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COMMITMENT TO DIFFERENTIATION IN FRIEND CELLS AND INITIATION OF GLOBIN mRNA SYNTHESIS OCCURS DURING THE G1 PHASE OF THE CELL CYCLE

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SUMMARY

We have measured the kinetics of specific globin mRNA and Friend virus (FV) RNA synthesis by hybridization to immobilized cDNA after induction of differentiation of two erythroleukemia cell lines (F4N, B8) by butyrate and Me_2SO. The induction with butyrate in these cell lines occurs very rapidly (16–24 h). Cell cycle analysis was made of the populations throughout induction by flow cytofluorometry. The kinetics of commitment of cell populations to terminal differentiation by butyrate was determined by removal of inducer at various times and scoring of benzidine staining cells (hemoglobin producing). In addition, the cell cycle dependence of commitment was determined by flow sorting out of G1 and S+G2 cells various times after addition of inducer and scoring benzidine-stained colonies after growth in methylcellulose. Cells exposed to inducer were also sorted by cell cycle phase using an elutriator rotor. The amount of globin mRNA synthesis in the different cell populations was then determined.

1. It was found that an 8–12 h period in butyrate was required before (*a*) globin specific mRNA was synthesized; and (*b*) commitment to differentiation occurred. The time course of globin mRNA synthesis was positively correlated with G1 arrest, as has been also found by others.

2. The increase of FV RNA synthesis was not found during G1 arrest. It occurred early and before commitment.

3. Commitment of cells to irreversible differentiation upon butyrate induction occurs only during the G1 phase of the cell cycle.

4. Globin mRNA synthesis occurs first only in G1 cells.

5. Globin mRNA is synthesized later in all phases of the cell cycle.

These data suggest that (*a*) commitment to differentiation and globin mRNA accumulation are coupled; and (*b*) that both events occur only in G1 cells after a pre-commitment phase of about 12 h.

The Friend cell system offers a good opportunity to study the sequence of events occurring in terminal erythroid differentiation starting from a homogeneous cell type. Induction of differentiation with a wide variety of inducers including Me_2SO and butyric acid [1–3] results in (*a*) changes in the cell membrane such as increased agglutinability with lectins [4], reduced permeability to small molecules [5], a decrease in H-2 histocompatibility antigen [6], and an increase in the inner-membrane protein

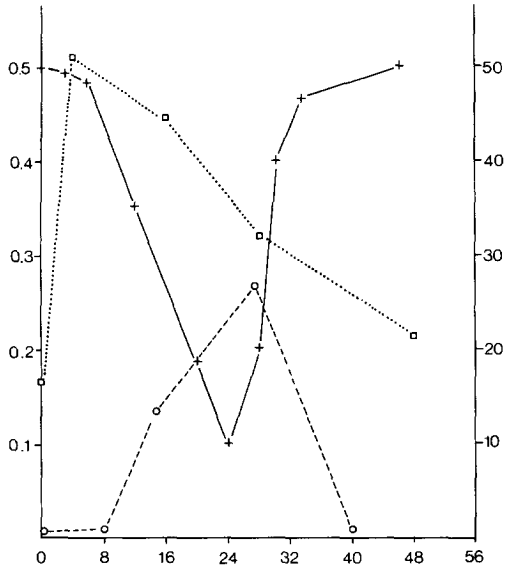

Fig. 1. Abscissa: time after butyrate addition (hours); *ordinate: (left)* % RNA hybridized; *(right)* % cells in S, G2 and M phase.

Time of synthesis of globin mRNA and viral RNA with respect to G1 arrest during butyrate induction of F4N cells. Viral RNA and globin mRNA synthesis were determined as described in Materials and Methods. The number of cycling cells was calculated from the number of cells in S and G2+M phases as determined by flow cytofluorometry (representative data are given in fig. 2): +—+, Cycling cells; O---O, globin mRNA synthesis; □---□, viral RNA synthesis. The data on globin mRNA synthesis are from other experiments than those referred to in table 1 and fig. 3. Globin synthesis is already increased to 8% of the total protein synthesis 16 h after butyrate induction. It increases to 15% 48 h after butyrate exposure and then declines (outgrowth of non-differentiating cells).

spectrin [7]; (*b*) synthesis of hemoglobin [8]; and, in some cell lines (*c*) the induction of an endogenous spleen focus-forming virus complex (SFFV) [9, 10].

The question as to whether DNA synthesis is required prior to the onset of globin mRNA synthesis is still controversial. One group of investigators has claimed that at least one cycle of DNA synthesis is required before the induction of hemoglobin synthesis [11, 12]. However, it has also been reported that induction of hemoglobin synthesis with butyric acid can take place under conditions such that DNA synthesis is largely inhibited by hydroxyurea or cytosine arabinoside [13]. More recently, evidence has been presented that Friend cells arrested at the single cell stage by inhibitors of DNA synthesis or by isoleucine deprivation fail to accumulate hemoglobin, in contrast to cells which are able to divide before becoming arrested [14]. Some of the studies cited above have resorted to artificial growth conditions, DNA synthesis inhibition, or cell synchronization, all of which probably change radically the kinetics of erythropoiesis as well as the response to and toxicity of the inducing chemicals (unpublished observations of the authors).

Many Friend cell lines accumulate globin mRNA over an appreciable time (more than one cell generation) after addition of inducer [15]. Several Friend cell lines do, however, show accumulation of globin mRNA and onset of globin synthesis relatively soon after induction [5, 16]. These cells are therefore more suitable for cell kinetic studies and were used in this report.

In a preliminary report we have shown that G1 arrest of Friend cells which occurs during exposure to most inducers of differentiation [11, 12, 17, 18] is coupled to the timing of globin mRNA synthesis [19]. This was recently confirmed [17]. We report here experiments which were designed to test in which phase of the cell cycle commitment to differentiate occurs and whether commitment and globin mRNA synthesis (accumulation) are linked.

MATERIALS AND METHODS

Preparation and labelling of RNA

Globin mRNA was extracted from mouse reticulocytes [20]. The globin mRNA was labelled in vitro with $Na^{125}I$ [21]. ^{32}P-labelled viral 60S RNA was prepared and purified as described [10].

Table 1. *Timing of globin specific mRNA synthesis after induction of erythroleukemia cells*

Data given are for a single experiment, but the hybridizations were performed at least 2–3 times for each cell line and inducer. Comparable results were obtained for each experiment (see figs 1 and 3). The time course of induction of globin mRNA is not always identical as shown by these data and those shown in figs 1 and 3

Cell line +inducer	Labelling time after addition of inducer (hours)	% RNA hybridized	Cell line +inducer	Labelling time after addition of inducer (hours)	% RNA hybridized
B8 butyrate	Control	0.02	F4N butyrate	Control	0.009
	4–7	0.02		5–8	0.009
	7–10	0.03			
	12–15	0.21		12–15	0.13
	24–27	0.31		24–27	0.26
	48–51	0.12		33–36	0.26
B8 Me_2SO	Control	0.02	F4N Me_2SO	Control	0.009
	0–4	0.01		0–4	0.009
	12–16	0.01		12–16	0.009
	24–28	0.11		24–28	0.10
	36–40	0.25		36–40	0.10
	72–76	0.13		72–75	0.44

Data given are for a single experiment but the hybridizations were performed at least 2–3 times for each cell line and inducer. Comparable results were obtained for each experiment (see figs 1 and 3).

Preparation of cDNA

Unlabelled globin cDNA was prepared as described previously [20]. Friend virus RNA from virus released by Me_2SO-induced Friend cells (F4–6) was used [16]. FVcDNA was synthesized by the lysed virion technique [22]. In a [^{32}P]RNA protection experiment, 70% of the ^{32}P 60S RNA was stabilized at a cDNA : RNA ratio of 1 : 1 indicating that the distribution of FV sequences in the FVcDNA is uniform. The plateau value of 87% obtained at cDNA excess showed that the cDNA constitutes a complete copy of the FV genome.

Coupling of cDNA to Sepharose

Approx. 10 μg of cDNA were coupled to 5 ml of packed cyanogenbromide-activated Sepharose (2B, 4B, or CL-4B) [23]. The Sepharose was washed successively with 10 vol of water, 1 M NaCl, formamide hybridization buffer (0.05 M Tris-HCl, pH 7.5; 0.75 M NaCl; 1 mM EDTA; 0.5% SDS; 50% formamide), formamide elution buffer (0.01 M Tris-HCl, pH 8.5; 0.1 M EDTA; 0.01% SDS; 98% formamide) and 0.1 N NaOH. The cDNA-Sepharose was exhaustively washed with hybridization buffer after NaOH treatment and stored at 4°C in this buffer. The coupling procedure was usually more than 90% efficient and less than 1% of the coupled cDNA was eluted during the hybridization assays.

Hybridization assays

The hybridization assays were set up as follows: 0.4 ml of a 50% slurry by volume of cDNA-Sepharose in hybridization buffer was transferred to a freeze-dried mixture of [^{3}H]RNA (10^6 cpm), 50 μg of *E. coli* or rRNA as carrier RNA, and 5 μg of poly A. The suspension was incubated 16 h at 40°C in a shaker water bath. The packed Sepharose was washed by resuspension and centrifugation (900 g) with 10 vol of hybridization buffer, resuspended in 1 vol of 2×SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7) and incubated for 30 min at room temperature with 25 μg/ml RNAse A which had been preheated to 80°C for 5 min to eliminate any residual DNAse activity. The cDNA Sepharose was pelleted by centrifugation and washed with 5 vol of 2×SSC and with hybridization buffer until no further radioactivity was eluted. The bound [^{3}H]RNA was eluted with 0.1 N NaOH (4 vol) and neutralized before the radioactivity was measured by scintillation counting.

Cell cycle analysis and sorting

A multiparameter automated computer-controlled cell sorter (MACCS) [6, 24] was used to make quantitative determinations of the distribution of cells within the cell cycle before and after induction of erythroleukemia cells and to effect separation of the cells on the basis of position in the cell cycle. The cells were stained for analysis of DNA content by either the acriflavin-Feulgen technique [25] or by mithramycin [26], or by Hoechst 33342 [27] staining after fixation. Viable cells in different parts of the cell cycle were sorted after staining for DNA content with the *bis*-benzimidazole dye Hoechst 33342 [27].

Elutriation of cells

An elutriator rotor for the Beckman J21-B centrifuge was used to isolate large quantities of cells in different

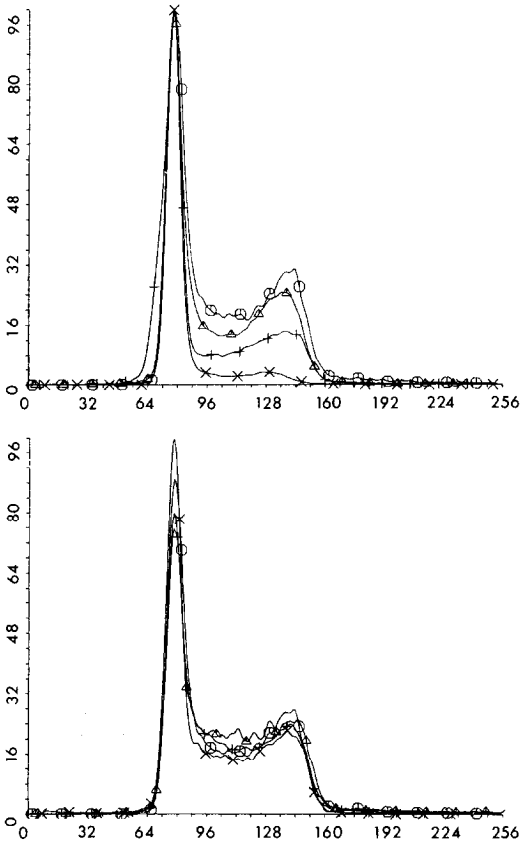

Fig. 2. Abscissa: DNA content (fluorescence); *ordinate:* cells $\times 10^{-2}$.

Cell cycle analysis (DNA content) of F4N cells during induction with butyrate. Cells were washed free of medium at the indicated time points, fixed in 5% formaldehyde, stained specifically for total DNA, and analyzed for DNA content by flow cytofluorometry in MACCS using 488 nm excitation. The frequency distributions of the fluorescence intensity (DNA content) of the cells normalized to 10^5 cells for each culture are overlaid for some of the times measured. (*a*) DNA distribution of cells. O—O, Before addition; – –, 4 h; +—+, 12.5 h; ×—×, 20 h after addition of butyrate. (*b*) DNA distribution of cells of half of the same cultures not treated with butyrate at the same time points.

phases of the cell cycle by counterflow centrifugation. Approx. 4×10^8 cells in log phase growth without inducer or with butyric acid were labelled for 3 h with [^{3}H]uridine and were centrifuged at 4°C in PBS containing 2% fetal calf serum (FCS) (to reduce cell clumping). Eleven fractions of cells with different densities were eluted by reducing the speed of the rotor with a 10 turn helipot on the speed control of the centrifuge while pumping at a constant rate of 8 ml/min. Fractions were spun down and RNA extracted for cDNA hybridization. An aliquot of cells from each fraction before extraction was fixed and analysed by flow cytometry in order to determine the percentage of cells in various parts of the cell cycle (for a review on methods of cell elutriation, see ref. [37]).

Induction of erythroleukemia cell cultures

Erythroleukemia cell lines F4N and B8 were grown as described previously [5, 8, 16]. The cells were labelled with [^{3}H]uridine, 50 μCi/ml with a specific activity of 42 Ci/mM for 3 or 4 h. RNA was extracted [16]. *N*-butyric acid was used at a concentration of 0.8 mM for F4N cells, 1.0 mM for B8 cells. Me_2SO was used at a concentration of 1.7% for B8 cells and 1.2% for F4N cells. Cells were kept in log phase of growth before and during induction. After sorting the cells were plated and grown in 1% methylcellulose-containing medium. They were stained 3 days later with benzidine and the number of benzidine positive and negative colonies were scored. Globin synthesis was measured as described previously [8]. Cells were labelled for 12 h with L-[4,5-^{3}H]leucine or L-[U-^{14}C]leucine (Radiochemical Centre, Amersham, Bucks.). The α and β chain labelling is presented as a % value of the total non TCA-precipitable cpm in Friend cell cytoplasm. Values for background cpm in the α and β globin chain region of the column eluate have been subtracted [8].

RESULTS

Kinetics of globin and globin mRNA synthesis after induction of Friend cells

Globin synthesis in F4N cells is maximal at 2 days but already much above background 8–20 h after butyrate exposure (fig. 1). We have quantitated the kinetics of synthesis of globin mRNA during the early period of induction using tritium-labelled cellular RNA and globin cDNA immobilized on Sepharose. The specificity of the hybridization technique was established by measuring the binding of tritiated heterologous RNA and iodinated globin mRNA. All three types of cDNA-Sepharose (2B, 4B or CL4B) bound 80–90% of iodinated globin mRNA at saturation under conditions of cDNA excess. cDNA-Sepharose 2B or 4B bound 0.01–0.02% non-globin RNA, whereas cDNA-

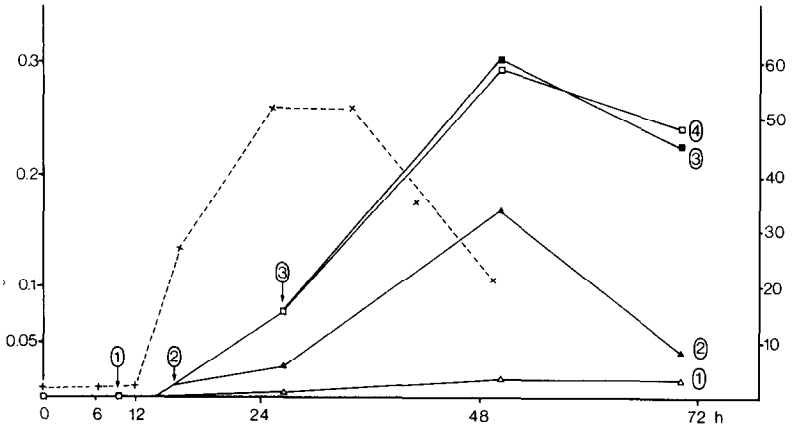

Fig. 3. Abscissa: time after butyrate addition (hours); *ordinate: (left)* % RNA hybridized; *(right)* % benzidine positive cells.

Commitment to terminal differentiation and timing of globin mRNA synthesis in F4N cells during butyrate induction. The number of benzidine positive cells was used as a measure of terminal differentiation. F4N cells were grown as described in Materials and Methods either in butyrate throughout the experiment or butyrate was removed after the times indicated by the arrows and the cells were then grown in butyrate-free medium. The percentage of benzidine positive cells in the total culture at each point, 0, 8, 25.5, 50, and 72 h is plotted. The absolute number of B^+ cells was determined in each experiment. We assumed that the number of all divisions after commitment was the same regardless of the presence or absence of the inducer. The absolute numbers were then related to the controls (continuous treatment with butyrate). Percent of benzidine positive cells after removal of butyrate at △—△, 8 h; ▲,—▲, 16 h; ■—■, 25.5 h; □—□, grown in the presence of butyrate for 72 h. Globin mRNA synthesis was measured as described and is plotted for the same time period (×---×).

Sepharose CL4B bound only 0.005%. Excess cDNA conditions were used during the kinetic assays described below.

Table 1 and fig. 1 show the percentage of cytoplasmic RNA labelled in 3 or 4 h pulses which was globin specific mRNA, as determined by hybridization to globin cDNA-Sepharose for two cell lines, B8 and F4N, and using two different inducing compounds, butyric acid and dimethylsulfoxide. Data for butyrate induction of F4N cells from other experiments is shown in fig. 1.

Following butyric acid addition, there was an abrupt increase in globin mRNA production beginning at 12 h post induction, continuing for some 20 h, and then decreasing in both B8 and F4N cells. The F4N cells induced with Me_2SO did not show any increase in globin specific mRNA until some 24 h after induction, and continued to produce messenger as late as 3 days post induction, whereas B8 cells reached maximum synthesis earlier.

Kinetics of viral RNA synthesis

Using the same samples taken for the analysis of globin mRNA synthesis, we measured the kinetics of viral RNA synthesis during butyrate-induced differentiation in both cell lines. Cell line F4N is a virus positive line which shows induction of an endogenous Friend spleen focus forming virus complex during differentiation [9]. We have established previously that cell line B8 can be induced to differentiate, but no induction of FV could be detected [9, 16]. This cell line appears to have a defect in the processing of the major internal viral protein p30 [28], but viral RNA synthesis may occur in a normal fashion followed by rapid breakdown, thus accounting for the lack of

Table 2. *Clonal analysis of commitment for differentiation induced by butyrate with B8 cells in different phases of the cell cycle*

Cells were exposed for 0, 8, or 16 h to butyrate and then sorted into G1 and G2+S phase cells. Cells in the region where G2+S and G1 may overlap were not used. Cells were then plated on methylcellulose, incubated 3 days without butyrate, and the resulting colonies stained with benzidine. Colonies consisting of a single cell, 2 cells, 3 and 4 cells, and 5 or more cells were counted separately. The last column lists the percentage of B^+ cells derived from the sum of B^+ cells in all colonies of the previous columns. Questionable cells were counted as negative. B8 cells rather than F4N cells were used in this experiment since survival of B8 cells is much superior to that of F4N cells during sorting and cloning of cells

Cells sorted			Colonies							
			of 1 cell		of 2 cells		of 3–4 cells		of 5+cells	
Time of exposure to butyrate (hours)	Phase	No. of colonies examined	% of colonies	$\%B^+$	% of colonies	$\%B^+$	% of colonies	$\%B^+$	% of colonies	$\%B^+$
0	G1	515	21	1.9	14	1.4	23	2.5	42	0.9
	G2+S	451	12	8.0	8	2.9	12	0	67	1.2
	All	966	16	4.0	11	1.9	18	1.7	55	1.1
8	G1	609	17	1.0	17	1.0	27	1.8	40	0.4
	G2+S	571	15	2.4	13	2.8	27	0.7	45	0.8
	All	1 180	16	1.6	15	1.8	27	1.3	42	0.6
16	G1	869	22	39	20	38	25	22	34	6
	G2+S	367	30	2.0	17	4.8	17	4.2	36	0.7
	All	1 236	24	26	19	30	23	19	34	4.1

accumulation of viral RNA during differentiation [16].

The kinetics of viral RNA synthesis was measured as described for globin mRNA, and the data are shown in fig. 1 for F4N cells. In cell line F4N, very early increase was observed in viral RNA synthesis prior to the observed burst of globin mRNA and globin synthesis. However, no change in viral RNA synthesis was seen in cell line B8, although a constant and relatively high rate of viral RNA synthesis was maintained (data not shown).

DNA content and cell cycle during induction of erythroleukemia cells

Using an automated cell analyzer-sorter (MACCS), we analyzed the DNA content of both F4N and B8 cultures with and without induction using Me_2SO or butyric acid. The culture was split at the time of induction and half of the cells were grown without inducing agent to serve as control culture. Fig. 2 shows representative data for four time points after butyric acid induction of F4N cells. It can be seen that the number of cells in S and G2+M phases decreased beginning at 12 h after induction and reached a minimum at about 19 h post induction. The DNA content of the control cells was also analyzed, and data for the same time points as in fig. 2*a* are plotted in fig. 2*b*. A constant number (45–55%) of cells in S and G2+M phases (cycling cells) was observed. Cycling cells reappeared at about 26 h after induction, and by 36 h the number of cycling cells was similar to the control.

The correlation of the DNA content data

%B+ colonies/ % all colonies	%B+ cells
1.6	0.9
2.0	0.6
1.7	0.8
1.0	0.7
1.2	0.8
1.1	0.8
19	12
2.6	2.1
15	10

with the kinetics of globin mRNA synthesis is shown in fig. 1. There was a striking decrease in the number of cells entering S and G2 and mitosis at the time of the onset of globin mRNA synthesis. Thus, an arrest of the cells in G1 phase appeared during the increased production of globin mRNA. In the case of Me_2SO induction with F4N cells, there was a decrease in cells entering S and G2+M beginning at about 20 h after induction. However, the induction of mRNA and increase in hemoglobin-containing cells (as seen by the number of benzidine positive cells) was not as synchronous as with butyrate induction. Likewise, the G1 arrest was also not as dramatic and consisted of a reduced number of cycling cells over several cell generations starting at 20 h.

Commitment of cells to differentiation as a function of time spent in inducer

In order to determine the role of inducer in the commitment of erythroleukemia cells to differentiation, we have correlated the number of cells which ultimately produced hemoglobin in cultures which were incubated for various times with butyrate. F4N cells were incubated in 0.8 mM butyrate at time zero. At 8, 16 and 25.5 h, some cultures were washed twice with medium not containing butyrate and grown further in complete medium without butyrate. A cell culture with no added inducer served as a control. At 0, 8, 25.5, 50 and 70 h, cells were counted in all cultures, and the number containing hemoglobin scored by benzidine staining. The latter (corrected for outgrowth at the various times) are plotted in fig. 3 along with the kinetics of mRNA production for the F4N cells determined by hybridization. Cultures which were grown in the presence of inducer for 25.5 h showed the same time course and extent of induction as those which were grown continuously in the presence of butyrate for the full 72 h. Those which were grown in inducer for only 16 h produced half the number of hemoglobin-containing cells at 50 h, whereas those which had inducer for only 8 h produced only 3% hemoglobin-containing cells (fig. 3). Almost identical results were obtained with B8 cells.

Only G1 cells are committed to differentiate

In order to show that the G1 cell cycle phase and commitment to differentiation are functionally linked, we sorted viable cells according to their position in the cell cycle using MACCS and the *bis*benzimidazole dye 33342 Hoechst, and performed outgrowth and cloning studies with these

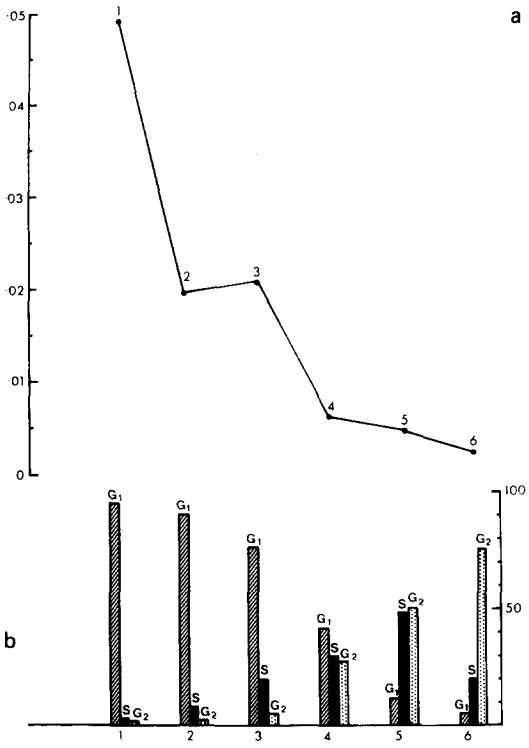

Fig. 4. Abscissa: fraction no.; *ordinate:* (*a*) % RNA hybridized; (*b*) % cells in S, G1, G2 and M phase.

Globin mRNA synthesis in sorted F4N cells populations 20–23 h after induction of differentiation. Cells were sorted by elutriation in a Beckman centrifuge. (*a*) The % globin mRNA synthesis in the fractions 1–6 (●—●) was determined as described. The background count as measured in HeLa cells was 0.009% and is subtracted from the values shown. The unfractionated cell population showed 0.024% globin mRNA synthesis. (*b*) Distribution of cell cycle phases in each sorted population was determined by flow cytometry as described in Materials and Methods.

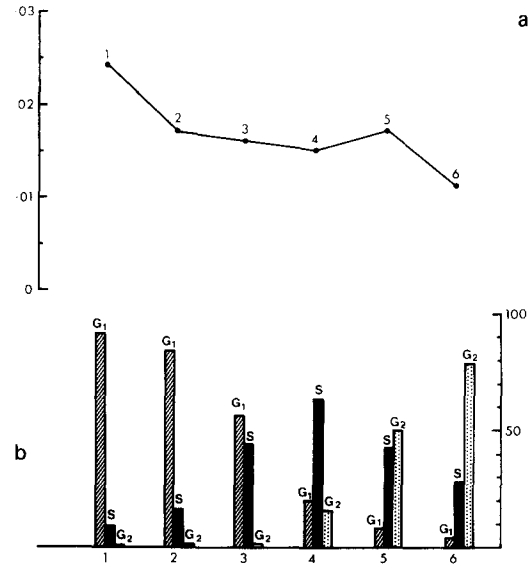

Fig. 5. Abscissa: fraction no.; *ordinate:* (*a*) % RNA hybridized; (*b*) % cells in S, G1, G2 and M phase.

Globin mRNA synthesis in sorted F4N cells populations 32–35 h after induction of differentiation. Cells were sorted by elutriation in a Beckman centrifuge. (*a*) The % globin mRNA synthesis in the fractions 1–6 (●—●). The background count as measured in HeLa cells was 0.006% and is subtracted from the values shown. The unfractionated cell population showed 0.018% globin mRNA synthesis. (*b*) Distribution of cell cycle phases in each sorted population determined by flow cytometry as described in Materials and Methods.

cells. Log phase cells were induced with butyrate as described above. Cells sorted 8 and 16 h after butyrate treatment were placed in growth medium without the inducer, and the number and size of benzidine positive colonies plated in methylcellulose was determined 3 days later (table 2).

The data show clearly that (*a*) after 8 h of induction by butyrate none of the phases of the cell cycle showed an increase in differentiating cells above the untreated level; and (*b*) after 16 h of butyrate, 22–39% of the colonies of 4 cells or less growing from the G1-sorted population were benzidine staining (B^+), whereas those sorted from the G2 and S phases were only 2–5% B^+, i.e., similar to the control uninduced cells (0–8% B^+ colonies). Overall, 2% of the control G2+S and 1.6% of the G1 cell population were committed (B^+ colonies), whereas 2.6% of the 16 h butyrate-treated G2+S and 19% of the G1 cells yielded benzidine positive colonies.

Only G1 phase cells initiate globin mRNA synthesis and accumulation

In order to show whether synthesis and accumulation of globin mRNA occurs in spe-

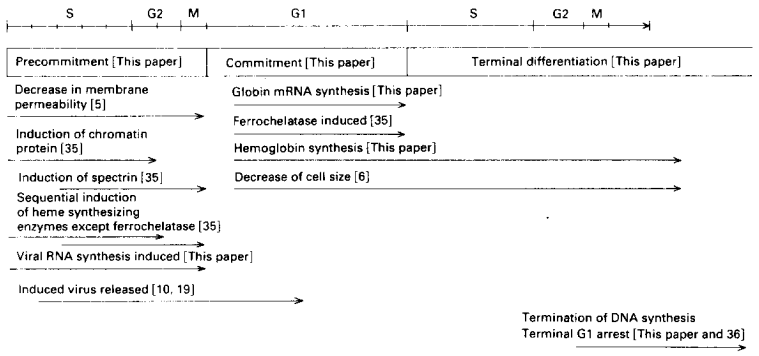

Fig. 6. Schematic diagram of differentiation events in F4N cells during exposure to butyrate or Me_2SO. Published data have been collated together with the data in this paper to give a scheme of events during Friend cell differentiation.

cific parts of the cell cycle, we labelled cells as described and sorted viable cells according to their density, which is correlated to the position in the cell cycle using a Beckman elutriator rotor. RNA of cells fractionated in this way was extracted and the amount of labelled globin mRNA was determined as described before.

The percentage of cells for each fraction in G1, S or G2 was determined by flow cytometry. Data for cells exposed to 23 h and 35 h butyrate are shown in figs 4 and 5. Fig. 4 shows globin mRNA synthesis in cells exposed 23 h to butyrate: Only cells in G1 are synthesising globin mRNA.

In order to determine whether globin mRNA synthesis is generally restricted to G1 phase cells we sorted cells 35 h after butyrate exposure and determined globin mRNA synthesis as shown in fig. 5. Globin mRNA synthesis is at that time not restricted to G1 phase cells and occurs throughout the cell cycle.

DISCUSSION

We have investigated the kinetics of globin mRNA synthesis and FV RNA synthesis after induction of erythroleukemia cells with butyric acid and Me_2SO. In both a virus positive and a virus negative cell line, the kinetics of globin specific mRNA synthesis could be described as a burst starting at 12 h after addition of butyrate or Me_2SO [16, 30] and declining again after further 24–26 h, in some cases reaching background values at 40 h post induction. In contrast, the kinetics of mRNA synthesis after induction with Me_2SO in the same lines, especially for cell clone F4N, results in a slower increase of the rate of globin mRNA synthesis which persists for longer times.

The burst of globin specific mRNA produced after butyrate induction suggested that the cells were induced semi-synchronously thus enabling a study of the cell cycle dependence on induction (fig. 6). As we show in fig. 3, the number of cells entering S and G2+M phases is drastically reduced beginning at 12 h after addition of butyrate. This extension of G1 we have termed G1 arrest, which may be synonymous with G0. G1 arrest during induction of erythroleukemia cells has now been reported by several groups. Earlier studies by Terada et al. [12] showed G1 arrest but the cells were not in log phase, i.e. G1 arrest may have been the result of pseudo-starvation conditions since the cells were allowed to get into plateau phase growth. They were then diluted with fresh medium so that steady state conditions did not apply. In a preliminary report we have con-

firmed Terada's findings for cells grown continuously under log phase growth conditions [19]. Recently, other groups have shown some degree of G1 arrest of cells induced with Me_2SO, butyrate or hexamethylene *bis*acetamide [12, 17–19, 29] but not with some other inducers [18]. The time of globin mRNA synthesis as studied by pulse labelling of the mRNA in our cells correlates positively with the arrest in G1 of the majority of cells. Using butyrate induction for varying lengths of time, we determined from the number of benzidine positive or hemoglobin-containing cells (fig. 3) that by 16 h at least 50% of the cells were committed to terminal differentiation. By 25.5 h after addition of butyrate, all susceptible cells were recruited, as compared with cells exposed to butyrate for 72 h or longer. A more refined analysis of the correlation of terminal commitment of the cell cycle phase and the time in inducer was determined by clonal growth of single cells in methylcellulose after cell sorting by flow cytofluorometry. We stained the colonies with benzidine to allow a quantitation of those cells committed to differentiate following a procedure similar to that previously employed by Housman's group [32]. The data is given in table 2. We found that after 16 h of induction, only G1-arrested cells were committed to differentiate, whereas none or very few of the cells in S and G2 were committed. The relationship of G1 (G1 arrest) and the commitment process thus indicates that a large fraction of the G1 cells 16 h after addition of butyrate are physiologically in a different state as compared to G1 cells of uninduced cultures and to S and G2 cells 16 h after butyrate exposure. The trigger to terminal differentiation and commitment thus only occurs during passage of cells through G1 (fig. 6).

A big proportion of the cells in G1 phase and not those cells in other phases of the cell cycle after exposure to the inducer for at least 8 h are committed to terminal differentiation and synthesise globin mRNA. G1 arrest would thus increase the proportion of cells entering the critical phase required for differentiation. Commitment during G1 may take place with all inducers of Friend cell differentiation but may not require G1 arrest. This explanation would be consistent with data published by Schildkraut [18].

Rastl & Swetly [31] show a correlation of G1 arrest of F4N cells coincident with an increase in poly (ADP-ribose) polymerase activity. Gusella et al. [32] and Geller et al. [29] have made clonal analyses of Me_2SO induction and suggested that commitment and globin mRNA synthesis are coupled, but only measured the accumulation of globin mRNA. Thus, it is not clear from their data how commitment is correlated to other cellular functions.

The cycle time of the uninduced cells in our study is 12–14 h. The strong positive correlation of globin mRNA synthesis and commitment occurring with a lag of 12 h suggest that passage of the cells through a specific phase of the cell cycle (possibly S phase [11]) in the presence of inducer is necessary for commitment (fig. 6). Commitment occurs only during G1 (see above). Most of these committed G1 cells cycle again yielding growth-restricted 2–8 cell colonies which are benzidine positive. The lack of hemoglobin-synthesizing colonies derived from S and G2 phase cells after 16 h induction and the coincident increase in globin and globin mRNA synthesis and G1-arrested cells indicated that actual commitment during the G1 phase of the cell cycle may require globin mRNA synthesis.

We therefore separated cells according to cell cycle stage 23 h after butyrate induc-

tion and determined the level of globin mRNA synthesis. Only G1 cells synthesize globin mRNA (fig. 4). Globin mRNA synthesis may thus occur at the earliest and perhaps exclusively during the passage of cells through G1. We also separated cells in different cell cycle stages 35 h after butyrate induction to see whether globin mRNA synthesis occurs exclusively in the G1 phase of the cell cycle (fig. 5). Cells in G2 and S phase also synthesize globin mRNA at that period of induction. The somewhat higher levels of globin mRNA synthesis in G1 phase cells even at that time point of induction is most likely an indication that previously uncommitted cells get committed in G1 (see above) and synthesize globin mRNA first in G1.

The precommitment phase, which shows reversibility of differentiation and lack of commitment, is characterized by other early parameters of Friend cell differentiation (fig. 6), such as induction of spectrin synthesis [7, 33, 35], induction of most of the heme-synthesizing enzymes except the last, ferrochelatase [35], increase in agglutinibility of the cells by lectins [4], early increase in the viscosity of the hydrocarbon layer [6], and an early decrease in membrane permeability [5]. The increase in Friend viral RNA synthesis as observed here is a very early change [16, 19] but is found only in those cells (F4N) which are also inducible for endogenous spleen focus-forming virus release, but not in B8 cells, which are virus negative and not inducible for virus. The early induction of viral RNA synthesis and of virus release which is coupled to differentiation is similar to that observed during induced differentiation of granulocytic leukemia cells [34]. It may be of general significance [10] but is not required for differentiation and commitment to occur.

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