Proc. Nat. Acad. Sci. USA
Vol. 69, No. 12, pp. 3620–3623, December 1972

Globin Messenger-RNA Induction During Erythroid Differentiation of Cultured Leukemia Cells

(Friend leukemia virus/synthetic globin DNA/DNA-RNA hybridization)

JEFFREY ROSS*, YOJI IKAWA†, AND PHILIP LEDER*

* Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, and † Viral Leukemia and Lymphoma Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by C. B. Anfinsen, October 10, 1972

ABSTRACT **A cloned line of murine proerythroblastoid cells (T-3-Cl-2), transformed by Friend leukemia virus, undergoes changes associated with erythroid differentiation when treated with dimethylsulfoxide in culture. This line, which does not undergo spontaneous differentiation, develops specific erythrocyte-membrane antigen and accumulates detectable amounts of heme within four days of dimethylsulfoxide treatment. In the present study, we have followed the phenotypic expression of the globin genes by measuring globin mRNA in differentiating cells. Our hybridization probe for this purpose is [^{3}H]DNA, which is complementary to purified globin mRNA, synthesized by viral RNA-directed DNA polymerase. This probe is sufficiently sensitive to detect less than 1 ng of globin mRNA. Using it, we find little or no hybridizable globin mRNA in either uninduced cells or in treated control lymphoid cells. In contrast, globin mRNA can be detected in T-3-Cl-2 cells 2 days after induction by dimethylsulfoxide; it reaches a maximum concentration four days after induction. At this time, cells that stain positively for heme appear. The hybridizable cytoplasmic RNA induced in these cells has the sedimentation properties of 9S globin mRNA. Considering the stable character of globin mRNA, our results are most readily explained in terms of a transcriptional activation of the globin genes.**

Erythroid differentiation can be studied in proerythroblastoid tissue culture cells transformed with Friend leukemia virus (1, 2). Morphologically, these cells have some of the characteristics of immature erythroblasts, but they can be induced to initiate erythroid differentiation by the addition of dimethylsulfoxide (Me_2SO) to the culture medium (3). Several biochemical and morphologic markers of partially or fully differentiated erythroid cells have been identified after induction, including erythrocyte membrane antigen, heme, hemoglobin, and an increase of iron uptake (3, 4). Inasmuch as globin gene frequency and globin mRNA can be assayed with synthetic DNA as a hybridization probe (5), the phenotypic expression of globin genes can be followed as a function of these parameters in cultured leukemia cells.

Most recent studies indicate that globin genes are not highly reiterated in cells committed to globin production (5, 6, 20). The number of globin genes does not appear to differ between globin-producing cells and those committed to other lines of differentiation (5). If a relatively constant level of globin gene representation in DNA is assumed, induction of globin synthesis in cultured cells might be accompanied by an induced increase of cellular globin mRNA content or by the induced translation of a large pre-existing population of globin mRNA. Our present studies, designed to examine these possibilities, indicate that globin mRNA is virtually undetectable in undifferentiated cells, but increases markedly after Me_2SO induction. Taken together with other data, this result suggests the operation of a transcriptional control mechanism.

Abbreviation: Me_2SO, dimethylsulfoxide.

MATERIALS AND METHODS

Growth and Preparation of Friend Leukemia Cells. The mouse leukemia cell line, T-3-Cl-2, was derived from a transplantable ascites line that had been established by syngeneic transplantation of a focal splenic lesion of Friend virus-induced leukemia (7) and had been cloned in soft agar. This cloned line showed erythrocytic maturation during diffusion chamber culture in a mouse peritonium, and became positive for erythrocyte-membrane antigen (2). By use of the technique developed by Friend *et al.* (3), these cells became benzidine-positive and developed a hemoglobin-like color upon treatment with Me_2SO (4). For the present studies, T-3-Cl-2 cells and control lymphoid cells transformed by Moloney murine leukemia virus, Lybf-2-7, were grown in 250-ml plastic flasks (Falcon) containing 50 ml of HAM F-12 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated calf serum (GIBCO) in 5% CO_2 at 37°. 1 Day before the addition of Me_2SO, the cells were passed in fresh medium at 1×10^5 cells per ml. Unsterilized Me_2SO (Baker Chemical Co.), 1%, was added and incubation was continued as indicated in the relevant experiments. Cells were harvested by centrifugation, washed twice in buffer containing 0.01 M phosphate (pH 8.0)–0.14 M NaCl, and processed immediately or stored in liquid nitrogen.

Preparation of Globin mRNA, Synthetic DNA, and Total Leukemia Cell RNA. Mouse globin mRNA was purified from the reticulocytes of phenylhydrazine-treated (anemic) mice by oligo(dT)–cellulose chromatography and sucrose gradient centrifugation (8). This mRNA directed the synthesis of globin in an ascites cell-free extract and migrated as a single band during polyacrylamide gel electrophoresis (8). Radioactive DNA complementary to mouse globin mRNA was synthesized with RNA-directed DNA polymerase from avian myeloblastosis virus with the purified globin mRNA as template (9). This DNA, containing [^{3}H]dGMP (9800 mCi mmol) and [^{3}H]dCMP (30,000 mCi/mmol) demonstrated appropriate hybridization specificity, hybridizing with purified

9S mouse globin mRNA (Fig. 5) and polysomal RNA derived from mouse reticulocytes (Fig. 3), but not with similar RNA derived from duck reticulocytes or mouse BALB/3T3 tissue culture cells (J.R. and P.L., unpublished result). Total cellular RNA and cytoplasmic RNA were prepared from washed T-3-Cl-2 and Lybf-2-7 cells by phenol–chloroform–isoamyl alcohol extraction at 60°, and total RNA by DNase treatment, essentially as described by Penman (10).

Assays and Sucrose Gradient Centrifugation Analyses. DNA–RNA hybridization was performed in 0.2-ml reaction mixtures containing 15 mM Tris·HCl (pH 7.2), 0.15 M NaCl, 0.5 mM EDTA, 38% formamide, [^{3}H]DNA (about 1500 cpm), and RNA as indicated. [^{3}H]DNA was heated in 76% formamide at 60° for 10 min before use. The complete reaction was incubated at 40° for 24–48 hr. Reaction mixtures were diluted with 2 ml of buffer containing 0.03 M sodium acetate–0.1 M NaCl–1.2 mM zinc sulfate–30 μg/ml of calf-thymus DNA, and hybrid formation was assayed by resistance (Cl_3CCOOH-precipitable radioactivity) to digestion with 10 μg of nuclease S_1 from *Aspergillus oryzae* at 45° for 60 min (11 R. Benveniste, personal communication). Zero-time control reactions indicated that no inhibitors of nuclease were present in extracted RNA. Cytoplasmic RNA was analyzed on a 15–30% sucrose gradient containing 0.01 M Tris·HCl (pH 7.4)–0.1 M NaCl–1 mM EDTA by centrifugation in a Spinco SW41 rotor at 40,000 rpm for 13 hr at 4°. 0.5-ml Fractions were collected and 0.1-ml aliquots were taken for hybridization assay as noted above. RNA was also analyzed by centrifugation through a linear gradient of 89% Me_2SO–10% [*U*-^{2}H]Me_2SO–0 g/100 ml sucrose–1% H_2O to 89% [*U*-^{2}H]Me_2SO–10% Me_2SO–10 g/100 ml sucrose–1% H_2O containing 1 mM EDTA (12). Centrifugation was in a Spinco SW65 rotor at 60,000 rpm for 13 hr at 27°. 0.11-ml fractions were collected and 0.04-ml aliquots were taken for hybridization assay. Hybridization was done in 0.1-ml reaction mixtures containing the components noted above, except that formamide was omitted and the final concentration of Me_2SO was 40%. Cells were stained with benzidine by Ralph's method (13).

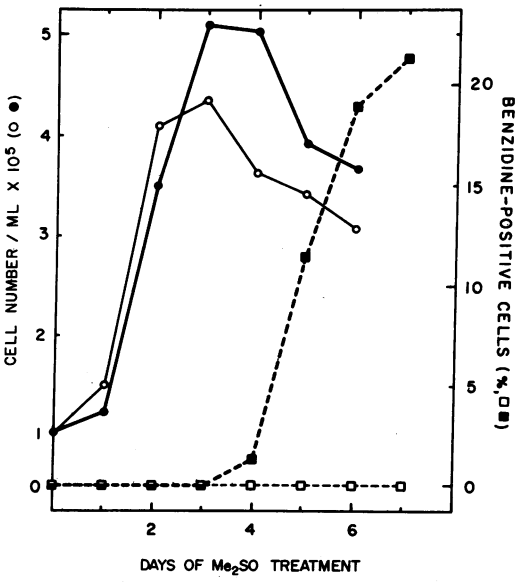

FIG. 1. Effect of Me_2SO on T-3-Cl-2 cell growth and heme production. Cells were grown, treated, and stained with benzidine. Only clearly positive cells were scored positive. Over 95% of the cells remained viable throughout the initial 4 days of treatment, as tested by trypan blue exclusion. *Closed symbols* indicate the presence of Me_2SO, *open symbols* its absence.

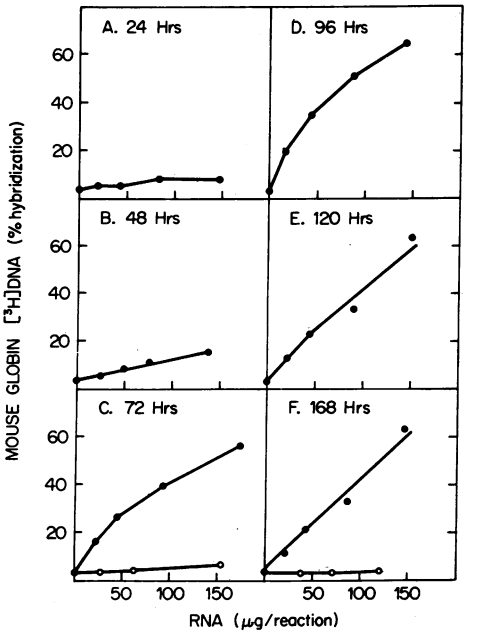

FIG. 2. Time course of induction of globin mRNA after treatment with Me_2SO. Hybridization assays were performed on total RNA extracted from cells grown and treated as indicated under *Methods*. *Closed circles* indicate the presence of Me_2SO, *open circles* its absence.

RESULTS

Induction of erythroid changes and appearance of globin mRNA sequences

Relatively undifferentiated Friend virus-transformed cells undergo various erythroid changes after treatment with Me_2SO (3, 4). The addition of a relatively low concentration of Me_2SO (1%) to the culture medium of T-3-Cl-2 cells (Fig. 1) has little effect upon cell growth or viability during the first 4 days of treatment. It does result in the appearance of benzidine-positive cells (indicating the presence of heme) after 4 days of incubation, and the conversion of about 20% of the cells to benzidine positive after 7 days. This conversion does not occur among untreated control cells (Fig. 1). A pink color could readily be discerned in the pelleted cells by the seventh day, and, indeed, Friend *et al.* (3) find that cells treated in this way produce hemoglobin.

To determine whether this erythroid change is accompanied by accumulation of globin mRNA, total cellular RNA was prepared from T-3-Cl-2 cells at various times after treatment with 1% Me_2SO, and assayed for RNA that would hybridize with [^{3}H]DNA complementary to purified mouse globin mRNA (Fig. 2). Control hybridization studies were performed with total cellular RNA derived from untreated T-3-Cl-2 cells (Fig. 2) and treated and untreated lymphoid Lybf-2-7 cells. Untreated T-3-Cl-2 cells contain virtually no hybridizable RNA (Fig. 2, 72 and 168 hr). RNA from both treated and untreated Lybf-2-7 cells gave identical negative results (data not shown). In contrast, hybridizable globin sequences begin to appear between 24 and 48 hr after treat-

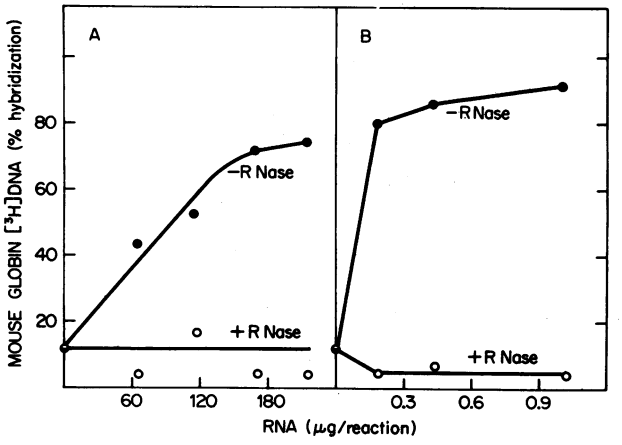

FIG. 3. RNAse sensitivity of hybridizable globin mRNA from Me_2SO-treated cells. Total RNA was extracted from cells grown 4 days in the presence of Me_2SO. This RNA (*A*) or mouse reticulocyte polysomal RNA (*B*), was incubated at 37° for 30 min with (*open circles*) and without (*closed circles*) 40 μg/ml of DNase-free pancreatic RNase. Aliquots were then incubated with globin [³H]DNA.

ment of T-3-Cl-2 cells. The relative concentration of globin RNA sequences reaches a maximum at 96 hr and remains relatively constant for the next 3 days.

Characterization of hybridizable RNA induced in treated cells

That the hybridizable globin sequences are present in an RNA molecule is illustrated by their sensitivity to digestion with ribonuclease (Fig. 3). Hybridization is abolished when both total T-3-Cl-2 cellular RNA or control mouse reticulocyte RNA are exposed to RNase before hybridization. The sedimentation properties of the hybridizable RNA can also be determined by aqueous and nonaqueous (Me_2SO) sucrose gradient sedimentation (Fig. 4). Most of the hybridizable cytoplasmic RNA sediments at about 9 S under both conditions. A small amount of hybridizable RNA sediments faster than 18 S in aqueous sucrose (Fig. 4*A*), but does not appear

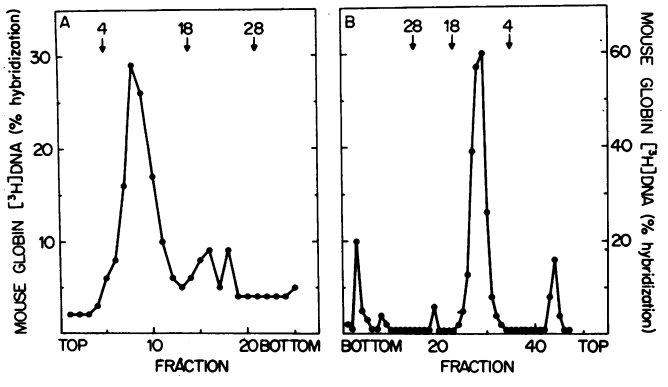

FIG. 4. Velocity sedimentation of Me_2SO-induced globin mRNA. RNA was prepared from the cytoplasm of cells treated with Me_2SO for 4 days. About $8A_{260}$ units of RNA was applied to the aqueous sucrose (*A*) and Me_2SO (*B*) gradients, which were prepared, centrifuged, and assayed as indicated under *Methods*. The *arrows* indicate the position of cellular 4, 18, and 28S RNAs.

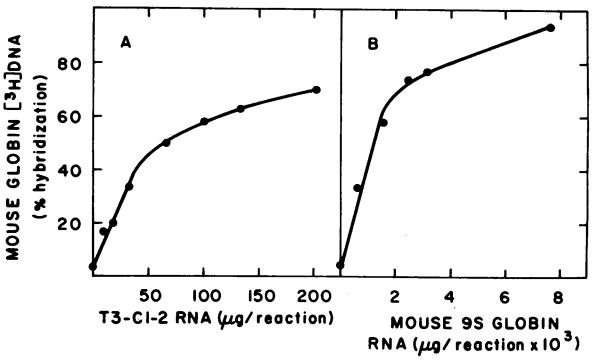

FIG. 5. Calibrated hybridization of purified 9S globin mRNA from mouse and RNA derived from Me_2SO-treated cells. Total cellular RNA was derived from pooled cells treated 72–120 hr with Me_2SO.

in the gradient centrifuged under nonaqueous conditions, which minimize hydrogen-bonded aggregation.

Quantitation of globin mRNA sequences in fully induced cells

The amount of hybridizable RNA in induced (72–120 hr) cells can be roughly estimated by calibration of the amount of [³H]DNA hybridized with purified mouse globin mRNA (Fig. 5). About 0.8 ng of purified globin mRNA hybridizes with about 30% of the [³H]DNA. Under the same conditions, this degree of hybridization occurs in the presence of about 25 μg of total T-3-Cl-2 cellular RNA, suggesting that about 0.003% of the total RNA of the entire treated cell population corresponds to globin mRNA sequences.

DISCUSSION

The Friend virus-transformed cell line T-3-Cl-2 and other similarly transformed leukemia cells can be induced to undergo changes that are analogous to those involved in the differentiation of erythrocytic precursors (1–4). They develop erythrocyte membrane-specific antigen, accumulate heme and hemoglobin, and undergo morphologic changes akin to normoblastic maturation (3, 4). The fact that these cells can be induced by treatment with Me_2SO to undergo erythroid changes makes them a convenient model for studying globin gene expression (3). While the mechanism by which Me_2SO induces these changes is unclear (3), the cell line in which these observations were originally made exhibited a limited degree of spontaneous erythroid differentiation throughout serial passage (14). For our purposes, the T-3-Cl-2 line has the advantage of not undergoing spontaneous differentiation in culture (4). The fact that erythrocyte-membrane antigen can be induced in this line by several conditions in addition to Me_2SO treatment (16) make it particularly suitable for detailed studies of the inductive mechanism.

It is convenient to think of mechanisms that might control the phenotypic expression of specific genes during differentiation in terms of increasing the dosage of a specific set of genes, or in terms of inducing their transcription or the translation of their corresponding mRNAs. With respect to erythrocytic development, evidence suggests that the globin genes are not highly reiterated (5, 6) and that mRNA in reticulocytes is relatively stable (15). These observations

make it likely that the most important control steps in the committment of a cell to globin production affect either (or both) the transcription or (and) translation processes. We have determined globin mRNA levels in cultured T-3-Cl-2 cells as they undergo erythroid differentiation in response to Me_2SO treatment by the use of highly radioactive DNA complementary to purified mouse globin mRNA as a specific hybridization probe (5, 9, 17, 18). Our assay detects very small amounts of RNA that have the hybridization and sedimentation properties of globin mRNA (see Figs. 4 and 5). Using this assay, we find little or no hybridizable RNA in uninduced T-3-Cl-2 cells. However, 2 days after Me_2SO induction, globin mRNA sequences can be detected; they reach a relatively stable level within 4 days of induction (Fig. 2). This result makes less likely a control step involving the translational activation of a large, reserve population of globin mRNA. Still, our studies do not allow us to distinguish between control mechanisms involving increased synthesis or decreased degradation to account for the accumulation of globin mRNA. Since little or no detectable globin mRNA is present in uninduced cells, and globin mRNA is relatively stable (15), it seems more likely that globin mRNA accumulates as a result of transcriptional activation of the globin genes. Indeed, very recent studies indicate that a newly labeled species of 9S RNA appears in a similar cell line treated with Me_2SO (19).

Our results also indicate that globin mRNA sequences constitute only a very small fraction of the total cellular RNA of induced cells (Fig. 5). While it is clear that cells undergoing erythroid differentiation require the phenotypic expression of many genes in addition to those directing the synthesis of globin, it is not clear whether this mRNA is being produced in large amounts by a small proportion of the treated cells, or whether about 0.003% of the RNA of each treated cell corresponds to the globin messenger. Previous studies have indicated that erythrocyte-membrane antigen appears in about 60% of T-3-Cl-2 cells by the fourth day of Me_2SO treatment (2). In these studies, we have conservatively scored about 20% of the treated cells as benzidine positive (Fig. 1), suggesting that a significant proportion of the treated cells are undergoing differentiation. A further question arises regarding the role of the Friend leukemia virus in this process. Me_2SO treatment of T-3-Cl-2 cells induces a 100-fold increase of the virus titer (4). While it is likely that the virus is involved in maintaining the neoplastic character of these cells, it might also have some direct effect on the genes responsible for erythroid differentiation.

It is a pleasure to acknowledge the expert technical assistance of James Dotson and the advice of Drs. R. Benveniste and E. Scolnick about the preparation and use of S_1 nuclease. We are also grateful to Catherine Kunkle for her expert assistance in the preparation of this manuscript. Y.I. was supported in part by a USPHS International Research Fellowship (1 F05 TW-1704-01) while on leave from the Cancer Institute, Tokyo.

1. Friend, C., Patuleia, M. C. & de Harven, E. (1966) *Nat. Cancer Inst. Monogr.* **22,** 505–520.
2. Furusawa, M., Ikawa, Y. & Sugano, H. (1971) *Proc. Jap. Acad.* **47,** 220–224.
3. Friend, C., Scher, W., Holland, J. G. & Sato, T. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 378–382.
4. Ikawa Y., Furusawa, M. & Sugano, H. (1972) "Collected Papers of 5th Int. Symp. on Comparative Leukemia Research," Padova, Italy, Sept. 14–16, (1971) *Bibl. Haematol.*, in press.
5. Packman, S., Aviv, H., Ross, J. & Leder, P. (1972) *Biochem. Biophys. Res. Commun.*, in press.
6. Bishop, J. O., Pemberton, R. & Baglioni, C. (1972) *Nature New Biol.* **235,** 231–234.
7. Ikawa, Y. & Sugano, H. (1966) *GANN* **57,** 641–643.
8. Aviv, H. & Leder, P. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 1408–1412.
9. Ross, J., Aviv, H., Scolnick, E. & Leder, P. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 264–268.
10. Penman, S. (1966) *J. Mol. Biol.* **17,** 117–130.
11. Sutton, W. D. (1971) *Biochim. Biophys. Acta* **240,** 522–531.
12. Acheson, N. H., Buetti, E., Scherrer, K. & Weil, R. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 2231–2235.
13. Lo Bue, J., Dornfest, B. C., Gordon, A. S., Hurst, J. & Quastler, H. (1963) *Proc. Soc. Exp. Biol. Med.* **112,** 1058–1062.
14. Scher, W., Holland, J. G. & Friend, C. (1971) *Blood* **37,** 428–437.
15. Grasso, J. A., Woodward, J. W. & Swift, H. (1963) *Proc. Nat. Acad. Sci. USA* **50,** 134–140.
16. Sugano, H., Furusawa, M., Kawaguchi, T. L. & Ikawa, Y. (1972) "Collected Papers of 5th Int. Symp. on Comparative Leukemia Research," Padova, Italy, Sept. 14–16, (1971) *Bibl. Haematol.*, in press.
17. Kacian, D. L., Spiegelman, S., Bank, A., Terada, M., Metafora, S., Dow, L. & Parks, A. A. (1972) *Nature New Biol.* **235,** 167–169.
18. Verma, I. M., Temple, G. F., Fan, H. & Baltimore, D. (1972) *Nature New Biol.* **235,** 163–167.
19. Ostertag, W., Melderis, H., Steinheider, G., Kluge, N. & Dube, S. *Nature New Biol.*, in press.
20. Harrison, P. R., Hell, A., Birnie, G. D. & Paul, J. (1972) *Nature,* **239,** 219–221.