Effects of 5-Fluorouracil on Globin mRNA Synthesis in Murine Erythroleukemia Cells†

Tadatoshi Iwata, Tetsu Watanabe, and Donald W. Kufe*

Laboratory of Clinical Pharmacology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

Received October 18, 1985; Revised Manuscript Received December 16, 1985

ABSTRACT: Although 5-fluorouracil (FUra) incorporates in all species of RNA, the molecular basis for producing cytotoxicity by RNA-related mechanisms remains unclear. We have studied the effects of FUra on the synthesis of α- and β-globin mRNA in murine erythroleukemia cells. An analysis of equivalent amounts of RNA from FUra-treated cells suggested that this agent causes a relative increase in globin mRNA. However, when RNA obtained from the same number of cells was analyzed, thus taking into account decreases in ribosomal RNA, the amount of intracellular α- or β-globin mRNA actually decreased following FUra treatment. The decrease in globin mRNA was associated with decreases in both intracellular globin and hemoglobin content. The findings thus indicate that FUra inhibits the accumulation of α- and β-globin transcripts. This effect of FUra could occur by decreasing transcriptional rate or, as in the case of ribosomal RNA, by interfering with the processing of these nucleic acids. Either mechanism could contribute to the RNA-related cytotoxicity induced by this agent.

Several mechanisms of action are probably responsible for the cytotoxic effects of the pyrimidine analogue FUra.[1] This agent is converted to FdUMP which binds irreversibly to thymidylate synthase and thereby inhibits DNA synthesis (Heidelberger, 1975). More recently, FUra residues have been detected in eukaryotic DNA (Kufe et al., 1981; Danenberg et al., 1981; Ingraham et al., 1981; Major et al., 1982), and excision of these residues may contribute to the cytotoxicity and mutagenicity of this agent (Herrick et al., 1982; Kufe et al., 1983). However, correlations between the extent of FUra incorporation into RNA and cytotoxicity (Cadman et al., 1979; Spiegelman et al., 1980; Kufe & Major, 1981; Glazer & Lloyd, 1982; Dolnick & Pink, 1983) have suggested that this mechanism may be primarily responsible for the effects of this agent. The relative contribution of these cytotoxic mechanisms, however, are probably related to different intracellular patterns of FUra metabolism (Laskin et al., 1979).

Although FUra incorporates to a significant extent in all species of RNA, the molecular basis for producing cytotoxicity by an RNA-related mechanism remains unclear. FUra inhibits 28S and 18S rRNA synthesis by a posttranscriptional effect on the processing mechanism (Wilkinson & Pitot, 1973; Wilkinson et al., 1975). The extent of FUra incorporation into prRNA (45S and 32S) correlates with inhibition of maturation to cytoplasmic 28S and 18S rRNA (Herrick & Kufe, 1984). The transcription of the 45S rRNA precursor is also inhibited by FUra (Wilkinson et al., 1975; Herrick & Kufe, 1984), although the extent of FUra substitution in prRNA is a better correlate of cell lethality (Herrick & Kufe, 1984). FUra also incorporates into tRNA and inhibits methylation of this nucleic acid (Tseng et al., 1978; Glazer & Hartman, 1980). The misincorporation of FUra into bacterial tRNA results in a reduction in the rate of amino acid acceptance and of ribosomal binding, presumably due to FUra-induced changes in the anticodon regions of these molecules (Ramberg et al., 1978).

The incorporation of FUra into poly(A) RNA results in little if any change in quantitative and qualitative translational activity (Glazer & Hartman, 1983). These results suggest that the formation of (FUra)mRNA is not associated with cytotoxicity. Recent studies, however, indicate that FUra increases DHFR mRNA and that, although DHFR enzyme activity increases per cell, there is a decrease of enzyme activity per milligram of protein (Dolnick & Pink, 1983). Furthermore, the rate of translation in vitro is unaffected by FUra incorporation into DHFR mRNA (Dolnick & Pink, 1985). The finding that an altered DHFR is produced in the presence of FUra supports a mechanism of translational miscoding during protein synthesis (Dolnick & Pink, 1985). Thus, FUra misincorporation into RNA appears to alter the maturation of rRNA and the function of both tRNA and mRNA.

In these studies, we have monitored the effects of FUra on the synthesis of α- and β-globin mRNA. Although a relative increase in these mRNAs is observed following FUra treatment, there is actually a decrease in the intracellular content of each mRNA. The decrease in intracellular α- and β-globin mRNA is associated with a decrease in both globin and hemoglobin production. These findings would suggest that, in contrast to increases in DHFR mRNA synthesis, FUra treatment inhibits the accumulation of globin mRNA. This inhibition of globin transcripts could occur at a transcriptional level or by defects in the processing mechanisms.

Materials and Methods

Cell Culture and Chemicals. Friend erythroleukemia cells (murine erythroleukemia cells, MEL), clone PC4 (provided

† This investigation was supported by U.S. PHS Grant CA28488 awarded by the National Cancer Institute and by an American Cancer Society Faculty Research Award (D.W.K.).

* Address correspondence to this author at the Dana-Farber Cancer Institute.

[1] Abbreviations: FUra, 5-fluorouracil; FdUMP, 5-fluorodeoxyuridine monophosphate; prRNA, preribosomal RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; mRNA, messenger RNA; DHFR, dihydrofolate reductase; MEL, murine erythroleukemia; dThd, thymidine; Me_2SO, dimethyl sulfoxide; α-MEM, minimum Eagle's medium, type α; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-2703$01.50/0

by Dr. D. Housman, Massachusetts Institute of Technology, Cambridge, MA), were grown in suspension culture in α-MEM (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco), 100 units of streptomycin/mL, 100 μg of penicillin/mL, and 2 mM L-glutamine at 37 °C in a 5% CO_2 atmosphere. Cell density was maintained between 2×10^4 and 5×10^5 cells/mL for continuous logarithmic growth.

MEL cells were grown in suspension culture in the presence of varying FUra (Sigma Chemical Co., St. Louis, MO) concentrations (10^{-6}–10^{-3} M), 10^{-4} M dThd (P-L Biochemicals, Milwaukee, WI), or 1.25% Me_2SO (Fisher Scientific, Boston, MA). Cultures were scored for benzidine-positive cells by the wet benzidine method (Orkin et al., 1975). Heme concentration was determined by utilizing a Perkin-Elmer MPF-4 fluorescence spectrophotometer (Sassa, 1976).

Northern Blot Analysis. MEL total cellular RNA was purified by the guanidine thiocyanate–cesium chloride method (Chirgwin et al., 1979). The RNA was dissolved in H_2O and the concentration determined by monitoring the optical density at 260 and 280 nm. The RNA was then dissolved in 40% formamide, 2.2 M formaldehyde, 40 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 10 mM sodium acetate, and 1 mM EDTA. Samples were heated to 55 °C for 15 min and then quickly chilled at 0 °C. Gel electrophoresis was performed in 1% agarose containing 2.2 M formaldehyde, 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, and 0.1% ethidium bromide at 40 V for 16 h. The gel was then washed and transferred to nitrocellulose filters (Thomas, 1980).

The filters were prehybridized at 42 °C for 8–12 h in buffer consisting of 50% formamide, 5 × SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% SDS, 5 × Denhardt's solution, and salmon sperm DNA (200 μg/mL). The RNA blots were then hybridized at 42 °C for 24 h in the same buffer containing nick-translated ^{32}P-labeled cloned mouse β-major globin DNA (Tilghman et al., 1977) or α-globin DNA (Leder et al., 1978) provided by Dr. P. Leder (Harvard Medical School, Boston, MA). The filters were then washed twice with 0.1 × SSC and 0.1% SDS at 50 °C. The filters were exposed to X-ray film at −70 °C with an intensifying screen.

Protein Gel Electrophoresis. MEL cellular proteins were separated by electrophoresis in a 5–20% SDS–polyacrylamide gel (Laemmli, 1970) and stained by the silver nitrate method (Merril et al., 1981).

Results

We have previously monitored the incorporation of FUra into MEL cell RNA and DNA (Herrick & Kufe, 1984). The incorporation of FUra into MEL RNA was dependent upon time of exposure and drug concentration. In the present studies, we have similarly exposed MEL cells to FUra concentrations ranging from 10^{-6} to 10^{-3} M. The experiments have been performed in the presence of 10^{-4} M dThd to bypass possible inhibition of thymidylate synthase through the formation of FdUMP. At this concentration, dThd had no detectable effect on the proliferation of MEL cells (data not shown). The effect of FUra on MEL cell proliferation is illustrated in Figure 1. Thus, higher FUra concentrations (10^{-4} and 10^{-3} M) resulted in a partial slowing of MEL growth. Figure 1 also illustrates the effects of increasing FUra concentrations on the level of intracellular RNA. Increasing FUra concentrations resulted in decreases in total intracellular RNA. On the basis of previous studies (Herrick & Kufe, 1984) which demonstrated inhibition of the synthesis and maturation of prRNA by similar concentrations of FUra (10^{-7}–10^{-3} M), we attributed the decline in total intracellular RNA to decreases

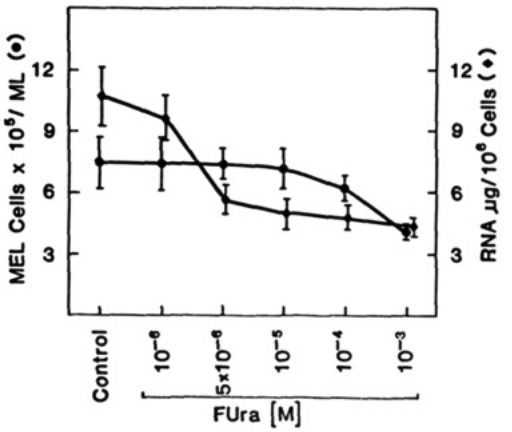

FIGURE 1: Effects of FUra on MEL cell growth and RNA content. MEL cells in logarithmic growth phase were seeded at 2×10^5 cells/mL in the presence of 10^{-4} M dThd. Varying concentrations of FUra were added at seeding. Cell counts and RNA content were determined at 72 h. Each point represents the mean ± the standard deviation (SD) of three separate experiments. Viability was over 95% in each group.

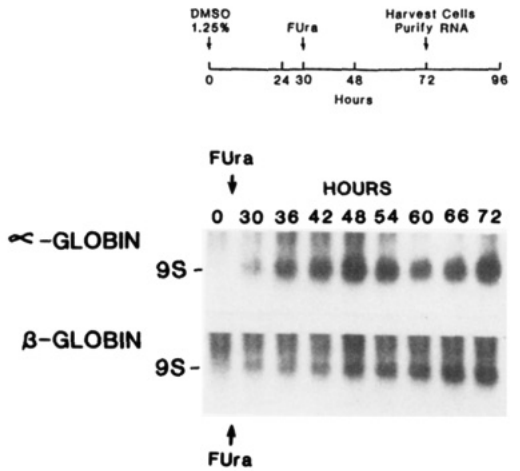

FIGURE 2: Induction of globin mRNA synthesis in MEL cells treated with Me_2SO and FUra. MEL cells were grown in the presence of 1.25% Me_2SO and 10^{-4} M dThd. FUra (10^{-5} M) was added at 30 h, and the cells were harvested at various times up to 72 h for purification of RNA. The RNA was purified by the guanidine thiocyanate–cesium chloride method and analyzed by electrophoresis through a 1% agarose–formaldehyde gel, followed by Northern blot transfer to nitrocellulose. Samples (15 μg) were hybridized by using ^{32}P-labeled α-globin or β-globin DNA.

in rRNA. As analyzed by gel electrophoresis, total cellular RNA obtained from MEL cells treated with increasing FUra concentrations demonstrated progressive declines in the level of 28S and 18S RNA (data not shown).

In order to ensure that all of the transcripts misincorporated FUra, we have monitored the effects of this agent on globin RNA induced by treatment of MEL cells with Me_2SO. In this type of experiment, FUra can be added upon induction of globin mRNA, and thus these transcripts are synthesized in the presence of drug. As illustrated in Figure 2, FUra was added 30 h after treatment with Me_2SO (1.25%) and the RNA harvested every 6 h up to 72 h. Under these experimental conditions, α-globin RNA was just detectable at the time of FUra addition, while β-globin transcripts became detectable 6–12 h later (Figure 2). Thus, the synthesis of both α- and β-globin transcripts occurs in the presence of FUra. The effects of varying FUra concentrations (10^{-6}–10^{-3} M) on α- and β-globin RNA were monitored at 72 h according to the schema in Figure 2. The results obtained are shown in Figure 3. Equal amounts of total cellular RNA (15 μg) were applied

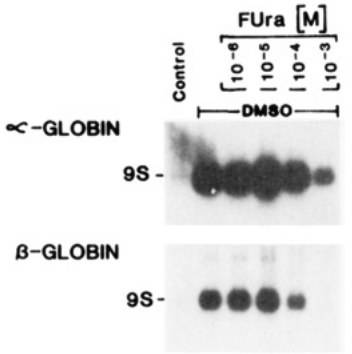

FIGURE 3: Effect of varying concentrations of FUra on globin mRNA. MEL cells were grown in the presence of 1.25% Me_2SO and 10^{-4} M dThd. Varying concentrations of FUra were added at 30 h, and the cells were harvested at 72 h for purification of RNA. Samples (15 μg) were analyzed by Northern blots using ^{32}P-labeled α-globin or β-globin DNA probes.

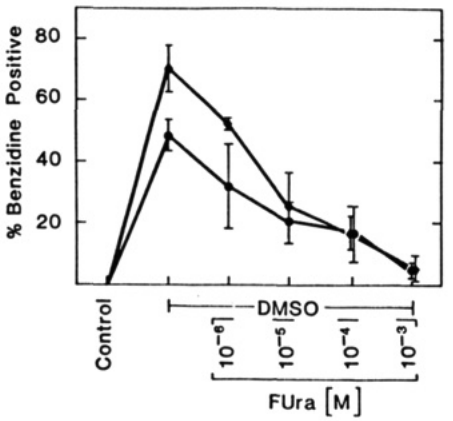

FIGURE 4: Effects of Me_2SO and FUra on percent benzidine-positive MEL cells. MEL cells were seeded at 2×10^5 cells/mL. Me_2SO (1.25%) and dThd (10^{-4} M) were added at seeding. Varying concentrations of FUra were added at 30 h, and the cells were monitored for benzidine staining at 72 (●) and 96 h (◆). Results are expressed as the mean ± the standard deviation (SD) of two separate determinations.

to each lane. Increases in the level of both α- and β-globin transcripts were observed following treatment with 10^{-5} M FUra. In contrast, at higher FUra concentrations, there was a progressive decline in both α- and β-globin RNA (Figure 3).

We have also monitored the effects of FUra on production of hemoglobin. Benzidine-positive MEL cells decreased upon exposure to increasing FUra concentrations at both 72 and 96 h after addition of Me_2SO (Figure 4). These findings were in contrast to the increases in the level of globin transcripts which, for example, increased following treatment with 10^{-5} M FUra. In order to reconcile these differences, we analyzed the transcripts present in total RNA from the same number of cells rather than applying the same amount of RNA to each lane. This approach takes into account any decreases in rRNA that occurred as a result of FUra treatment. Under these experimental conditions, a progressive decrease in 28S and 18S RNA was also observed in cells treated with Me_2SO and increasing concentrations of FUra (Figure 5). Furthermore, an analysis of α- and β-globin RNA in the same number of cells demonstrated a decrease in the level of transcripts compared to that obtained in cells treated with Me_2SO alone (Figure 6). Similar results have been obtained in three separate experiments (legend to Figure 6). Thus, these findings would suggest that applying the same amount of RNA per lane resulted in an apparent, but not actual, increase in globin RNA due to decreases in the synthesis of 28S and 18S RNA.

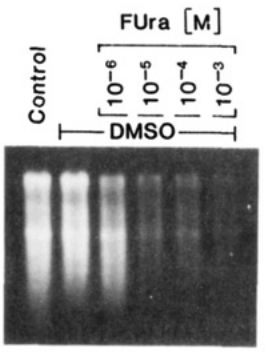

FIGURE 5: Ethidium bromide stained gel of RNA from MEL cells grown in Me_2SO and FUra. MEL cells were treated according to the schema in Figure 4. Total cellular RNA was purified from 3×10^6 cells and analyzed in a 1% agarose–formaldehyde gel, followed by ethidium bromide staining.

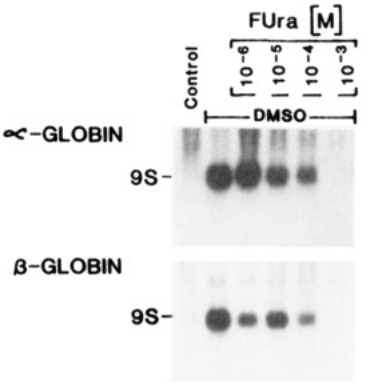

FIGURE 6: Effect of FUra on globin mRNA accumulation in the same number of cells. MEL cells (3×10^6) were treated according to the schema in Figure 2. Total cellular RNA was purified, analyzed by gel electrophoresis, and transferred to nitrocellulose. The Northern blots were then hybridized with ^{32}P-labeled α-globin or β-globin DNA probes. The effects of FUra were quantitated by densitometric tracings of autoradiographs obtained from three separate experiments for both α- and β-globin RNA. The intensity of the signals for α- and β-globin RNA from FUra-treated cells as compared to that from cells treated with Me_2SO alone (mean ± SD) were as follows: 10^{-6} M FUra, 74.5 ± 18.1%; 10^{-5} M FUra, 71.8 ± 7.4%; 10^{-4} M FUra, 49.1 ± 16.5%; 10^{-3} M FUra, 7.2 ± 7.1%.

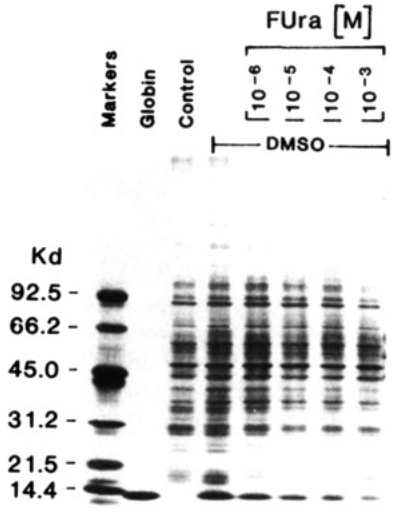

FIGURE 7: Effects of FUra on intracellular globin content. MEL cells were treated according to the schema in Figure 2. Total cellular protein was purified from 1.2×10^5 cells, separated by electrophoresis in a 5–20% SDS–polyacrylamide gel, and stained by the silver nitrate method.

We have also monitored intracellular globin content by gel electrophoresis. The results obtained demonstrate decreasing intracellular levels of globin in cells treated with increasing concentrations of FUra (Figure 7). In these experiments, extracts from the same number of cells were applied in each lane. Thus, decreases in globin transcripts (Figure 6) were accompanied by decreases in intracellular globin content (Figure 7).

Discussion

Recent studies have examined the effects of FUra on a specific mRNA in methotrexate-resistant DHFR gene amplified KB cells (Dolnick & Pink, 1983). The level of DHFR RNA increases in the FUra-treated KB cells. Furthermore, the size distribution of the DHFR RNA species is unaffected, thus suggesting that mRNA processing is not affected by FUra incorporation into RNA. Translation of purified DHFR mRNA in vitro has demonstrated that the rate of translation is unaffected by FUra incorporation into mRNA (Dolnick & Pink, 1985). However, the DHFR synthesized from the FUra-containing mRNA alters binding to methotrexate and a monospecific polyclonal antibody (Dolnick & Pink, 1985). These findings support the hypothesis of translational miscoding during protein synthesis as a mechanism of FUra-mediated cytotoxicity.

In order to assure that all of the mRNA transcripts misincorporated FUra, we performed studies that added FUra after induction of globin mRNA synthesis. Thus, under these experimental conditions, globin transcripts were synthesized in the presence of drug. Both the α- and β-globin mRNA appeared to increase following exposure to increasing FUra concentrations. However, the interpretation of these results was complicated by decreases in 28S and 18S RNA. Thus, when RNA obtained from the same numbers of cells was applied, the amount of globin RNA actually decreased following FUra treatment. These decreases in globin mRNA were associated with decreases in intracellular globin and hemoglobin content, thus suggesting that decreases in benzidine-positive cells were related to decreased globin mRNA. We have also measured heme synthesis using a fluorometric assay and found less pronounced decreases in intracellular heme as compared to globin content (data not shown). Finally, as in the case of DHFR mRNA, in vitro translation of mRNA synthesized in the presence of FUra showed no significant decrease in globin synthesis except after treatment with 10^{-3} M FUra (data not shown).

The present findings are thus in contrast to those previously reported for the effects of FUra on DHFR mRNA. We were unable to find detectable increases in globin mRNA when taking into account decreases in the synthesis of 28S and 18S RNA. Treatment of KB cells with FUra failed to result in significant decreases in total cellular RNA or rRNA (Dolnick & Pink, 1983, 1985). Another explanation for the discrepancy in terms of effects of FUra on specific transcripts might be related to inhibition of cellular proliferation. Thus, FUra inhibits growth of both KB and MEL cells. A partial arrest of cell proliferation could result in enhanced synthesis of enzymes involved in deoxynucleotide biosynthesis, such as DHFR. In contrast, it would seem less likely that cells would respond to partial slowing of growth by an increase in the synthesis of globin mRNA. An approach to the resolution of some of these issues would involve measuring effects of FUra on rates of mRNA transcription.

Finally, the studies with DHFR (Dolnick & Pink, 1983, 1985) and globin mRNA might suggest that FUra has no detectable effect on the processing of these nucleic acids. These conclusions are based on the absence of a detectable higher molecular weight mRNA precursor during treatment with FUra. The finding that FUra decreases synthesis of globin mRNA could be secondary to either decreases in transcriptional rate or a processing defect without the accumulation of a precursor mRNA. Indeed, FUra inhibits processing of 45S and 32S RNA in the absence of accumulation of these higher molecular weight prRNA species (Herrick & Kufe, 1984). Incorporation of FUra into small nuclear RNA could interfere with the function of this RNA species in mRNA processing and splicing (Hernandez & Keller, 1983; Moore & Sharp, 1984). A defect in splicing could result in decreases in the level of a particular transcript without accumulation of the pre-mRNA. Taken together, the effects of FUra incorporation into prRNA, tRNA, mRNA, and small nuclear RNA may all contribute to induction of cell lethality.

Registry No. FUra, 51-21-8.

References

Cadman, E., Heimer, R., & Davis, L. (1979) *Science (Washington, D.C.) 205*, 1135–1137.

Chirgwin, T., Przybyla, A., MacDonald, R., & Rutter, W. (1979) *Biochemistry 18*, 5294–5299.

Danenberg, P. V., Heidelberger, C. A., Mulkins, M. A., & Peterson, A. R. (1981) *Biochem. Biophys. Res. Commun. 102*, 617–623.

Dolnick, B., & Pink, J. (1983) *J. Biol. Chem. 258*, 13299–13306.

Dolnick, B., & Pink, J. (1985) *J. Biol. Chem. 260*, 3006–3014.

Glazer, R., & Hartman, K. (1980) *Mol. Pharmacol. 17*, 245–249.

Glazer, R., & Lloyd, L. (1982) *Mol. Pharmacol. 21*, 468–473.

Glazer, R., & Hartman, K. (1983) *Mol. Pharmacol. 23*, 540–546.

Heidelberger, C. (1975) *Antineoplastic and Immunosuppressive Agents* (Sartorelli, & Johns, D., Eds.) pp 193–321, Springer-Verlag, New York.

Hernandez, N., & Keller, W. (1983) *Cell (Cambridge, Mass.) 35*, 89–99.

Herrick, D., & Kufe, D. (1984) *Mol. Pharmacol. 26*, 135–140.

Herrick, D., Major, P., & Kufe, D. (1982) *Cancer Res. 42*, 5015–5017.

Ingraham, H. A., Tseng, B. Y., & Goulian, M. (1981) *Mol. Pharmacol. 21*, 211–216.

Kufe, D., & Major, P. (1981) *J. Biol. Chem. 256*, 9802–9805.

Kufe, D., Major, P., Egan, E. M., & Loh, E. (1981) *J. Biol. Chem. 256*, 8885–8888.

Kufe, D., Scott, P., Fram, R., & Major, P. (1983) *Biochem. Pharmacol. 32*, 1337–1340.

Laemmli, K. (1970) *Nature (London) 227*, 680–685.

Laskin, J. D., Evans, R. M., Slocum, H. D., Burke, D., & Hakala, M. T. (1979) *Cancer Res. 39*, 383–398.

Leder, A., Miller, H., Hamer, D., Seidman, J., Norman, B., Sullivan, M., & Leder, P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 6187–6191.

Major, P., Egan, E. M., Herrick, D., & Kufe, D. (1982) *Cancer Res. 42*, 3005–3009.

Merril, R., Dunau, L., & Goldman, D. (1981) *Anal. Biochem. 110*, 201–207.

Moore, C., & Sharp, P. (1984) *Cell (Cambridge, Mass.) 36*, 581–591.

Orkin, S., Swan, D., & Leder, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 98–102.

Ramberg, E., Mohammad, I., Rulf, S., Moeller, B., & Horowitz, J. (1978) *Biochemistry 17*, 3978–3985.

Sassa, E. (1976) *J. Exp. Med. 143*, 305–315.
Spiegelman, S., Sawyer, R., Nayak, R., Ritzi, E., Stolfi, R., & Martin, D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4966–4970.
Thomas, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201–5205.
Tilghman, S., Tiemier, D., Polsky, F., Edgell, M., Seidman, J., Leder, A., Enquist, L., Norman, B., & Leder, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4406–4410.
Tseng, W., Medina, D., & Randerath, K. (1978) *Cancer Res. 38*, 1250–1257.
Wilkinson, D., & Pitot, H. (1973) *J. Biol. Chem. 248*, 63–68.
Wilkinson, D., Tlsty, T., & Hanas, R. (1975) *Cancer Res. 35*, 3104–3020.

Study of the Interaction between Uncharged Yeast tRNA^{Phe} and Elongation Factor Tu from *Bacillus stearothermophilis*†

A. Heerschap,‡ J. A. L. I. Walters, J.-R. Mellema, and C. W. Hilbers*
Laboratory of Biophysical Chemistry, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Received August 19, 1985; Revised Manuscript Received December 3, 1985

ABSTRACT: Proton NMR studies are presented on the interaction of nonaminoacylated yeast tRNA^{Phe} and elongation factor Tu·GTP from *Bacillus stearothermophilis*. From experiments in which transfer of magnetization is observed between proton spins of tRNA and the protein, it is concluded that complex formation takes place. Amino acid residues of the protein come into close contact with the base pair A5U68 and/or U52A62 of the acceptor TΨC limb of the tRNA molecule. From the line broadening of tRNA resonances, associated with complex formation, an association constant of 10^3–10^4 M^{-1} is estimated. The NMR experiments do not monitor a significant conformational change of the tRNA molecule upon interaction with the protein. However, at times long after the onset of complex formation, spectral changes indicate that the upper part of the acceptor helix becomes distorted.

In the elongation cycle of protein synthesis, the specific binding of aminoacyl-tRNA (aa-tRNA) to the programmed ribosome is mediated by elongation factor Tu (EFTu) (Miller & Weissbach, 1977; Kaziro, 1978; Bosch et al., 1983). In this process, the EFTu protein and guanosine 5′-triphosphate (GTP) form a complex (EFTu·GTP) which preferentially binds to aminoacylated noninitiator tRNAs. The thus formed ternary complex then interacts with the ribosome in such a way that the tRNA is positioned in the ribosomal A site. The molecular mechanism underlying the formation of the ternary complex and also the why and wherefore of its existence are still a matter of conjecture. For instance, EFTu·GTP does not appear to discriminate among various aminoacylated elongation tRNAs and therefore must obviously recognize structural features common to all of these tRNAs. However, the characteristic features of the common structural elements that are recognized by the protein are not well-known. Conformational changes both in the tRNA and in the protein as a result of their mutual interactions have been postulated as being necessary for proper ribosome binding and codon recognition, but so far, no definite experimental evidence is available to prove or disprove such proposals.

A variety of experiments have been performed to detect which sites on aa-tRNA interact with EFTu and how this interaction influences the conformation of tRNA. These include (a) probing of tRNA structure in the ternary complex by enzymatic modifications (Jekowsky et al., 1977; Boutorin et al., 1981; Wikman et al., 1982), (b) chemical modification (Bertram & Wagner, 1982; Douthwaite et al., 1983; Riehl et al., 1983), (c) spin-labeling of tRNA (Kruse et al., 1978; Sprinzl et al., 1978; Weygand-Durasevic et al., 1981), (d) cross-linking between EFTu and tRNA (Kao et al., 1983), (c) oligonucleotide binding (Kruse et al., 1980), (f) binding of other tRNAs with a complementary anticodon (Yamane et al., 1981), and (g) measurement of GTPase activity as a function of aa-tRNA fragment binding (Guesnet et al., 1983). The conclusions from these experiments can be summarized as follows: (1) The 3′-aminoacylated end of tRNA is most important for the interaction with EFTu·GTP. (2) EFTu·GTP covers the helix formed by the acceptor and T stem and most of the variable loop. (3) Anticodon stem and loop are not in close contact with the protein. This is probably also true for most parts of the D stem, D loop, and T loop. (4) Structural rearrangements as a result of complex formation occur in several regions of the tRNA molecule, including the anticodon loop and stem. The nature and extent of these changes have remained unclear.

Also, proton NMR studies have been carried out to investigate whether conformational changes occur in tRNA molecules upon complex formation with EFTu·GTP (Shulman et al., 1974). The imino protons in tRNA, of which the majority are involved in hydrogen bonds in the base pairs, resonate in the low-field part of the ^1H NMR spectrum between 9 and 15 ppm (Hilbers, 1979). Because EFTu, like other proteins, hardly contributes any resonance intensity to this spectral region, it is possible to observe the hydrogen-bonding network of tRNA through this spectroscopic window while tRNA interacts with the elongation factor. From this NMR work,

†Experiments were recorded at the Dutch National 500/200 hf NMR Facility Nijmegen, which is sponsored by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).
‡Present address: Philips Research Laboratories, 5600 JA Eindhoven, The Netherlands.

0006-2960/86/0425-2707$01.50/0