IN SITU LOCALIZATION OF GLOBIN MESSENGER RNA FORMATION

II. After Treatment of Friend Virus-Transformed Mouse Cells with Dimethyl Sulfoxide

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ABSTRACT

Globin messenger RNA (mRNA) levels in Friend virus-transformed mouse cells have been estimated by *in situ* hybridization of DNA copy (cDNA) to fixed preparations of cells and by hybridization of cDNA to extracted cytoplasmic RNA in true solution. The results obtained by both methods agree in showing that a low level of globin mRNA can be detected in untreated Friend cells. The levels of hemoglobin and globin mRNA have also been correlated after treatment of Friend cells with dimethyl sulfoxide (DMSO). The results obtained by both experimental approaches show that there is a minimum period of treatment with DMSO required in order that Friend cells may become hemoglobinized, and that this period coincides with the time when globin mRNA accumulates. Moreover, bromodeoxyuridine prevents both hemoglobin and globin mRNA accumulation.

In the accompanying paper (1) *in situ* hybridization of globin DNA copy (cDNA) to fetal liver was used to elucidate the process of globin messenger RNA (mRNA) formation in erythroid precursor cells. The essential conclusions were also confirmed by conventional hybridization studies. In the present paper, the same dual approach is applied to a different experimental system in which hemoglobin synthesis in Friend virus-transformed mouse cells is induced by treatment with dimethylsulfoxide (DMSO) (2). The results obtained by *in situ* and conventional hybridization agree in showing that there is a minimum period of treatment with DMSO required in order that globin mRNA and hemoglobin may accumulate.

MATERIALS AND METHODS

Cell Culture

Friend cells (clone 707) and lymphoma cells (L 5178Y) were grown in Ham's F12 medium (Flow Laboratories, Irvine, Scotland), supplemented with minimal essential medium amino acids, penicillin (60 U/ml), and 10–15% horse serum and buffered with HEPES or bicarbonate. LS cells were grown in Eagle's minimal essential medium plus 5% calf serum.

All other methods are fully described in the accompanying paper (1).

RESULTS

Formation of Hemoglobin

Friend cells normally grow from about 10^4 cells/ml to 10^6 cells/ml with a doubling time of about 12 h. Friend cells grow in 1.5% DMSO at about the same rate as untreated cells for about 3 days (six to seven generations); then cell division ceases and cells begin to accumulate hemoglobin, so that the percentage of hemoglobinized cells increases rapidly after the 3rd day of exposure to DMSO (Table I). However, it is not necessary for cells to undergo a critical number of divisions in

order to begin to synthesize hemoglobin: if cells from a stationary culture are seeded at low density into medium containing 1.5% DMSO, then the cells do not divide; nevertheless, most cells become hemoglobinized after 6 days. However, the question remains as to whether Friend cells require to be cultured continuously for 5 days in DMSO in order to become fully hemoglobinized. This question can be investigated by measuring the percentage of Friend cells hemoglobinized after culturing in DMSO for various times followed by culture in absence of DMSO for the remainder of the 6-day period (Table II). These results show that it is necessary to treat Friend cells with DMSO for the first 3 days in order to obtain the maximum percentage of hemoglobinized cells after 6 days; however, treatment of Friend cells with DMSO for more than 3 days does not increase the yield of hemoglobinized cells.

TABLE I

Hemoglobin Formation in DMSO-Treated Friend Cells

Cell type	Time spent in 1.5% DMSO	Hemoglobinized cells
	Days	%
Friend 707 (inducible line)	0	1
	1	1
	2	1
	3	35
	4	61
	5	81
	6	85
Friend 707 R (noninducible line)	6	0
LS	0	0
	6	0
Lymphoma LY	0	0
	6	0

The Friend 707 R cell line was obtained from Friend 707 cells by continuous culture in 1.5% DMSO for about 2 mo, then grown in DMSO-free medium before culture again in DMSO for 6 days.

Formation of Globin mRNA

It is obviously important to relate changes in the hemoglobin content of DMSO-treated Friend cells with any changes in globin mRNA content. Further, it is important to determine whether there is any heterogeneity in response to DMSO in the individual cells in the total Friend cell population. It is possible to elucidate these questions by a combination of the two approaches described in the accompanying paper, namely by *in situ* hybridization of globin cDNA to fixed slide preparations of Friend cells at various stages after treatment with DMSO, or by titration of cytoplasmic RNA extracted from such cells with globin cDNA. Fig. 1 *a–f* shows how the extent of *in situ* hybridization of globin cDNA increases during treatment of Friend cells with DMSO; these results are quantified in Table III. The precentage of the Friend cells showing increased *in situ* hybridization is about 90–95%. By contrast, neither LS cells nor lymphoma cells show *in situ* hybridization to globin cDNA, whether or not they are treated with DMSO (See Table III and Figs. 1 and 2 of accompanying paper). Moreover, a noninducible

TABLE II

Effect of Time of Exposure to DMSO on Hemoglobin Formation

Time in DMSO	Cell concentration $\times 10^{-6}$	Hemoglobinized cells at end of period of culture in DMSO	Subsequent time in culture without DMSO	Final cell concentration $\times 10^{-6}$	Final percent hemoglobinized cells
Days		%	*Days*		%
1	0.22	1	5	2.8	17
2	0.66	1	4	2.5	36
3	1.43	32	3	2.3	78
4	2.71	58	2	2.8	84
5	2.92	86	1	2.6	84
6	2.1	84	–	–	–

Cells were inoculated from exponentially growing cultures at 10^5 cells/ml, and DMSO was added to a final concentration of 1.5%.

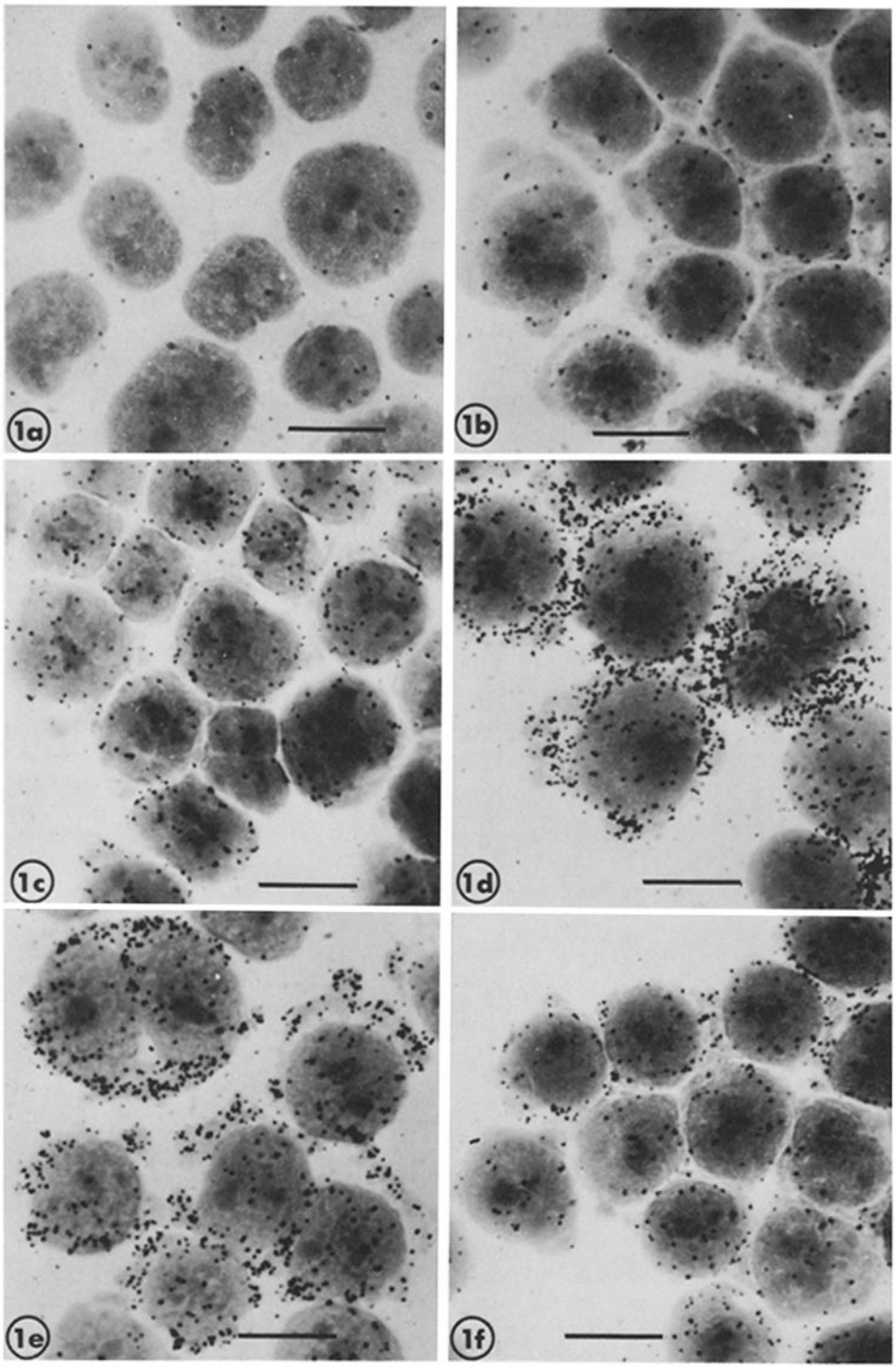

FIGURE 1 *In situ* hybridization of globin cDNA to Friend cells after treatment for (*a*) 0 days, (*b*) 1 day, (*c*) 2 days, (*d*) 3 days, (*e*) 4 days, (*f*) 5 days with 1.5% DMSO. Autoradiographic exposure, 7 wk. The bar represents 10 μm. × 1,400.

TABLE III

Globin mRNA Formation in DMSO-Treated Cells

Cell type	Time spent in 1.5% DMSO	Globin mRNA in cytoplasmic RNA × 10^2	*In situ* hybridization
	Days	%	
Friend 707 Cells	0	1.6	8 ± 3
	1	2.1	36 ± 11
	2	—	32 ± 8
	3	7.2	72 ± 22
	4	8.7	79 ± 28
	5	7.5	56 ± 20
	6	5.8	33 ± 19
Friend 707 R	6	—	15
LS Cells	0	0.1	6
	6	—	8
Lymphoma cells	0	0.1	8
	6	0.1	12

The concentration of globin mRNA in extracted RNA was determined by titration with globin cDNA. Hybrids formed between cDNA and cytoplasmic RNA from DMSO-treated Friend cells banded in CsCl at a density of 1.78 after treatment with ribonuclease, and had a melting temperature of 67°C in 0.5 M NaCl/50% formamide, i.e. the same as that of reticulocyte 9S RNA/cDNA hybrid under the same conditions (see accompanying paper for description of the methods). *In situ* hybridization (average number of grains per cell ± standard error) refers to an autoradiographic exposure of 6 wk. In order to compare the *in situ* hybridization results (measuring the amount of cytoplasmic globin mRNA per cell) with those quoted in column 4 (measuring the concentration of globin mRNA in total cytoplasmic RNA), allowance must be made for the reduction in content of cytoplasmic RNA in Friend cells. (See legend to Table IV).

cell line derived from Friend cells no longer accumulates globin mRNA on growth in DMSO, as judged by *in situ* hybridization of cDNA (Tables I and III). Although with the usual autoradiographic exposure of 7–10 wk the untreated Friend 707 cells show a similar slight extent of *in situ* hybridization as do LS cells, after an autoradiographic exposure of 5 mo, most untreated Friend cells show a significantly greater *in situ* hybridization than LS cells exposed for the same time (Fig. 2). Therefore most cells in the untreated Friend cell population contain very small, but detectable, amounts of globin mRNA.

The experiments in which the globin mRNA content of Friend cells was estimated by titration of extracted cytoplasmic RNA with globin cDNA confirm these conclusions obtained by *in situ* hybridization (Table III). Whereas no globin mRNA can be detected in either untreated or DMSO-treated LS or lymphoma cells, a small amount of globin mRNA is present in untreated Friend cells. After treatment of Friend 707 cells with DMSO, the globin mRNA content increases, reaching a maximum in cells treated for about 4 days with DMSO, as was obtained by *in situ* hybridization. Similar results have been obtained by other workers, estimating globin mRNA by conventional hybridization methods (3, 4) or by the ability to direct globin synthesis in oocytes (5).

Effect of Bromodeoxyuridine

Bromodeoxyuridine (BUdR) prevents the formation of hemoglobinized cells in cultures of Friend cells treated with DMSO, provided that it is added before addition of DMSO (Table IV). Similar results have been reported recently by other workers (6, 4). Estimation of globin mRNA levels by *in situ* hybridization shows that this reduction in hemoglobin formation is due to greatly reduced levels of globin mRNA in cells treated with DMSO and BUdR as compared with DMSO alone (Table IV). Similar conclusions concerning the effect of BUdR have been reported recently by Friend and coworkers, estimating the levels of globin mRNA by titration with globin cDNA (4).

DISCUSSION

The results presented in this paper show that Friend cells begin to accumulate hemoglobin during culture in DMSO: the proportion of hemoglobinized Friend cells increases sharply after 2–3 days in DMSO, reaching a maximum of about 85% after culture in DMSO for 5–6 days. Paul and Hickey (7) have shown by cloning experiments that the proportion of Friend cells (15%) which do not become hemoglobinized after treatment with DMSO does not represent a noninducible population of cells. The reason why certain cells in the population fail to respond to DMSO is not yet clear.

It is not necessarily valid to interpret the sudden increase in hemoglobinized cells (as detected by benzidine staining) to mean that hemoglobin synthesis is initiated 2–3 days after treatment of Friend cells with DMSO. However, the experi-

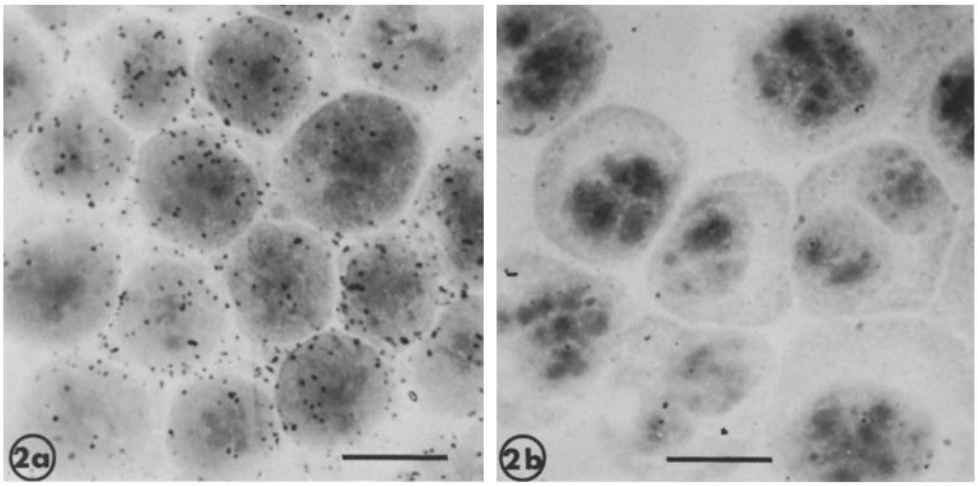

FIGURE 2 *In situ* hybridization of globin cDNA to (*a*) untreated Friend cells and (*b*) mouse L cells. Autoradiographic exposure, 20 wk. The bar represents 10 μm. × 1,400.

TABLE IV

Effect of BUdR on Hemoglobin and Globin mRNA Formation in Friend Cells

Treatment	Hemoglobinized cells	Globin mRNA content
	%	*grains/cell*
Nil	1	12
1.5% DMSO	75	115
10^{-5} M BUdR	1	15
1.5% DMSO + 10^{-5} M BUdR	1	20

Globin mRNA content was estimated by *in situ* hybridization after treatment for 3 days; the percent of haemoglobinized cells was measured after treatment for 6 days. BUdR was only effective in preventing formation of hemoglobinized cells if added 12 h before addition of DMSO. Under these conditions, BUdR reduced the growth of cells slightly. The relative amounts of cytoplasmic RNA per cell after treatment with DMSO and/or BUdR for 6 days were: control, 100%; DMSO, 65%; BUdR, 95%; DMSO + BUdR, 80%.

ments in which Friend cells were treated with DMSO for varying lengths of time do indicate that the maximum proportion of hemoglobinized cells is achieved by treatment of Friend cells with DMSO for a minimum period of 3 days followed by culture in DMSO-free medium for a further 3 days. Clearly, the processes which lead ultimately to hemoglobin formation must be stabilized within the first 3 days of treatment with DMSO. This is particularly interesting in view of the fact that the level of cytoplasmic globin mRNA increases about 10 times during the first 3 days of treatment of Friend cells with DMSO; furthermore, this rise in globin mRNA content is prevented by low concentrations of BUdR. This conclusion is derived from the novel *in situ* hybridization studies, confirmed also by more conventional hybridization procedures. One of the main effects of DMSO is, therefore, to enable the Friend cells to accumulate cytoplasmic globin mRNA during the first 3 days of treatment; thereafter its continued presence in the culture medium is not necessary.

The results obtained by the conventional hybridization approach also show that populations of untreated Friend cells contain detectable amounts of globin mRNA. The *in situ* hybridization studies extend this conclusion by showing that most cells in the untreated Friend cell population contain a low level of globin mRNA. This must mean that there is some globin mRNA synthesis in untreated Friend cells. Clearly, further experiments are required to elucidate in detail the mechanism whereby this constitutive level of synthesis globin mRNA is increased and leads to stabilization and translation of globin mRNA in the cytoplasm. This will be the subject of a further communication (8, 9).

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