

Materials and Methods

Chemicals. Restriction endonucleases were obtained from Promega Biotec (Madison, WI), New England Biolabs (Beverly, MA), or GIBCO (Grand Island, NY)/BRL (Gaithersburg, MD). SP6 and T7 RNA polymerases were obtained from New England Biolabs. Moloney murine leukemia virus reverse transcriptase and Amplitaq DNA polymerase were obtained from Promega Biotec and United States Biochemicals (Cleveland, OH), respectively. S1 nuclease, guanidine thiocyanate, thymidine, and FUra were purchased from Sigma Chemical Co. (St. Louis, MO). MTX was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. [^{3}H]UTP (35.2 Ci/mmol), [6-^{3}H]FUra (20 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), and [α-^{32}P]CTP (3000 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA) and DuPont/New England Nuclear (Boston, MA). Ultrapure cesium chloride, RPMI 1640 medium, and dialyzed horse serum were obtained from GIBCO/BRL.

Cell culture. The properties and maintenance of the human KB cell line and its MTX-resistant subline 1BT, used for this and previous studies, have been described previously (9, 18). Both cell lines were maintained in RPMI 1640 medium containing 5% dialyzed horse serum, at 37° in a 5% CO_2/air atmosphere; 1BT cells were maintained in the presence of 50 μM MTX. Cell lines were assayed every 3 months for *Mycoplasma* contamination with a GEN-probe (San Diego, CA) *Mycoplasma* rapid detection kit and consistently tested negative.

Nucleic acid preparation. Total cellular RNA was isolated from KB and 1BT cells by extraction and CsCl gradient centrifugation as described (22, 23). Poly(A)$^+$ RNA was purified from total cellular RNA using the PolyATract mRNA isolation system from Promega Biotec and was quantitated spectrophotometrically (24).

Oligonucleotides. DNA oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer, using phosphoramidite chemistry in the trityl-off mode. After cleavage and deprotection at 55° overnight with concentrated ammonium hydroxide, oligonucleotides were dried under vacuum, redissolved in 50 mM triethylammonium acetate, and purified by high pressure liquid chromatography as described previously (25).

Quantitative PCR. Quantitative PCR was performed based upon the competitive template approach (19–21). The primers and three competitive template RNAs (i.e., internal standards) used for quantitation of DHFR mRNA, pre-mRNA, and pre-mRNA plus splicing intermediate, and their orientations to their respective target RNAs, are shown schematically in Fig. 1.

The competitive template cDNAs were generated by site-specific mutagenesis using PCR, as described by Perrin and Gilliland (26) and Ho *et al.* (27). cDNA fragments (generated by PCR) corresponding to the target RNAs to be amplified for quantitative PCR were mutated to introduce novel *Sma*I restriction sites at the locations indicated (Fig. 1). For the pre-mRNA plus splicing intermediate internal standard, TTA (positions 1112–1114 of the human DHFR gene) was mutated to CCG to introduce a *Sma*I site. For the pre-mRNA internal standard, two thymidine residues (at positions 1169 and 1172) were mutated to cytidine and guanosine, respectively, to introduce a *Sma*I site. The pre-mRNA plus splicing intermediate internal standard cDNA was cloned into the *Eco*RI/*Hind*III site of pGEM-I to generate construct pHDI$_2$E$_3$-I, and the pre-mRNA internal standard cDNA was cloned into the *Eco*RI/*Pvu*II site to generate construct pHDI$_2$E$_3$. The DHFR mRNA internal standard has been described previously (25). To generate RNAs to be added as internal standards to the reverse transcription reactions, plasmids containing mutant inserts were linearized by restriction enzymes (*Pvu*II for the pre-mRNA internal standard and *Hind*III for the pre-mRNA plus intermediate internal standard). ^{3}H-labeled RNAs were then generated from each by *in vitro* transcription with either SP6 (for pHDI$_2$E$_3$) or T7 RNA polymerase (for pHDI$_2$E$_3$-I), in the presence of [^{3}H]UTP. After digestion of the plasmid DNA with DNAse, ^{3}H-labeled RNAs were purified by Sephadex G-75 column chromatography. ^{3}H-labeled RNAs were quantitated by determining [^{3}H]UTP incorporation, with confirmation by UV spectrophotometry (25). The size and integrity of the internal standard RNAs were confirmed by Northern blot analysis (data not shown).

Fig. 1 shows the general process for eukaryotic splicing of pre-mRNA (28, 29). The sense primer 277 (5′-CTGGCCAGGAATTGATTAT-3′, positions 1002–1020 of the human DHFR gene) (30), designed for amplification of DHFR pre-mRNA, corresponds to a region contained within intron II, 115 bases 5′ to the intron II-exon 3 junction. Because most branch points occur 19–30 bases upstream of a splice-accepting exon (28), primer 277 was expected to correspond to a region upstream of the branch point. The antisense primer 278 (5′-GAGTTCTCTGCT-

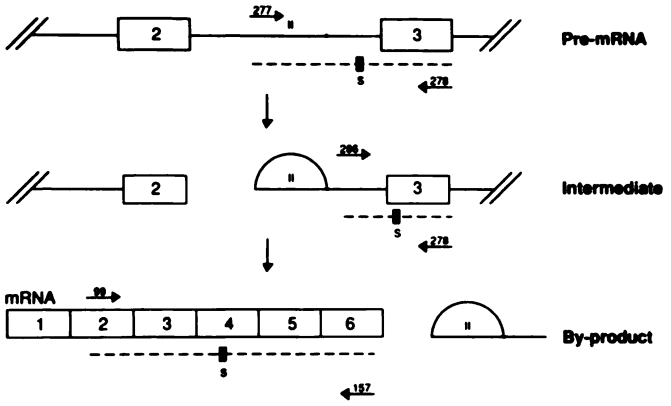

Fig. 1. Strategy for DHFR RNA species quantitation by PCR. The process of splicing, with relevant exons and introns, is depicted. *Open boxes (arabic numerals)*, exons; *solid line*, intron II. *Dashed lines below each RNA species*, regions mutated to generate internal standard RNAs (*vertical bars*, novel *Sma*I restriction sites) and the regions bordered by the primers used for quantitative PCR. *Rightward arrows above RNAs*, sense primers; *leftward arrows below RNAs*, antisense primers. Each primer is indicated by its respective numerical designation. *, Branch point for lariat formation. A detailed explanation of the strategy for the determination of each DHFR RNA species (pre-mRNA, splicing intermediate, and mRNA) by quantitative PCR is given in Materials and Methods. *S*, *Sma*I. Not drawn to scale.

GAGAACTAAAT-3′, positions 1317–1337 of the human DHFR gene) is complementary to a region within exon 3. Thus, PCR using the primer pair 277 and 278 should amplify only DHFR pre-mRNA. Amplification of the lariat splicing intermediate should be prevented by blockage of reverse transcriptase by the branch point during generation of cDNA before PCR. The expected products from amplification of the pre-mRNA and its internal standard (using primer pair 277 and 278) are both 238-bp long, whereas the internal standard RNA amplification product yields 111- and 127-bp fragments after *Sma*I digestion. The sense primer 286 (5′-GCTTTCTTTGTGATTTTATAGGT-3′, positions 1115–1137 of the human DHFR gene) corresponds to a region in intron II, 3′ to the suspected branch point. Therefore, the primer pair consisting of primers 286 and 278 should detect both the pre-mRNA and the lariat form splicing intermediate. The PCR products resulting from amplification of either cellular RNA or the pre-mRNA plus splicing intermediate internal standard using primer pair 286 and 278 are each 125-bp long. However, after digestion with *Sma*I, the PCR product derived from the internal standard yields DNA fragments that are 57- and 68-bp long. The level of the lariat splicing intermediate is derived by subtracting the calculated amount of pre-mRNA (determined by quantitative PCR) from the calculated amount of pre-mRNA plus splicing intermediate. Primer pair 99 (sense, 5′-CAGAGAATGACCACAACCTC-3′, corresponding to a region within exon 2) and 157 (antisense, 5′-CACCTTCATATTAATCATTCTTCTC-3′, complementary to a region within exon 6) (25) were used for quantitative PCR of mRNA, as described previously (25). The use of this primer pair does not allow amplification of pre-mRNA reverse transcripts, probably because the PCR product is too long to be amplified under the conditions of the assay. The DNA fragment resulting from the PCR product derived from the reverse transcript of DHFR mRNA using primer pair 99 and 157 is 453 bp, and those arising from the internal standard are 282 and 171 bp, after *Sma*I digestion (25).

For cDNA synthesis and subsequent PCR, 100 ng of RNA were combined with various amounts of an internal standard ^{3}H-labeled RNA and a reaction cocktail to obtain a final concentration of 1× PCR buffer (50 mM Tris·HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001%, w/v, gelatin), 1 mM levels of each of the four deoxynucleoside triphosphates, 0.5 μM antisense primer, additional $MgCl_2$ to 5 mM final concentration, and 10 units of Moloney murine leukemia virus reverse transcriptase, in a 10-μl volume. Reactions were incubated at 42° for 10 min and then at 95° for 2 min. Reactions were stored at 4° until used for PCR.

PCR was performed by combining 1 μl of a reverse transcription reaction with 9 μl of a PCR cocktail to yield a final concentration of 1× PCR buffer, 0.1 mM levels of each of the four deoxynucleoside triphosphates, 0.5 μM levels of each of the sense and antisense primers, 2.8 mM $MgCl_2$, 1 μCi of [α-^{32}P]dCTP, and 0.03 units/μl Amplitaq DNA polymerase. Reactions were overlaid with mineral oil and amplification was carried out by sequential cycling at 95° (30 sec), 55° (1 min), and 72° (3 min), for a total of 40 cycles, in a Perkin-Elmer Cetus thermal cycler (31). At the end of the last cycle the reactions were incubated at 72° (7 min). The reactions were then adjusted to contain 7 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 9 units of *Sma*I, in a final volume of 12.5 μl, and were incubated at room temperature overnight (≥16 hr). After *Sma*I digestion, 10 μl of each reaction were resolved by electrophoresis as described previously (25). The gel was then dried under vacuum (80° for 30 min) and scanned with an AMBIS radioimager to quantitate each ^{32}P-labeled DNA species. Each experiment included two control reactions in which the reverse transcription reactions (without cellular RNA) were established with or without internal standard RNA. The amounts of cellular DHFR RNA species were determined by comparing the relative radioactive contents of PCR products derived from DHFR RNA with those of the respective internal standard PCR products in each reaction tube after *Sma*I digestion. This approach provides a control for the intertube variability commonly observed with PCR (19–21, 25).

S1 nuclease mapping. S1 nuclease mapping studies were carried out as previously described by Durnam and Palmiter (32), with minor modifications (16). The probe used for these studies was generated by PCR, using the primer pair 277 and 378 (i.e., primer 278 with a T7 promoter sequence at its 5′ end) to amplify the appropriate region of the DHFR gene with KB genomic DNA as a template, followed by *in vitro* transcription of the amplified DNA with T7 RNA polymerase (33).

Results

DHFR RNA quantitation by PCR. Solution hybridization and/or nuclease mapping have been used to determine the relative amounts of mRNA and pre-mRNA present in DHFR gene-amplified 1BT cells, which overexpress the various DHFR RNA species (17, 18). However, these methods do not allow detection of the DHFR splicing intermediate or DHFR pre-mRNA in the parental KB cells, due to their limited sensitivities. Quantitative PCR has been shown to be a rapid and sensitive method for RNA quantitation, and the ability to determine the absolute number of molecules of gene transcripts allows a more insightful analysis of gene expression. We thus attempted to develop a PCR-based assay to quantitate the various species of DHFR RNA expected to be present throughout the splicing process and to use this assay to study the effect of FUra on DHFR pre-mRNA splicing.

To test the feasibility of using the quantitative PCR strategy, each DHFR RNA species of interest in KB and 1BT cells was quantitated by PCR and the results were compared with previous data obtained by other means. Results from the reverse transcription and PCR for each DHFR RNA species (pre-mRNA, pre-mRNA plus splicing intermediate, and mRNA) and its respective internal standard are shown in Fig. 2A. After *Sma*I digestion, PCR products derived from DHFR RNA remained intact due to the absence of *Sma*I restriction sites, whereas products derived from amplification of the internal standards each generated two fragments of the expected sizes (see Materials and Methods). Fig. 2, B and C, demonstrates a typical quantitative PCR result, which in the figure is used to estimate the amount of splicing intermediate found in poly(A)$^+$ RNA from 1BT cells. Various amounts of internal standard RNA were mixed with a constant amount (i.e., 10 ng) of poly(A)$^+$ RNA. After reverse transcription, PCR, and *Sma*I digestion, the resultant ^{32}P-labeled DNAs were resolved by polyacrylamide gel electrophoresis and the individual bands were quantitated. The percentage of total cpm per lane, derived from cellular RNA or internal standard, was plotted as a function of the amount of internal standard added per reaction. When the amount of input internal standard RNA added to the reverse transcription reaction equals the amount of target RNA, the amount of ^{32}P-labeled product arising from the PCR for each is also equal. Because the number of input standard RNA molecules is known, a direct determination of the absolute number of molecules can be assessed from the graph (Fig. 2C). In general, PCR quantitation was found to be most reliable if the amount of the internal standard RNA in the reverse transcription reaction was within 10-fold of the amount of the RNA being quantitated.

The quantitative PCR strategies to measure the amounts of either DHFR pre-mRNA or the combination of pre-mRNA plus splicing intermediate used the same antisense primer (primer 278) but different sense primers. Although both sense primers corresponded to regions within intron II, primer 277

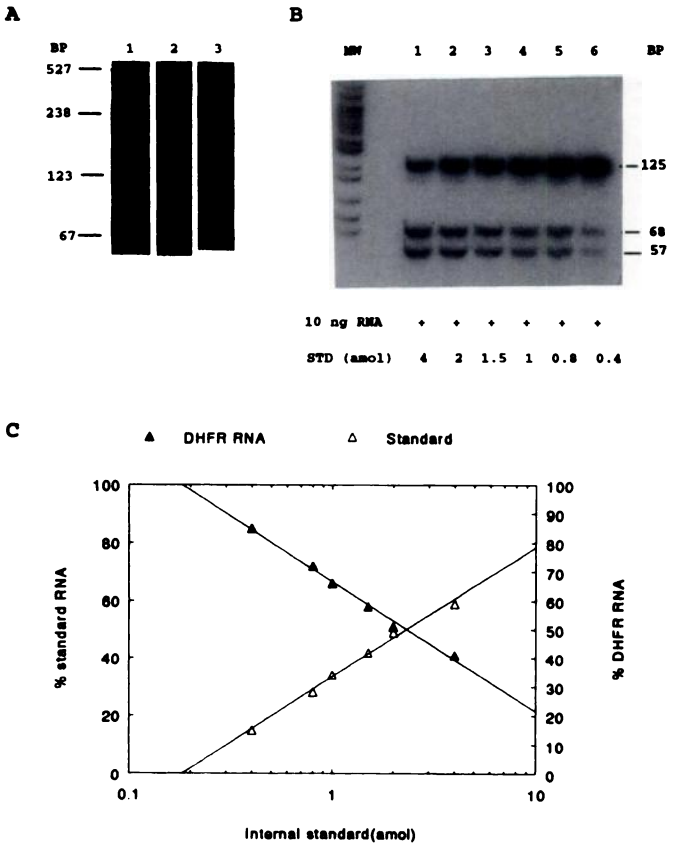

Fig. 2. PCR of DHFR RNA. A, Autoradiogram of the ^{32}P-labeled PCR products corresponding to each RNA species and their respective internal standards after *Sma*I digestion. The autoradiograms shown were overexposed to allow demonstration of minor RNA species protected from nuclease digestion. *Lane 1*, ^{32}P-labeled PCR products of DHFR pre-mRNA (238 bp) and its internal standard (111 and 127 bp); *lane 2*, PCR products of the DHFR pre-mRNA plus splicing intermediate (125 bp) and its internal standard (57 and 68 bp); *lane 3*, PCR products of DHFR mRNA (453 bp) and its internal standard (282 and 171 bp). B, Autoradiogram of a gel used for quantitation of the DHFR splicing intermediate. The amount of internal standard RNA (*STD*) added to each reverse transcription reaction was varied as indicated and the amount of poly(A)$^+$ RNA was maintained constant at 10 ng. C, Graphic representation of the data from B. The cpm of each band was determined by radioimaging and the percentage of total cpm per lane for the PCR products from the internal standard and the DHFR pre-mRNA plus splicing intermediate is plotted as a function of the amount of internal standard added. The DNA molecular weight marker (*MW* in B) is ^{32}P-labeled, *Msp*I-digested pBR322.

was chosen to be upstream of the suspected branch point to amplify only pre-mRNA, and primer 286 corresponded to a region downstream of the suspected branch point to allow amplification of the lariat intermediate in addition to the pre-mRNA (Fig. 1). If the hypothesis used for primer design is correct, then quantitative PCR using primer pair 286 and 278 should indicate that there is more DHFR RNA (i.e., pre-mRNA plus splicing intermediate) than does quantitative PCR using primer pair 277 and 278 (i.e., pre-mRNA only). The difference in the observed amount of DHFR RNA determined by these two sets of reactions should reflect the amount of splicing intermediate. As indicated in Table 1, in both KB and 1BT poly(A)$^+$ RNA the calculated amounts of DHFR pre-mRNA plus splicing intermediate were 2–4-fold higher than that of pre-mRNA. This suggests that we can now distinguish a DHFR pre-mRNA from a splicing intermediate by the choice of primers and we can measure the amounts of each RNA species.

TABLE 1

Quantitation of DHFR RNA species by PCR

RNA from KB and 1BT cells was prepared and quantitative PCR was performed as described in Materials and Methods. Each value represents the average ± standard deviation obtained from two to three different RNA preparations. See text for details.

RNA species	RNA amount		1BT/KB
	KB	1BT	
	amol/μg poly(A)$^+$ RNA		
Pre-mRNA	$1.25 \pm 0.2 \times 10^{-1}$	$3.25 \pm 0.35 \times 10^{1}$	260
Intermediate + pre-mRNA	$4.2 \pm 1.2 \times 10^{-1}$	$1.67 \pm 0.55 \times 10^{2}$	
Intermediate	2.95×10^{-1}	1.35×10^{2}	457
mRNA	$1.34 \pm 0.35 \times 10^{-2}$	$3.28 \pm 0.13 \times 10^{4}$	243

Table 1 shows the results of quantitation by PCR of each DHFR RNA species from KB and 1BT cells. Absolute levels of both pre-mRNA and mRNA were determined directly by quantitative PCR. Based upon the rationale presented above, the amount of the splicing intermediate was derived by subtracting the amount of pre-mRNA from the amount of pre-mRNA plus splicing intermediate. As indicated in Table 1, the ratio of DHFR mRNA content between 1BT and KB cells was 243:1, which compares well with the 240–250:1 ratio previously determined by solution hybridization and quantitative PCR (18, 25). The ratio of mRNA to pre-mRNA within each cell line was approximately 10^3:1. The similarity of these ratios for DHFR RNA species present before, during, and after splicing in each cell line suggests that, under steady state conditions, the overproduction of DHFR RNA in 1BT cells does not alter DHFR RNA processing. The quantitative PCR results indicate there is approximately 50-fold more DHFR mRNA/μg of RNA in the poly(A)$^+$ fraction, compared with unfractionated cellular RNA, for both KB and 1BT cells (data not shown). This also agrees with previous data indicating that DHFR RNA species are primarily polyadenylated in both cell lines.

Confirmation of PCR results by S1 nuclease mapping. S1 nuclease mapping analysis of total cellular RNA and poly(A)$^+$ RNA from 1BT cells was carried out to independently quantitate each RNA species and to substantiate results obtained by quantitative PCR. The results of this study are presented in Fig. 3. The observed nuclease-protected fragments of 238, 127, and 104 bp are the sizes expected to be protected by the DHFR pre-mRNA, splicing intermediate, and mRNA, respectively. The presence of additional nuclease-protected fragments of approximately 244 and 80 bp in length likely results from the heterogeneity of amplified DHFR genes present in the 1BT cell line, giving rise to DHFR RNA species with minor sequence heterogeneity (18). Thus, the 104- and 80-bp nuclease-protected fragments were both included for the quantitation of DHFR mRNA, the nuclease-protected 127-bp fragment was used for quantitation of the splicing intermediate, and the nuclease-protected 238- and 244-bp fragments were both used for quantitation of pre-mRNA. The results confirmed the existence of three RNA species in 1BT poly(A)$^+$ RNA, corresponding to the three RNA species assayed by quantitative PCR. The ratio of mRNA- to pre-mRNA-protected fragments was approximately 1400:1 in the poly(A)$^+$ RNA fraction, and the ratio of splicing intermediate- to pre-mRNA-protected fragments with poly(A)$^+$ RNA was approximately 2:1 (as determined by comparing the radioactive content of protected frag-

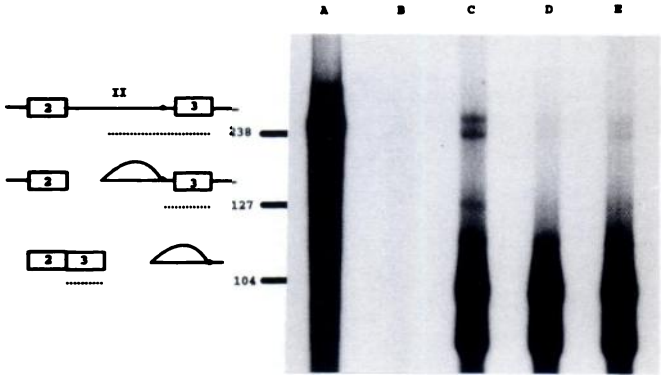

Fig. 3. S1 nuclease mapping. *Left*, schematic diagram showing each DHFR RNA species and the ^{32}P-labeled RNA fragments they are expected to protect from nuclease digestion (*dashed lines*). For an explanation of symbols see the legend to Fig. 1. The probe length is 244 bp. The length of the fragment protected by each RNA species is shown beside its respective dashed line. *Right*, autoradiogram of the ^{32}P-labeled RNA species protected from nuclease digestion after electrophoresis. Visualization of the low levels of pre-mRNA and splicing intermediate species required overexposure of bands corresponding to the undigested probe and mRNA species. *Lane A*, ^{32}P-labeled probe only without S1 nuclease treatment; *lane B*, ^{32}P-labeled probe only treated with S1 nuclease; *lane C*, 100 μg of total cellular 1BT RNA after probe hybridization and nuclease digestion; *lane D*, 1 μg of 1BT poly(A)$^+$ RNA after hybridization and digestion; *lane E*, 10 μg of poly(A)$^+$ RNA after probe hybridization and digestion.

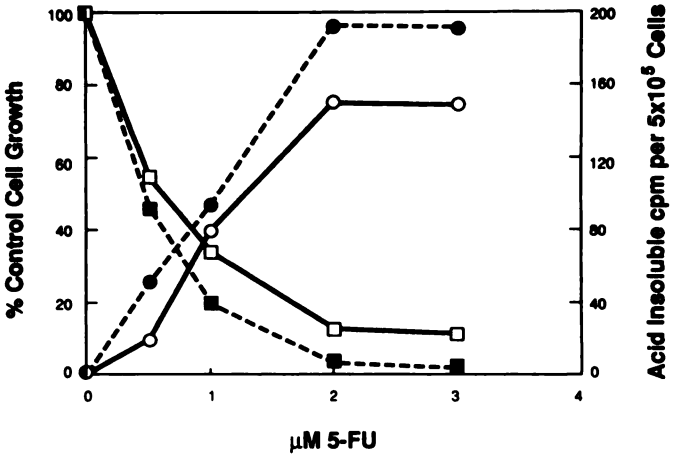

Fig. 4. Correlation between growth inhibition and FUra incorporation into RNA. 1BT cells were seeded in 25-cm^2 flasks on day 0, as described in Materials and Methods. Thymidine (30 μM) was included in the culture medium with or without 50 μM MTX. On day 1, [^{3}H]FUra (4 μCi/μmol) was added at the concentrations indicated. The cells were allowed to grow until day 6 and then were harvested, and an aliquot was counted. The remaining cells were pelleted by centrifugation and extracted with 10% ice-cold trichloroacetic acid. The acid-insoluble precipitates were collected by filtration through nitrocellulose filters, washed with 5% ice-cold trichloroacetic acid and 70% ethanol, dried, and counted in 2 ml of Aquasol. *Squares*, percentage of control cell growth (□, without MTX; ■, with MTX); *circles*, acid-insoluble cpm/5 × 10^5 cells (○, without MTX; ●, with MTX).

ments after normalization for molecular weight differences). The ratios of the various RNA species in 1BT poly(A)$^+$ RNA, determined by S1 nuclease mapping, are similar to those determined by quantitative PCR, indicating that PCR yields accurate quantitative data for the RNA species being studied.

Relationship of growth inhibition and DHFR RNA splicing to incorporation of FUra into RNA. Previous studies showed a relationship between growth inhibition and incorporation of FUra into RNA (7–9), a finding confirmed in the present study (Fig. 4). Pretreatment of cells with MTX is known to increase the incorporation of FUra into RNA (34). We therefore used the combination of MTX and FUra to increase FUra incorporation into RNA. Fig. 4 shows that FUra incorporation into 1BT RNA was increased in the presence of MTX. Because FUra incorporation into RNA correlated with growth inhibition of FUra and this incorporation could be enhanced by the addition of MTX, we asked whether these two methods of increasing FUra incorporation into RNA (i.e., FUra dose variation, with or without MTX) have an effect on DHFR pre-mRNA splicing. Results shown in Fig. 5 show a comparison of the levels of the different DHFR RNA species in 1BT cells found at three different FUra concentrations, in the presence or absence of MTX. 1BT cells were used for this study to facilitate detection of the various DHFR RNA species. As indicated, there was a concentration-dependent decrease in the levels of both DHFR mRNA and the assayed splicing intermediate with FUra treatment but little change in the DHFR pre-mRNA level. Whereas MTX at the concentration used had no effect upon the levels of any DHFR RNA species by itself, the addition of MTX to the culture medium before FUra resulted in an additional decrease in the levels of DHFR mRNA and the splicing intermediate but little change in the DHFR pre-mRNA level. Cells exposed to 1 μM FUra plus MTX for 6 days had 50% reduced levels of mRNA and 55% reduced levels of the splicing intermediate, compared with untreated cells.

Discussion

Previous studies in our laboratory, using solution hybridization and nuclease mapping assays, demonstrated an increase in pre-mRNA levels, relative to mRNA levels, and a decreased rate of conversion of nuclear DHFR mRNA to cytoplasmic mRNA after exposure of 1BT cells to FUra (18). Two possible explanations, i.e., that FUra may inhibit processing of pre-mRNA and/or affect the stability of nuclear DHFR mRNA, were proposed. To further differentiate these two possibilities, it was necessary to quantitate levels of a DHFR splicing intermediate. The limited sensitivity of nuclease protection and conventional blotting techniques prevents the addressing of these questions in cell lines that do not overexpress the RNA species of interest.

A strategy using quantitative PCR (Fig. 1) to measure the absolute amounts of each DHFR RNA species (pre-mRNA, mRNA, and splicing intermediate) with enhanced sensitivity, compared with dot blot, solution hybridization, nuclease protection, and Northern blot analyses, has been developed. Using this method, we can detect RNA species of low abundance in non-DHFR gene-amplified KB cells. Three RNA species, each of which is expected to be diagnostic of a different stage of splicing, have been successfully detected and quantitated with high precision by quantitative PCR in both KB and 1BT cells (Table 1). Standard deviations for the quantitation of each RNA species from different RNA preparations ranged from 10 to 30%. The results indicate that quantitative PCR can be used to detect RNA species of low abundance in cells, with little variation. The similarity in the ratio of mRNA levels between 1BT and KB cells detected by either solution hybridization or quantitative PCR and the similar ratios for each RNA species in poly(A)$^+$ RNA detected by quantitative PCR and S1 nuclease mapping (Fig. 3) validate quantitative PCR as a suitable

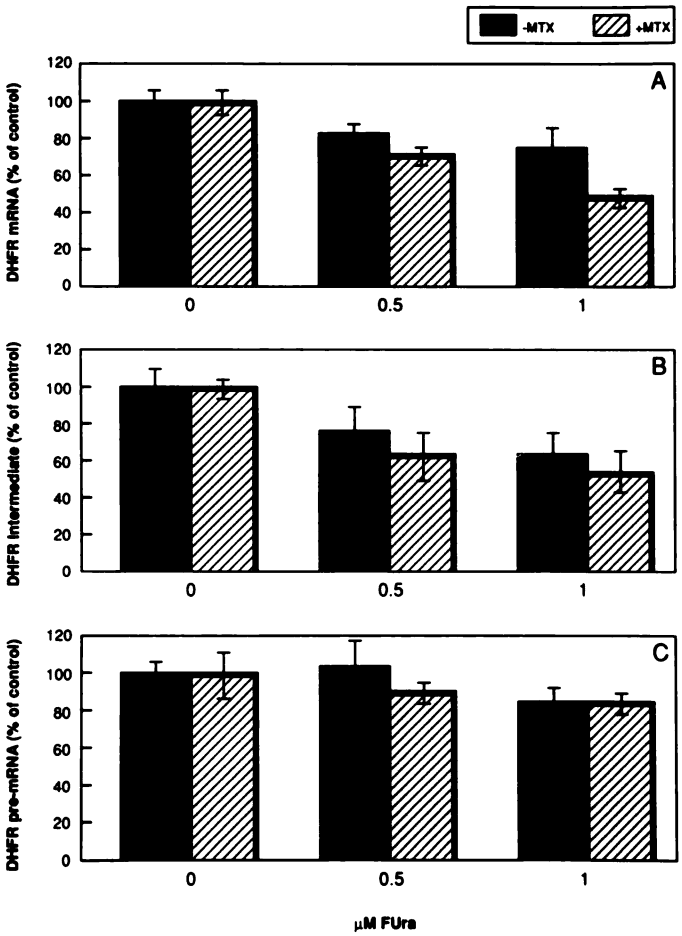

Fig. 5. Effect of FUra on DHFR RNA species, as determined by quantitative PCR. 1BT cells were seeded in 150-cm^2 flasks in medium containing 30 μM thymidine, in the absence or presence of 50 μM MTX. Cells were allowed to attach overnight and were treated the next day with various concentrations of FUra as indicated. After 6 days of treatment, cells were harvested and extracted. RNA preparation, purification, and quantitative PCR procedures are described in detail in Materials and Methods. A, Effect of FUra on DHFR mRNA levels; B, effect of FUra on levels of a DHFR splicing intermediate; C, effect of FUra on DHFR pre-mRNA levels. ■, Results obtained without MTX pretreatment; ▨, results obtained with MTX pretreatment. The bar heights represent the RNA levels relative to the control (i.e., no FUra); *error bars*, standard deviations derived from either two or three different measurements.

method to monitor gene expression. The similarity of ratios for each RNA species between KB and 1BT cells also suggests that overexpression of the DHFR gene probably does not significantly alter splicing of the overabundant DHFR pre-mRNA under steady state conditions and that 1BT cells are a suitable model for study.

Adventitious genomic DNA contamination in total cellular RNA preparations rendered the use of total RNA for quantitation by PCR impractical, and poly(A)$^+$ RNA was used. Trace amounts of genomic DNA were found to affect the results of pre-mRNA and splicing intermediate quantitation by PCR, due to the ability of the DNA to be significantly amplified along with the pre-mRNA and splicing intermediate cDNAs generated by reverse transcription.[1] This problem of genomic DNA contamination influencing the quantitative PCR results was undoubtedly exacerbated by the extremely low levels of DHFR pre-mRNA and splicing intermediate found in cells. Poly(A)$^+$ RNA purified using the PolyATract isolation system (Promega Biotec) appeared to be free of DNA contamination, because quantitation of pre-mRNA from poly(A)$^+$ RNA preparations remained the same with or without extensive DNase treatment (data not shown). Because it is not clear that all pre-mRNAs and splicing intermediates are polyadenylated, there is a risk that not all the pre-mRNA or splicing intermediate species are detected in the poly(A)$^+$ RNA fraction. Although detection of DHFR pre-mRNA and a splicing intermediate in KB and 1BT cell poly(A)$^+$ RNA (Table 1) indicates the presence of both DHFR pre-mRNA and splicing intermediate in poly(A)$^+$ RNA, there is a possibility that some of the splicing intermediate RNA corresponding to intron II and exon 3 may be present transiently in the non-poly(A)$^+$ fraction, because of the preexcision of downstream introns. An estimate of the DHFR splicing intermediate (on a per cell basis) by nuclease mapping revealed that >60% of the splicing intermediate (intron II-exon 3) was present in the poly(A)$^+$ RNA fraction (data not shown).

Growth inhibition of 1BT cells, after 6 days in the presence of FUra, appears to be mediated by incorporation of FUra into RNA (Fig. 4). Studies of the effect of FUra on DHFR RNA levels revealed a dose-dependent decrease in DHFR mRNA and a splicing intermediate but little change in pre-mRNA (Fig. 5). To further demonstrate the relationship between growth inhibition and incorporation of FUra into RNA, 50 μM MTX was included in the culture medium along with FUra. As demonstrated previously, MTX induces disruption of two early *de novo* purine biosynthesis steps, leading to the accumulation of 5′-phosphoribosyl-2′-pyrophosphate. This accumulation enhances the conversion of FUra to 5-fluorouridine monophosphate and its ultimate incorporation into RNA (34). Indeed, MTX pretreatment did increase the FUra incorporation into RNA in 1BT cells (Fig. 4). Because 50 μM MTX alone has a limited effect on 1BT cell growth, the increased growth inhibition by FUra in the presence of MTX is probably related to the increased incorporation of FUra into RNA. MTX pretreatment, which can increase incorporation of FUra into RNA and growth inhibition, further enhances the effect of FUra on the changes in the levels of DHFR mRNA and the splicing intermediate, although these effects are modest at the sublethal concentration of MTX used.

The steady state level of mRNA is determined by mRNA turnover and production. Because FUra has no effect on the half-life of DHFR mRNA (18), decreased mRNA levels after FUra treatment must be the result of decreased production. Because a reduced transcription rate would result in the decrease of all three RNA species (i.e., pre-mRNA, splicing intermediate, and mRNA) in FUra-treated cells, this cannot explain the selective decrease in mRNA and the splicing intermediate. Additionally, it was previously demonstrated that FUra does not affect transcription of the DHFR gene in this system (18). Previous studies from this laboratory suggested the possibility of decreased conversion of nuclear mRNA to cytoplasmic mRNA, possibly coupled with enhanced turnover of nuclear mRNA. If this were the case, the levels of splicing intermediate would be expected to remain constant, as would the levels of pre-mRNA, after FUra treatment. The concerted decrease in both mRNA and a splicing intermediate strongly suggests that splicing is directly affected by FUra incorporation. The lack of an expected increase in DHFR pre-mRNA levels in the FUra-

[1] X.-P. Wu and B. J. Dolnick, unpublished observations.

treated cells cannot be explained by altered turnover (18). A more likely explanation for the unaltered levels of DHFR pre-mRNA in drug-treated cells is that cleavage at the donor exon-intron is unaffected but one of the two other components of the splicing process (i.e., lariat formation and exon-exon ligation) is affected and one or more of the two splicing intermediate species are unstable.

From previous studies it is known that FUra replacement of approximately 2% of uridine residues in RNA correlates with the inhibition of cell growth by 50% but that at least 84% replacement of uridine by FUra in pre-mRNA is required for detection of abnormal splicing products *in vitro* (35). Inhibition of splicing at low levels of FUra substitution in RNA *in vivo* likely does not derive from FUra incorporation into pre-mRNA alone but probably is a result of incorporation into or effects upon other components of the splicing machinery, such as snRNAs, which are uridine-rich in sequence and participate in splicing (35, 36). In fact, alterations in U1, U2, U4, and U6 snRNAs have been observed when cells are exposed to fluoropyrimidines (14, 35, 37). According to our data, 5′ exon-intron cleavage is probably not affected, because pre-mRNA levels do not change in response to FUra under conditions that do not affect DHFR pre-mRNA turnover (18). Previous studies have shown that U1 and U2 RNAs engage in splicing by binding to the regions around the 5′ exon-intron junction and branch point, respectively (39), and are responsible for cleavage of the 5′ intron that occurs simultaneously with lariat formation. Alterations in U1 and U2 RNAs observed after fluoropyrimidine exposure can thus be related to effects on splicing that probably involve lariat formation, because both the splicing intermediate and mRNA decrease after drug exposure. Sierakowska *et al.* (37) have demonstrated that nuclear extracts prepared from cells exposed to 5-fluorouridine are unable to splice exongenously supplied pre-mRNA, in part due to the loss of U2 RNA. In that study, only partial restoration of splicing activity was restored by supplementation of nuclear extracts with snRNPs. That result and a lack of correlation of lost splicing activity with a decrease in cellular U2 RNA indicated that additional splicing factors were affected by 5-fluorouridine, implicating a number of possible sites of fluorinated pyrimidine action at the level of splicing when examined in cell-free systems, but did not identify actual changes in splicing *in vivo*.

In summary, we conclude that FUra incorporation into RNA can lead to inhibition of pre-mRNA splicing. This can result in reduced mRNA and corresponding protein levels that may be critical for cell growth. Thus, inhibition of pre-mRNA splicing could be an additional mechanism of RNA-mediated cytotoxicity of FUra.

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