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Effects of 5-Bromo-2′-Deoxyuridine on Production of Globin Messenger RNA in Dimethyl Sulfoxide-Stimulated Friend Leukemia Cells

(differentiation/Friend leukemia virus)

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ABSTRACT **Friend leukemia cells grown in the presence of dimethyl sulfoxide show enhanced erythroid differentiation and hemoglobin synthesis. The effects of dimethyl sulfoxide stimulation are inhibited by BrdU. To determine the effect of BrdU on the amount of globin mRNA present in cells treated and not treated with dimethyl sulfoxide, molecular hybridization between total cell RNA and [^{3}H]DNA complementary to mouse globin mRNA was used. Cells treated with BrdU and dimethyl sulfoxide had 70% less globin mRNA than cells treated with dimethyl sulfoxide alone. The size and base sequence of the residual globin mRNA in the cultures treated with BrdU and dimethyl sulfoxide were unaltered. Cells treated with BrdU alone contained slightly more globin mRNA than did the untreated controls, suggesting that BrdU may have a dual effect in transcription of messenger RNA.**

5-Bromo-2′-deoxyuridine (BrdU) provides a tool for investigating some of the molecular events involved in ontogenesis as well as oncogenesis, for it affects tissues as dissimilar as muscle cells of chick embryo (1), mammary tissue of pregnant mice (2), and malignant cells (3–7). The effects on each of these systems are considered to be specific and nonrandom. From these studies, where low doses which did not significantly interfere with cell replication had been used, synthesis of proteins characteristic of the final differentiated state was inhibited. For example, hemoglobin synthesis by chick embryo was inhibited during culture in the presence of BrdU (8, 9).

We previously reported that dimethyl sulfoxide [$(CH_3)_2SO$] enhances heme synthesis and erythroid differentiation of Friend leukemia cells *in vitro* (10) and that inhibition of these effects by BrdU was dependent on its incorporation into DNA (7). Similarly, other investigators have observed inhibition by BrdU of $(CH_3)_2SO$-induced stimulation of globin synthesis (11). Since appreciable amounts of globin mRNA were detected in $(CH_3)_2SO$-stimulated, but not in control, Friend leukemia cells cultures, it appeared that $(CH_3)_2SO$ might act at a transcriptional level (12, 21.)

The present studies were designed to determine whether BrdU might also affect transcription of globin mRNA by Friend leukemia cells. We found that a decrease in the amount of globin mRNA accompanied inhibition of $(CH_3)_2SO$-induced differentiation by BrdU. Further, we demonstrated that the residual globin mRNA present in Friend leukemia cells treated with BrdU plus $(CH_3)_2SO$ was indistinguishable in size and base sequence from globin mRNA present in Friend leukemia cells treated only with $(CH_3)_2SO$.

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; $(CH_3)_2SO$, dimethyl sulfoxide; cDNA, complementary DNA.

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MATERIALS AND METHODS

Tissue culture conditions

Clone 745 A of Friend leukemia cells was grown in Eagle's basal medium supplemented with 15% fetal-calf serum, 250 units/ml of penicillin, and 0.2 mg/ml of streptomycin, as described (10). Tightly stoppered 2-liter flasks containing 1×10^8 cells in 1 liter of medium were placed in a 37° walk-in incubator. Gentle mixing was provided by a magnetic stirrer. The cells used in these studies were grown for 4 days under one of the following conditions: (*1*) control—untreated cells cultured in basal medium, (*2*) $(CH_3)_2SO$-treated—cells cultured in the presence of 2% $(CH_3)_2SO$ (v/v), (*3*) BrdU-treated—cells cultured in the presence of 3 μg/ml of BrdU, and (*4*) BrdU- and $(CH_3)_2SO$-treated—cells cultured in the presence of both 2% $(CH_3)_2SO$ and 3 μg/ml of BrdU. The cultures in each experiment were seeded simultaneously and maintained in the dark.

Preparation of RNA

RNA was prepared by a modification of the method of Natta (29). All glassware was treated with 0.1% ethyl oxidiformate (Eastman Kodak Co.) and then autoclaved; all plastic was treated with 2.0% Na dodecyl sulfate. 1 to 2×10^9 cells were suspended in 10 ml of isotonic saline and added by drops to an extraction mixture consisting of 40 ml of water-saturated distilled phenol, 20 ml of buffer R [46 mM NaCl–20 mM sodium acetate–2 mM NaEDTA (pH 5.2) made 2% with Na dodecyl sulfate], and 50 mg of bentonite dry weight, prepared by the method of Fraenkel-Conrat *et al.* (13). After the mixture was shaken for 30 min at 4°, the aqueous and phenol phases were separated by centrifugation at $17,300 \times g$. 10 ml of phenol was added to the aqueous phase, which was kept on ice while the phenol phase and the interphase were again extracted with 20 ml of buffer R by shaking for 15 min at 4°. The aqueous and phenol phases were again separated by centrifugation. The aqueous phases were combined and extracted with 30 ml of additional phenol by shaking for 20 min at 4°. After centrifugation for 5 min, the phenol phase was discarded. The aqueous phase was centrifuged for 20 min at $30,900 \times g$ and the pellet was discarded. 0.1 Volume of 3 M NaCl and 2 volumes of absolute ethanol were added to the aqueous phase, and the mixture was allowed to stand over-

night at −20°. After recovery of the RNA by centrifugation at 17,300 × *g* for 20 min, the precipitate was dissolved in 4 ml of distilled water. The RNA was reprecipitated as above, dissolved in a minimal volume of water, and stored at −70° until use.

Assay of globin mRNA content

Globin mRNA was quantitated by determining the amount of hybridizable radioactivity after a constant amount of tritiated complementary DNA ([^{3}H]cDNA) was annealed to several different concentrations of total cell RNA.

Preparation of [^{3}H]cDNA. RNA-dependent DNA polymerase of avian myeloblastosis virus was partially purified as described (14, 15). $(dT)_{10}$ Was obtained from Miles Laboratories. [^{3}H]dATP (12 Ci/mmol) was obtained from New England Nuclear Corp. *Aspergillus oryzae* S1 nuclease was purified from amylase type IV-A (Sigma Chemical Co.) as described (16, 17). *Escherichia coli* tRNA was obtained from Schwarz–Mann and extracted with phenol before use. Mouse-globin mRNA was purified by the procedure of Benz and Forget (18) from reticulocytes obtained from randomly bred Swiss mice made anemic by phenylhydrazine treatment. A single-stranded radioactive DNA copy of mouse-globin mRNA was obtained by incubating the mRNA with RNA-dependent DNA polymerase of avian myeloblastosis virus [^{3}H]dATP (specific activity 8 Ci/mmol), and actinomycin D (19). The DNA product was incubated at 37° for 18 hr in 0.3 N KOH, then neutralized with HCl. *E. coli* tRNA (50 μg) was then added as carrier. The DNA was isolated by Sephadex G-150 gel filtration in 0.1 M ammonium bicarbonate solution, lyophilized, and suspended in H_2O.

RNA–[^{3}H]cDNA Hybridization. Hybridization assays contained 0.2 M sodium phosphate buffer (pH 6.8) and 0.5% Na dodecyl sulfate in addition to cDNA and RNA. Incubations were performed in sealed 5.0-μl capillary tubes (Rochester Scientific) for 40 hr at 70°. The amount of labeled DNA hybridized was determined by diluting the reaction mixture into 2 ml of a solution containing 0.1 M sodium acetate (pH 4.5), 1 mM $ZnSO_4$, and 10 μg/ml of calf-thymus DNA (heat denatured) (Worthington Biochemical Corp.). Digestion with *A. oryzae* S1 nuclease was performed for 30 min at 45° (20). The remaining nuclease-resistant trichloroacetic acid-precipitable counts per min were taken to represent [^{3}H]cDNA in hybrid structures. Background radioactivity was determined by a hybridization assay in the absence of cell RNA. The percent of [^{3}H]cDNA that was hybridized was calculated by subtracting the background cpm from the total hybridized cpm and dividing this number by the total cpm added.

Calculation of Globin mRNA Content. The amount of globin mRNA present in Friend leukemia cell cultures was quantitated by annealing different amounts of the total cell RNA to a constant amount of [^{3}H]cDNA for a sufficient period of time for complete hybridization to occur. Values for the percent of hybridization were plotted against the amount of RNA added on a linear scale, and the half-saturation value was taken as the most accurate measure of the globin mRNA content of each sample. The amount of globin mRNA was determined with reference to a standard curve for purified mouse-globin mRNA, and the percent of the total cell RNA that was globin mRNA was calculated.

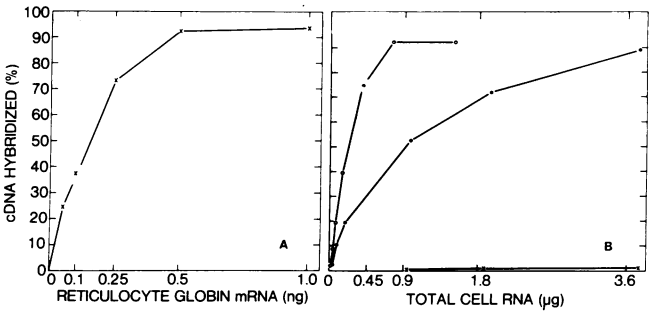

FIG. 1. RNA–mouse globin [^{3}H]cDNA hybridization curves. Hybridization was done as described in *Methods* for 40 hr at 70°. [^{3}H]cDNA added was 220 cpm per reaction mixture. A blank reaction mixture gave a value of 20 cpm. (*A*) Pure mouse-globin mRNA; (*B*) total cell RNA from treated and untreated Friend leukemia cells. ○, $(CH_3)_2SO$; ●, BrdU + $(CH_3)_2SO$; ×, BrdU or control.

Sucrose gradient studies

20 A_{260} units of total cell RNA from cultures treated with $(CH_3)_2SO$ and BrdU plus $(CH_3)_2SO$ were layered onto linear 5–20% sucrose gradients in 5 mM Tris·HCl (pH 7.4) and centrifuged at 27,000 rpm for 24 hr in a Spinco SW-27.1 rotor. The gradients were collected from below in 15-drop fractions through a Gilford recording spectrophotometer, and the amount of globin mRNA present in each fraction was determined by hybridization with cDNA.

RESULTS

The saturation curve of the hybridization of authentic Swiss mouse-globin mRNA and [^{3}H]cDNA (Fig. 1*A*) was used to calculate the amount of globin mRNA present in total cell RNA from treated cultures. The 50% saturation points of the hybridization of whole-cell RNA with [^{3}H]cDNA (Fig. 1*B*) revealed that the globin mRNA content of total cell RNA from $(CH_3)_2SO$-treated cultures was about 3.3-times greater than that from cultures treated with BrdU and $(CH_3)_2SO$. It is evident that RNA from cultures treated with $(CH_3)_2SO$ and with BrdU and $(CH_3)_2SO$ reached the same maximum hybridization value, indicating that BrdU treatment did not lead to a preferential loss of any base sequence normally present in globin mRNA derived from $(CH_3)_2SO$-treated cultures. Table 1 gives the results of two experiments on the effect of BrdU on the amount of globin mRNA in total cell RNA. There was about a 70% reduction in the percent of RNA hybridizable with cDNA in the cultures treated with BrdU and $(CH_3)_2SO$ compared to the cultures treated with $(CH_3)_2SO$ alone. The BrdU-treated cultures had 3- to 5-times the amount of globin mRNA as the untreated controls, although this difference was too small to be detected as plotted in Fig. 1*B*. In addition, there was a close correlation between the number of benzidine-positive cells and the globin mRNA content of each culture. The magnitude of the effects of BrdU was similar to that obtained when heme was used as a measure of erythroid differentiation (7), i.e., a 70% decrease in globin mRNA content in cultures treated with BrdU plus $(CH_3)_2SO$ was accompanied by a 70% decrease in the number of benzidine-positive cells as compared to the $(CH_3)_2SO$-treated cultures. However, such small increments in the amount of globin message as were obtained in the cultures treated

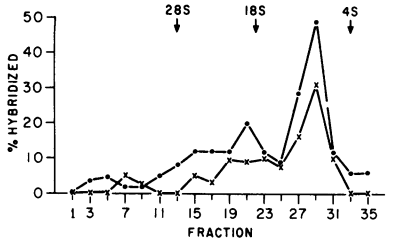

FIG. 2. Comparison of the size distribution of globin mRNA present in total cell RNA from Friend leukemia cells treated with $(CH_3)_2SO$ and with BrdU plus $(CH_3)_2SO$ as determined by sucrose density gradient analysis. For RNA obtained from cells treated with $(CH_3)_2SO$ but not BrdU (●), each hybridization mixture included material diluted 1:4 from the appropriate gradient fraction. For RNA obtained from cells treated with both $(CH_3)_2SO$ and BrdU (×), each hybridization mixture included material diluted 2:7 from the appropriate gradient fraction. 240 cpm of [^{3}H]cDNA was added. A blank reaction mixture gave a value of 9 cpm. S values were obtained from the A_{260} of the ribosomal RNAs present in the whole-cell RNA itself.

with BrdU alone were not always reflected in an increase in the percentage of benzidine-positive cells, since estimation by staining is less sensitive than that by hybridization. $(CH_3)_2SO$-treated cells contained 500-times more globin mRNA than control cells, an observation consistent with our previous studies on polyribosome patterns in control and $(CH_3)_2SO$-treated cells (21).

Further studies were directed at characterizing the globin mRNA synthesized in the presence of BrdU. Hybridization studies with total cell RNA that had been fractionated by sucrose density gradient centrifugation were conducted to determine if BrdU-inhibition of $(CH_3)_2SO$-induced differentiation was accompanied by appearance of globin mRNA of abnormal size. This abnormal size could result either if transcription of globin mRNA were altered by BrdU or if BrdU led to extensive degradation of globin mRNA. Fig. 2 illustrates that the distribution of hybridizable RNA obtained from cultures treated with $(CH_3)_2SO$ and with BrdU plus $(CH_3)_2SO$ was virtually identical. The maximum amount of hybridizable RNA was in the 9S region of the gradient and, at S values of less than 9, there was little material capable of hybridizing to globin cDNA.

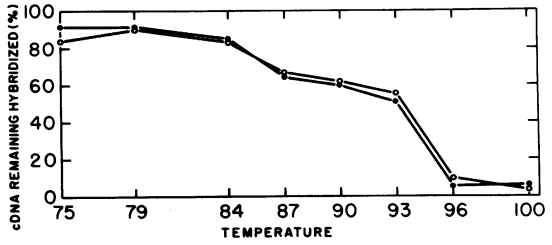

FIG. 3. Comparison of thermal stability of [^{3}H]cDNA–globin mRNA hybrids. A series of reaction mixtures containing 250 cpm of cDNA and 2.5 μg of total RNA from $(CH_3)_2SO$-treated Friend leukemia cells or 20 μg of total RNA from Friend leukemia cells treated with BrdU and $(CH_3)_2SO$ were prepared. Samples were incubated for 60 hr at 70° and cooled rapidly to room temperature. Individual samples were then heated for 3 min to the indicated temperatures and rapidly diluted into 2 ml of 0.1 M sodium acetate (pH 4.5), 1 mM $ZnSO_4$, and 10 μg/ml of calf-thymus DNA (heat denatured).

The possibility remained that BrdU was exerting a direct effect on transcription by causing synthesis of globin mRNA with an altered base sequence. To test this hypothesis, an analysis of the thermal stability of hybrids formed between [^{3}H]cDNA and mRNA from Friend leukemia cells treated with $(CH_3)_2SO$ and with BrdU plus $(CH_3)_2SO$ was done (Fig. 3). If globin mRNA synthesized by BrdU-treated cells contained frequent errors in transcription so that one base in 50 was incorrectly transcribed, a difference in the stability of duplexes formed between cDNA and globin mRNA from cells treated with $(CH_3)_2SO$ and with BrdU and $(CH_3)_2SO$ would have been detected (22). No such differences were found, indicating that, within the limits of this assay technique, the base sequences of the globin mRNAs synthesized by cells treated with $(CH_3)_2SO$ and with BrdU and $(CH_3)_2SO$ were the same.

DISCUSSION

These studies indicate that the proportion of total cell RNA that is globin mRNA is less in cultures containing BrdU and $(CH_3)_2SO$ than in cultures of Friend leukemia cells treated with $(CH_3)_2SO$ alone. The effects of BrdU on globin mRNA appear to be quantitative rather than qualitative, since our results tend to exclude the possibility that BrdU inhibits globin synthesis by altering an essential property of globin mRNA, such as its size or base sequence. Although BrdU may act in this system by accelerating globin mRNA decay, it appears more likely that BrdU interferes with synthesis of this mRNA or perhaps with the processing of globin mRNA in the nucleus.

An unusual observation has been the report that BrdU induces the differentiation of neuroblastoma cells *in vitro*, an effect independent of the incorporation of DNA (5). This latter observation is contrary to other studies which indicated that BrdU incorporation into DNA was essential for its biological effects (1, 2, 6).

The observations reported here may provide a resolution to these seemingly contradictory findings, since they are compatible with both processes. BrdU has been shown to decrease the amount of globin mRNA when Friend leukemia cells were being stimulated to differentiate in the presence of $(CH_3)_2SO$, as well as to cause a slight increase in globin mRNA when added to cells cultured in the absence of $(CH_3)_2SO$. Ostertag *et al.* (11) have demonstrated that BrdU actually augmented

TABLE 1. *Effect of BrdU on the amount of globin mRNA in total cell RNA of Friend leukemia cell cultures*

Culture conditions	RNA hybridized to cDNA/ total cell RNA (%) Exp. 1	Exp. 2
Control	0.0003	0.0002
+ BrdU	0.0015	0.0007
+ $(CH_3)_2SO$	0.149	0.144
+ BrdU + $(CH_3)_2SO$	0.041	0.036

Relative amounts of globin mRNA in total cell RNA from Friend leukemia cells. Data was calculated by estimating half-saturation values for each of the samples assayed in Fig. 1*B* and comparing these half-saturation values to values obtained for half saturation of mouse-globin mRNA.

globin synthesis in the presence of $(CH_3)_2SO$ in a mutant line of Friend leukemia cells deficient in thymidine kinase. Since this mutant would not be expected to incorporate BrdU into DNA, it appears that BrdU stimulation of globin mRNA synthesis by Friend leukemia cells may not be dependent upon the incorporation of BrdU into DNA. These observations suggest that two different mechanisms may be involved: one in which the *inhibition* of differentiation is *dependent on BrdU* incorporation into DNA and another, which is responsible for the *stimulation* of differentiation, is *independent* of DNA synthesis. These phenomena are currently under investigation.

Stellwagen and Tompkins (6), studying the effects of BrdU on tyrosine aminotransferase activity of cultured hepatoma cells, concluded that BrdU decreased the rate of tyrosine aminotransferase synthesis by decreasing the rate of synthesis of mRNA for this enzyme. These authors also suggested that BrdU probably affected the promoter region rather than the structural gene for this enzyme, thus lowering the rate of initiation of its transcription by RNA polymerase. These findings are compatible with those reported here.

The recent report of Lin and Riggs provides a possible explanation for the $(CH_3)_2SO$-induction of differentiation as well as for the BrdU-inhibition of this process (23). These investigators, studying the lac operon, demonstrated that the rate of dissociation of repressor from BrdU-substituted operator DNA is 10-times slower than from normal operator. They concluded that, for a given concentration of inducer, more repressor will remain bound to the operator when the operator contains BrdU. In Friend leukemia cells it is possible that transcription of the globin gene is inhibited by the presence of a repressor. $(CH_3)_2SO$ might stimulate differentiation by interfering with the binding of the repressor to the operator portion of a globin operon, either by decreasing the amouut of repressor present or by decreasing the affinity of repressor for operator. If this were the case, BrdU would then inhibit $(CH_3)_2SO$ stimulation of globin synthesis by being incorporated into the operator region, increasing the affinity of the repressor for operator, and thereby interfering with the effects of $(CH_3)_2SO$. There is also the possibility that in eukaryotic cells the hypothetical repressor may bind directly to the structural gene and, so, BrdU would act by increasing the affinity of the structural gene for the repressor.

Finally, BrdU induces the production of C-type virus particles (24–28), a function dependent upon incorporation of BrdU into DNA (28). In this situation, as with inhibition of $(CH_3)_2SO$-induced globin mRNA synthesis, BrdU may also interfere with the synthesis of an mRNA, i.e., an mRNA that codes for a repressor of viral synthesis.

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1. Bischoff, R. & Holtzer, H. (1970) *J. Cell Biol.* **44,** 134–150.
2. Turkington, R. W., Majumder, G. C. & Riddle, M. (1971) *J. Biol. Chem.* **246,** 1814–1819.
3. Silagi, S. & Bruce, S. A. (1970) *Proc. Nat. Acad. Sci. USA* **66,** 72–78.
4. Pasztor, L. M. & Hu, F. (1972) *Cancer Res.* **32,** 1769–1774.
5. Schubert, D. & Jacob, F. (1970) *Proc. Nat. Acad. Sci. USA* **67,** 247–254.
6. Stellwagen, R. H. & Tompkins, G. M. (1971) *J. Mol. Biol.* **56,** 167–182.
7. Scher, W., Preisler, H. D. & Friend, C. (1973) *J. Cell. Physiol.* **81,** 63–69.
8. Miura, Y. & Wilt, F. H. (1971) *J. Cell Biol.* **48,** 523–531.
9. Hagopian, H. K., Lippke, J. A. & Ingram, V. M. (1972) *J. Cell Biol.* **54,** 98–106.
10. Friend, C., Scher, W., Holland, J. . & Sato, T. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 378–382.
11. Ostertag, W., Crozier, T., Kluge, N., Melderis, H. & Dube, S. (1973) *Nature, New Biol.* **243,** 203–205.
12. Ross, J., Ikawa, Y. & Leder, P. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 3620–3623.
13. Fraenkel-Conrat, H., Singer, B. & Tsugita, A. (1961) *Virology* **14,** 54–58.
14. Verma, I. M., Meuth, N. L., Bromfeld, E., Manly, K. & Baltimore, D. (1971) *Nature New Biol.* **233,** 131–134.
15. Baltimore, D. & Smoler, D. F. (1972) *J. Biol. Chem.* **247,** 7282–7287.
16. Ando, T. (1966) *Biochim. Biophys. Acta* **114,** 158–168.
17. Sutton, W. D. (1971) *Biochim. Biophys. Acta* **240,** 522–531.
18. Benz, D. J., Jr. & Forget, B. G. (1971) *J. Clin. Invest.* **50,** 2755–2760.
19. Verma, I. M., Temple, G. F., Fan, H. & Baltimore, D. (1972) *Nature New Biol.* **235,** 163–167.
20. Housman, D., Forget, B. G., Skoultchi, A. & Benz, E. J. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 1809–1813.
21. Preisler, H. D., Scher, W. & Friend, C. (1973) *Differentiation* **1,** 27–37.
22. McCarthy, B. J. & Church, R. B. (1970) *Annu. Rev. Biochem.* **39,** 131–150.
23. Lin, S.-Y. & Riggs, A. D. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 2574–2576.
24. Lyons, M. S., Lasfargues, E. Y. & Came, P. E. (1966) *Nature* **212,** 100–101.
25. Lowy, D. R., Rowe, W. P., Teich, N. & Hartley, J. W. (1971) *Science* **174,** 155–156.
26. Aaronson, S. A., Todaro, G. J. & Scolnick, E. M. (1971) *Science* **174,** 157–159.
27. Klement, V., Nicolson, M. O. & Huebner, R. J. (1971) *Nature New Biol.* **234,** 12–14.
28. Teich, N., Lowy, D. R., Hartley, J. W. & Rowe, W. P. (1973) *Virology* **51,** 163–173.
29. Natta, C., Banks, J., Niazi, G., Marks, P. A. & Bank, A. (1973) *Nature New Biol.* **244,** 280–281.