Proc. Nat. Acad. Sci. USA
Vol. 72, No. 1, pp. 98–102, January 1975

Differentiation in Erythroleukemic Cells and Their Somatic Hybrids

(probability of differentiation/globin mRNA/dimethylsulfoxide/microspectrophotometry)

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Communicated by Saul Roseman, October 18, 1974

ABSTRACT **Clones of erythroleukemic cells differ in the extent to which they (*1*) undergo differentiation spontaneously and (*2*) can be induced to differentiate in the presence of dimethylsulfoxide. Here we demonstrate that relative differences in globin gene expression within and between clones largely reflect differences in the proportion of cells participating in differentiation rather than uniform differences in the extent to which all cells in these clones undergo differentiation. We call this phenotype of a clone its characteristic *probability of differentiation*, a property that reflects the likelihood that a cell of this clone will undergo erythrodifferentiation under given conditions. We have examined somatic hybrid cells formed between similar erythroleukemic lines, between phenotypically different erythroleukemic lines, and between erythroleukemic cells and mouse fibroblasts. Results of these experiments demonstrate that the spontaneous and induced probabilities of differentiation may be altered in an uncoupled fashion, suggesting that each is determined at *different* steps leading to a common pathway of globin gene expression.**

Murine erythroleukemic cells infected with Friend leukemia virus resemble proerythroblasts morphologically and mature in culture to a normoblast-like stage when treated with dimethylsulfoxide [$(CH_3)_2SO$] (1). This *in vitro* erythroid differentiation is characterized by the appearance of an erythrocyte membrane antigen (2), increases in heme synthesis and iron uptake (1), induction of hemoglobin synthesis (1, 2, 4), and the accumulation of globin mRNA (5, 6). The expression of the globin genes can be quantitated precisely and specifically in these cells by use of radioactive, synthetic globin complementary DNA (cDNA) (6).

Of paramount importance to all further studies on globin gene expression and differentiation in these cells is an assessment of the uniformity of the extent of differentiation in cells within cloned lines. Here we show that relative differences in apparent globin gene expression within and between lines largely reflect differences in the proportion of cells participating in erythroid differentiation rather than uniform differences in the extent to which all cells in clones undergo differentiation. For convenience we propose to call this phenotype of a clone its *probability of differentiation*, a property which reflects the likelihood that a cell of a clone will undergo differentiation under given conditions. This phenotype is preserved in subclones of cloned erythroleukemic lines, indicating that the probability of differentiation in these lines is controlled by relatively stable genetic or epigenetic factors. To assess the interactions of such factors we have also examined somatic hybrid cells formed between similar erythroleukemic lines, between phenotypically different erythroleukemic lines, and between erythroleukemic cells and mouse fibroblasts.

Abbreviations: $(CH_3)_2SO$, dimethylsulfoxide; globin mRNA, globin messenger RNA; cDNA, complementary DNA.

MATERIALS AND METHODS

Cells. Stock erythroleukemic cell lines consisted of T3-C12 (5) and GM 86 (clone 745) (7). Cells were routinely passed every 4 days in suspension culture at 1:25 dilution in F12 medium plus 10% heat-inactivated calf serum, and were inoculated directly into medium containing $(CH_3)_2SO$ when indicated.

Erythroleukemic cells form colonies in Microtest II dishes (Falcon Plastics) with efficiencies of 15–40% at inputs of less than 0.5 cell per well. Subclones suitable for cell hybridization using hypoxanthine–amethopterin–thymidine (HAT) selection (8) were prepared by ethylmethane sulfonate mutagenesis (9) and selection in either thioguanine at 40 μg/ml or bromodeoxyuridine (BrdU) at 30 μg/ml. A BrdU-resistant subclone of GM 86 (H-11) was made resistant to 1 mM ouabain (10) by similar methods. The subclones used in these studies were 2 thioguanine-resistant lines, T3-C12 TG D-3 and GM 86 TG D-5, and a BrdU–ouabain-resistant line, GM 86 H-11 OulA. Reversion frequencies of the thioguanine and BrdU markers of these lines were less than 10^{-6}. A9 mouse fibroblasts (8) were obtained from John W. Littlefield.

Somatic Hybridization. Cells were fused with the aid of β-propiolactone-inactivated Sendai virus (kindly supplied by John W. Littlefield). Independent hybrids were directly selected with HAT medium in Microtest dishes at 3000 to 5000 cells per well. Suspension hybrids appeared at a frequency of 10^{-5} within 14 days and were passed twice in HAT medium prior to passage under nonselective conditions. No hybrids (frequency less than 5×10^{-7}) were obtained without Sendai virus treatment of cell mixtures. All subclones and hybrids grew with a doubling time of 12–16 hr.

GM 86 and GM 86 H-11 were hybridized with A9 mouse fibroblasts by methods previously described for human lymphoblast–mouse fibroblast hybridizations (11).

Benzidine Staining. Cells in Microtest wells were stained with addition of two drops of 0.4% benzidine base, 2% hydrogen peroxide in 12% acetic acid to the culture medium (approximately 200 μl total).

Microspectrophotometry. Absorption spectra of individual cells were measured using a computer-assisted, wavelength scanning and recording microspectrophotometer (13). Cells

TABLE 1. *Globin mRNA in erythroleukemic cell lines*

Cell line	% Globin mRNA (×10³) in: Control	1% $(CH_3)_2SO$
T3-C12 (original stock)	0.1 (0.0–0.2)	10.0 (9.0–13.0)
T3-C12 (long term passage)	0.0	40.0
T3-C12 TG D-3	0.2 (0.1–0.3)	4.0 (3.0–4.5)
GM 86 (clone 745)	7.0 (2.0–10.0)	60.0 (45.0–100.0)
GM 86 H-11 Oula	7.0 (5.0–10.0)	63.0 (50.0–83.0)
GM 86 TG D-5	6.0 (5.0–8.0)	63.0 (40.0–85.0)

Cells were harvested after 4 days' growth in regular medium or medium containing 1.0% $(CH_3)_2SO$, and percentage of globin mRNA in total cytoplasmic RNA was determined as described in *Materials and Methods*. Values represent the average of several independent experiments with the complete range shown in parentheses. "Long term passage T3-C12" is a more inducible line of T3-C12 cells which arose after continuous growth of stock T3-C12 cells for a 10-month period. Cell growth in all lines was unaffected by treatment with 1% $(CH_3)_2SO$.

in a thin film between coverslips were randomly chosen. Each recording consisted of 32 bidirectional scans of the spectrum (325–695 nm) while a beam of light approximately 2 × 2 μm was centered on a cell. Control experiments demonstrated that adult mouse erythrocytes were a homogeneous population with a direct cellular absorptance (= 1 − transmittance) of 70% at 415 nm. No preferential absorption of linearly polarized light (i.e., linear dichroism) was observed for the absorption bands of erythrocytes or erythroleukemic cells.

RNA Extraction. Cytoplasmic RNA was prepared as described previously (6) without Potter–Elvejhem homogenization.

cDNA Probes, DNA·RNA and DNA·DNA Hybridization Assays. Preparation of mouse globin cDNA probe has been described (6, 12). Hybridization of cytoplasmic RNA samples to globin cDNA was performed at 45° in 0.45 M NaCl in 33% formamide for 21 hr in a total reaction volume of 30 μl. Input globin [³H]cDNA was 750–1000 cpm per assay tube. Percent hybrid formation was measured by the fraction of [³H]cDNA resistant to S_1 nuclease digestion for 75 min at 45° as before (6). Cellular RNA added per reaction was varied to yield at least two points within the linear range of hybridization (less than 35%) and the percentage of cytoplasmic RNA that was globin mRNA was calculated by reference to a standard curve for purified 9S mouse globin mRNA assayed under identical conditions. 30% hybridization under these conditions corresponded to 250 pg of pure globin mRNA.

Hybridization of globin [³H]cDNA to cellular DNA to estimate globin gene reiteration per haploid genome was performed as previously described (6).

RESULTS

Differentiation of Cloned Erythroleukemic Cell Lines. Cloned erythroleukemic lines differ in both their spontaneous and $(CH_3)_2SO$-induced levels of globin mRNA (Table 1). When $(CH_3)_2SO$-treated cells of these lines are stained with acid benzidine reagent to reveal hemoglobin accumulation after 5 days' induction, a nonuniform pattern of staining is evident. For example, in GM 86 and its subclones, a large proportion (about 50%) of cells stain positively; whereas in the thioguanine-resistant subclone of T3-C12 (TG D-3) only a small fraction (less than 5%) become positive under identical growth conditions. To exclude the possibility that this apparent heterogeneity of induction is an artifact due to insensitivity of benzidine staining, we examined the hemoglobin content of individual erythroleukemic cells using a computer-assisted microspectrophotometer (13). An average absorption spectrum of induced T3-C12 cells is shown in Fig. 1. The prominent Soret band with a peak at 415 nm is typical of oxyhemoglobin. Untreated T3-C12 cells show no absorption in this region. When T3-C12 cells are treated with 0.75, 1.0, and 1.5% (w/v) $(CH_3)_2SO$ for 5.5 days and then examined for globin mRNA, benzidine positivity, and hemoglobin detectable by microspectrophotometry, a direct correlation of globin mRNA with either estimate of the percentage hemoglobinized cells is observed (Fig. 2).

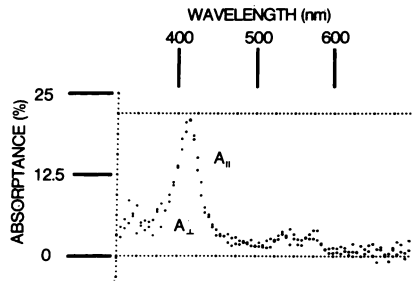

FIG. 1. Absorption spectrum of induced T3-C12 cells. Recordings from eight individual hemoglobinized cells were computer averaged. The two nearly identical traces ($A_{||}$, $A_{\perp}$) correspond to absorption of linearly polarized light. If one assumes a molar extinction coefficient of 125,000 M^{-1} cm^{-1} at 415 nm for hemoglobin (14) and an optical path length of 11 μm (= measured cell diameter), the absorptance of 20% (= optical density of 0.1) corresponds to a hemoglobin concentration of 0.72 mM.

No correlation of the hemoglobin content of individual *positive* cells measured by microspectrophotometry and either the $(CH_3)_2SO$ concentration used to induce them or cell diameter was observed. If cells with less than 4% absorptance are scored as negative, the microspectrophotometric estimate of the percentage positive cells is identical with that obtained by benzidine staining. When cells with barely detectable hemoglobin (between 1–4% absorptance) are included, microspectrophotometry indicates slightly more positive cells than benzidine staining for the same $(CH_3)_2SO$ treatment (Fig. 2). Since 1–2% absorptance is the lowest value easily discernible above background and the average induced T3-C12 cell has 10–20% absorptance, we estimate that cells negative by microspectrophotometry might contain, at a maximum, 5% the hemoglobin concentration of the average induced cell. Cells negative by benzidine staining might contain, at a maximum, 10–20% the hemoglobin content of the average induced cell. When our globin mRNA measurements are extrapolated to 100% benzidine positive cells, we estimate that a fully induced T3-C12 cell contains approximately 0.10–0.12% globin mRNA (Fig. 2). Since cultures of GM 86 cells grown in 1% $(CH_3)_2SO$ for 5 days contain approximately 50% benzidine positive cells and a globin mRNA content of 0.06%, both cell lines accumulate roughly equivalent concentrations of globin mRNA in fully induced cells.

The good correlation of globin mRNA content with these measurements of cell positivity for hemoglobin indicates that

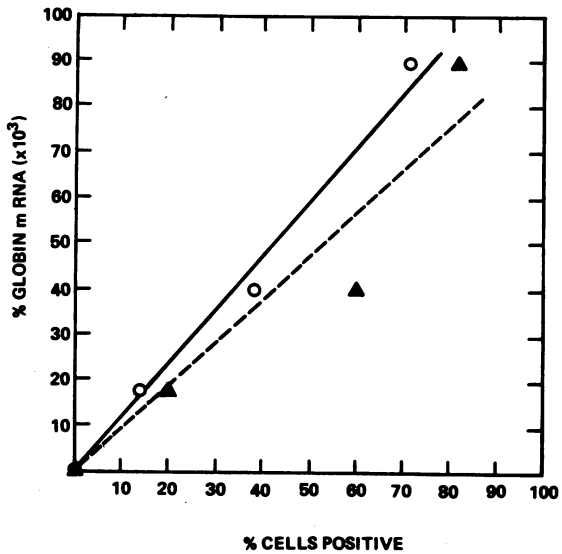

FIG. 2. Correlation of cytoplasmic globin mRNA with percentage of cells positive for hemoglobin determined by benzidine staining (O) and microspectrophotometry (▲). Long term passage T3-C12 cells were treated with 0, 0.75, 1.0, and 1.5% $(CH_3)_2SO$ for 5.5 days. Benzidine positivity within each culture was determined by counting more than 500 cells. 25 individual cells selected at random in each culture were examined for hemoglobin content by microspectrophotometry. Any cell with detectable absorptance at 415 nm was recorded as positive.

differences in apparent globin gene expression among erythroleukemic lines (Table 1) reflect the proportion of cells of each line that undergo differentiation upon $(CH_3)_2SO$ treatment rather than uniform differences in the extent to which all cells in these clones undergo differentiation under given conditions. We refer to this property of a clone as its characteristic *probability of differentiation*. The presence of benzidine positive cells at low frequency (about 1%) in untreated cell populations that contain appreciable globin mRNA (e.g., GM 86 and its subclones) similarly suggests that the "constitutive" globin mRNA measured in such cultures is derived from cells that have spontaneously differentiated or have arisen as daughter cells from previously differentiated cells. The probability of differentiation is low in untreated cultures, but is much higher in some lines (e.g., GM 86 derived lines) than others (e.g., T3-C12 TG D-3).

Clonal Stability of the Probability of Differentiation Phenotype. In view of this unusual pattern of differentiation in mass cultures, we examined two strikingly different erythroleukemic clones, GM 86 H-11 Oul A and T3-C12 TG D-3, more fully with respect to the stability of this phenotype at the clonal level. Independent, secondary subclones of these lines formed in $(CH_3)_2SO$ were stained with benzidine reagent *in situ*. In accord with the findings of Singer *et al.* (15) with GM 86 cells, greater than 90% of the colonies formed from GM 86 H-11 Oul A contained a majority of positively staining cells (Fig. 3A). In the remaining colonies, variable but significant (25–50%) numbers of benzidine positive cells were present. T3-C12 TG D-3, on the other hand, yielded *uniformly* subclones in $(CH_3)_2SO$ which contained scattered (less than 5%) positive cells (Fig. 3B). Compared with GM 86 and its subclones, T3-C12 TG D-3 and its progeny undergo limited erythroid differentiation in response to $(CH_3)_2SO$ treatment.

TABLE 2. *Chromosome complements of parents and hybrids*

Cell line	Mean chromosome number (range)
Parents:	
T3-C12 TG D-3	59 (57–63)
GM 86 H-11 Oul A	40 (36–42)
Hybrids:	
Clone 2	94 (85–103)
Clone 6	96 (86–110)
Clone 7	90 (82–96)
Clone 11	92 (89–100)

At least 10 metaphases of each parent and six of each hybrid are included. Hybrids were studied at 4–8 weeks after isolation.

Significantly, the cloning data demonstrate that the nonuniform response to $(CH_3)_2SO$ seen in mass cultures is a property of virtually *every* subclone, and therefore every cell, of a cloned line. Taken together, these findings indicate that the probability of differentiation in erythroleukemic lines is determined for the cells of each clone by stable genetic or epigenetic factors.

In spite of their strikingly different phenotypes, several similar properties of these lines should be emphasized. First, each contains approximately five copies of the globin genes per haploid content of cellular DNA as measured by hybridization kinetic analysis of globin cDNA and cell DNA (not shown), thereby excluding relative globin gene amplification in the cells more capable of extensive erythroid maturation with $(CH_3)_2SO$ treatment. Second, both cell lines synthesize hemoglobins of the diffuse type (16), which are not distinguishable by acrylamide gel electrophoresis. Third, the time course of globin mRNA accumulation during induction is similar in both clones, consisting of a 36-hr lag period followed by accumulation of globin mRNA until 96 hr of $(CH_3)_2SO$ treatment. Globin mRNA levels at 120 hr of $(CH_3)_2SO$ treatment equal those at 96 hr, consistent with our unpublished observations that globin mRNA accumulation ceases as cells enter the stationary phase of growth. This time course of induction and the necessity for the continuous presence of $(CH_3)_2SO$ for maximal induction (1) suggest that cells are continuously recruited for erythroid differentiation during the time of globin mRNA accumulation. In accord with this is the finding of a distribution of hemoglobin contents within induced cells extending from the minimum detectable to a maximum absorptance of approximately 30–35% as determined by microspectrophotometry.

Differentiation in Erythroleukemic Cell Hybrids. Quantitative differences in erythroid differentiation of T3-C12 TG D-3 and GM 86 H-11 Oul A cells (Table 1) reflect, ultimately, the end result of mechanisms that (*i*) permit or prevent spontaneous differentiation and (*ii*) limit or promote differentiation upon $(CH_3)_2SO$ treatment. We have employed somatic hybridization between these lines to examine the interaction of controlling elements as an initial approach to the genetic analysis of this *in vitro* form of erythroid differentiation.

As a control for these experiments two similar GM 86 subclones (GM 86 H-11 Oul A and GM 86 TG D-5, see Table 1) were hybridized to determine if cell culture manipulations, somatic hybridization itself, or phenotypic selection might artifactually affect differentiation observed for hybrid cells in culture. In such hybrid clones the average globin mRNA was

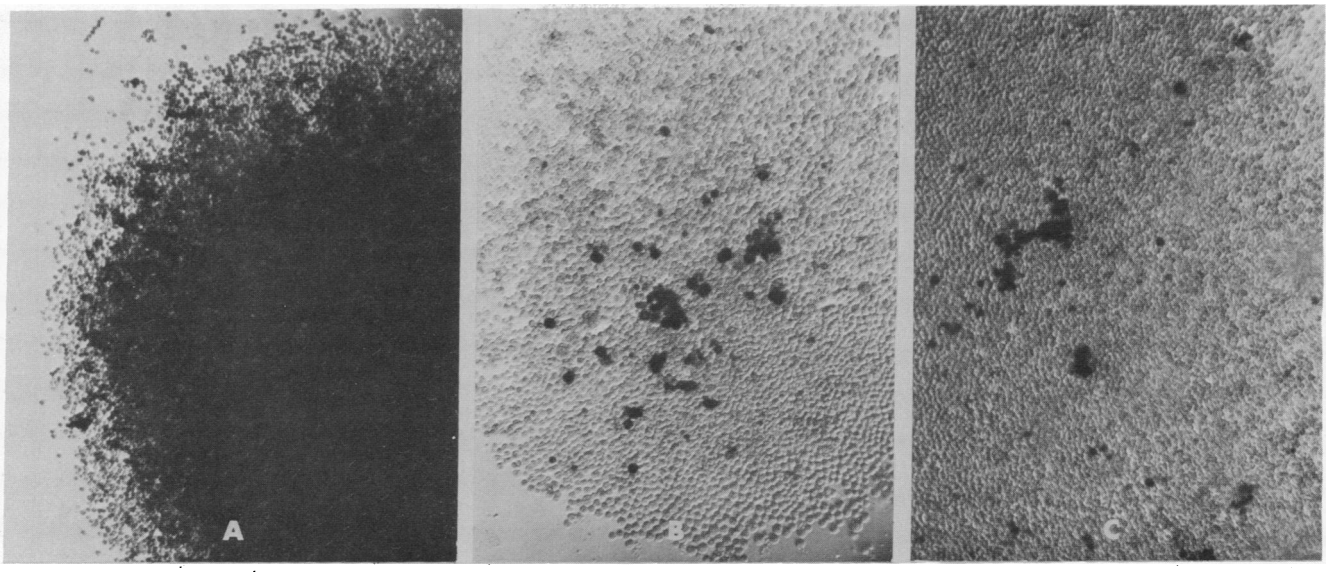

FIG. 3. Typical appearance of benzidine stained subclones of GM 86 H-11 Oula (A), T3-C12 TG D-3 (B), and hybrid clone 7 (C) after growth in 1.2% $(CH_3)_2SO$ for 14 days. Clones were stained *in situ* in Microtest wells. Magnification 125×.

0.01% for untreated populations and 0.06% for $(CH_3)_2SO$-treated cultures, indicating that the phenotype of the hybrid cells accurately reflects the parental phenotypes.

Hybrid clones of T3-C12 TG D-3 × GM 86 H-11 Oula were all quite similar. These hybrids contained intermediate levels of globin mRNA in the absence of $(CH_3)_2SO$ but were relatively resistant to further induction by $(CH_3)_2SO$ (Fig. 4). Thus, these hybrid cells had a level of spontaneous differentiation intermediate between parental lines, but a $(CH_3)_2SO$-induced extent of differentiation more nearly like that of the parent that differentiates less frequently. The specificity of these effects was demonstrated in separate experiments (not shown) in which the concentration of Friend leukemia virus RNA in cytoplasmic RNA of both parents and hybrids was shown to vary independently of globin mRNA. In addition, there is no appreciable change in total cytoplasmic RNA in these cells after $(CH_3)_2SO$ treatment. Further control experiments demonstrated that co-cultivation of parental lines does not mimic the phenotype observed in the hybrids and that the number of globin genes per haploid content of cell DNA extracted from one hybrid clone was indistinguishable from the low reiteration frequency obtained for the stock erythroleukemic lines.

The limited erythroid differentiation of these hybrids upon $(CH_3)_2SO$ treatment is reflected at the clonal level as well, as greater than 90% of subclones of hybrid clones grown in $(CH_3)_2SO$ resemble those of T3-C12 TG D-3 in having only scattered benzidine positive cells (Fig. 3C). The nearly complete chromosomal complement of these hybrids (Table 2) makes it unlikely, though not impossible, that material contributed by GM 86 H-11 Oula necessary for maximal induction has been lost in these hybrids. Instead, it is likely that factors contributed by T3-C12 TG D-3 to these hybrids result in suppression of cellular differentiation induced by $(CH_3)_2SO$. No significant progression in either chromosome number or globin mRNA levels was observed for two hybrid clones studied repeatedly over 20 passages (approximately 100 cell generations). The chromosomal stability of these suspension hybrids under normal growth conditions resembles that of intraspecific mouse fibroblast hybrids (8).

Apparent Extinction of Globin Expression in Mouse Fibroblast–Erythroleukemic Cell Hybrids. Fibroblast hybrids of A9 × GM 86 and A9 × GM 86 H-11 were prepared for comparison with the erythroleukemic cell hybrids described above. Of four such hybrids studied, none contained cytoplasmic RNA hybridizable to globin cDNA (less than 0.00005%) even after $(CH_3)_2SO$ treatment. Similar extinction of globin expression has been observed by A. Skoultchi and F. Ruddle (personal communication).

DISCUSSION

A Phenotype Reflecting the Probability of Differentiation. Our findings demonstrate that genetically homogeneous (cloned) populations of erythroleukemic cells do not respond homogeneously to $(CH_3)_2SO$ induction of erythroid differentiation. Differences in apparent globin gene expression between erythroleukemic cell lines largely reflect differences in the numbers of cells participating in erythroid differentiation in response to $(CH_3)_2SO$ rather than differences in absolute rates of globin gene transcription or globin mRNA accumulation attainable by induced cells within these lines. We have called this phenotype a clone's characteristic *probability of differentiation*, that is, the likelihood a cell of this clone will undergo differentiation under given conditions. Cloning experiments indicate that this phenotype is determined by rather stable genetic or epigenetic factors.

Differentiation in Hybrid Erythroleukemic Cells. Hybrid cells formed between erythroleukemic cells with similar phenotypes (GM 86 H-11 Oula and GM 86 TG D-5) resemble the parental lines with respect to their pattern of erythroid differentiation. This indicates that somatic hybridization alone does not introduce significant artifacts and further attests to the relative stability of the genetic or epigenetic elements that determine differentiation in the untreated and induced states in these cells.

In hybrids formed between dissimilar erythroleukemic lines (GM 86 H-11 Oula and T3-C12 TG D-3) the extents of differentiation in the untreated and induced states are uncoupled (Fig. 4). Spontaneous differentiation is intermediate

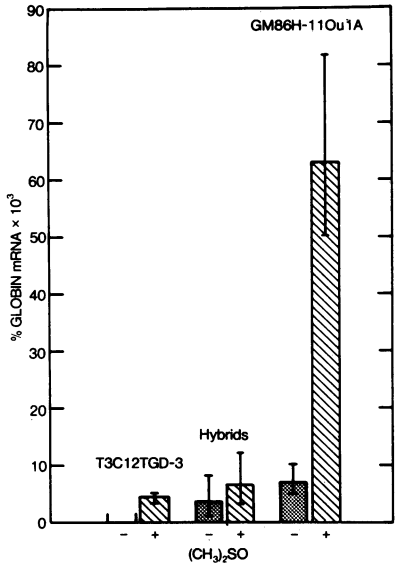

FIG. 4. Percentage of globin mRNA in total cytoplasmic RNA of parents and hybrids in control cultures (−, stippled) and after 4 days' treatment with 1.0% $(CH_3)_2SO$ (+, hatched). The total range of values is shown for each average. Thirteen independent hybrid clones, studied from 4 to 12 weeks after isolation, are represented. All but three were studied in at least two independent $(CH_3)_2SO$ treatments. Parental lines were passed in parallel and tested on four occasions during the period in which the hybrids were isolated and analyzed.

between parental values, whereas the extent of $(CH_3)_2SO$-induced differentiation is limited, presumably by material contributed by the T3-C12 TG D-3 cells. Recently Paul and Hichey (17) have reported results on hybrid cells formed between other dissimilar erythroleukemic lines. Their hybrids, probably affected by postfusion segregation, could be induced to form significant numbers of benzidine positive cells, but the relative quantitative aspects of differentiation in the untreated and induced states were not studied. Since our results show that the extents of differentiation in the untreated and induced states can be uncoupled, we conclude that they are ultimately determined at different steps along a sequence of events culminating in expression of the globin genes. As the effects we observe probably control the *participation* of cells in erythroid differentiation at an early step, we draw no conclusions from our experiments concerning the controls operating at subsequent steps, particularly those relating to direct control of globin gene transcription by diffusible activators or repressors.

Somatic hybrids formed between mouse fibroblasts and erythroleukemic cells demonstrated apparent extinction of globin expression, similar to that described in other hybrids formed between differentiated and "undifferentiated" cells (18). Although this extinction probably reflects fundamental differences between these cell types with regard to their commitment to different pathways of differentiation, the consequences of trivial factors (e.g., chromosomal loss or selection against globin expression in fibroblast hybrids) cannot be easily excluded until segregants with regained expression are isolated. The absence of globin mRNA in such hybrids, however, should not be used alone as evidence for the existence of diffusible repressors for globin gene transcription, as our studies suggest that several control steps precede this level. Indeed, the need to distinguish between controls affecting the probability of differentiation as opposed to those regulating the transcription of the globin genes is also essential when agents such as BrdU (19) are used to modulate gene expression.

Implications of the Probability of Differentiation Phenotype. These erythroleukemic cells provide a model of a special class of cells which differentiate continuously throughout the lifetime of the individual. Normal hematopoietic precursor cells form a constant, dividing population from which cells are continuously maturing. It is possible that the complex mechanisms that determine the probability of differentiation of erythroleukemic cells are those that also play a role in the maturation of cells of this class.

We thank Dr. Jeff Ross for helpful discussions and Dr. John W. Littlefield for the gift of the Sendai virus used in some of these experiments. The excellent assistance of Catherine Kunkle in the preparation of the manuscript is gratefully acknowledged.

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