Transient inhibition of initiation of S-phase associated with dimethyl sulfoxide induction of murine erythroleukemia cells to erythroid differentiation

(Friend cell/cell cycle/cell differentiation)

MASAAKI TERADA*, JERROLD FRIED†, URI NUDEL*, RICHARD A. RIFKIND*, AND PAUL A. MARKS*‡

*Departments of Human Genetics and Development, and of Medicine, and the Cancer Research Center, Columbia University, New York, N.Y. 10032; and
†Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021

ABSTRACT   The murine erythroleukemia cell (MELC) line in suspension culture can be induced to differentiate to erythroid cells by various compounds, including dimethyl sulfoxide (Me₂SO). Analysis of the cell cycle, during differentiation induced by Me₂SO, using thymidine incorporation, thymidine labeling index, and relative DNA content per cell as measured by flow microfluorometry, demonstrates a transient inhibition of entry of cells into S-phase of the cell cycle which is detected as early as 5 hr and is maximal about 20 hr after beginning of nonsynchronous cultures. Furthermore, in the presence of Me₂SO there is restricted binding of the intercalating dye propidium iodide to chromatin from MELC in G_1 phase of the cell cycle, as early as 10 hr of culture. This restricted binding of propidium iodide to chromatin is observed in MELC cultured with other inducing agents, such as butyric acid and dimethylacetamide, but is not detected with an Me₂SO-resistant cell line cultured with Me₂SO.

The present study examines the relationship between the cell cycle and the expression of a new pattern of genetic information in progeny of cells induced to differentiate (1). The murine erythroleukemia cell (MELC) line established by Friend et al. (2) shows a low level (<1%) of spontaneous erythroid differentiation in culture; inclusion of 280 mM dimethyl sulfoxide (Me₂SO) in the medium causes a high proportion of the cells to express a program of erythroid differentiation, including morphological changes (2), accumulation of globin mRNAs (3–6), α and β globin synthesis (7), increase in heme synthesis (8), synthesis of erythrocyte-specific proteins (9), loss in capacity for cell division (2, 10), and the appearance of erythrocyte-specific membrane proteins (11).

The rate of DNA synthesis, proportion of cells in S-phase, and the pattern of DNA accumulation have been measured and compared in MELC grown in the presence and absence of Me₂SO and other inducing agents, e.g., butyric acid (12) and dimethylacetamide (13). DNA synthesis has been measured using incorporation of isotopically labeled thymidine in pulse-labeling experiments; cells in S-phase were assayed by autoradiography of thymidine pulse-labeled cells; DNA accumulation per cell was determined using propidium iodide staining with flow microfluorometric analysis. By these assays for DNA synthesis or DNA content, there is evidence that Me₂SO and other inducing agents transiently inhibit the entry of cells into S-phase of the cell cycle. This inhibition is detected in nonsynchronous cultures as early as 5 hr and is maximal about 20 hr after beginning of culture.

In addition, we have observed a difference in the pattern of binding of intercalating dye (propidium iodide) to the chromatin of cells in G_1 phase, for MELC cultured without or with Me₂SO. In cells cultured without inducer there is an increase in binding of intercalating dye as early as 10 hr, which is not observed in cells cultured with Me₂SO. These results may reflect a difference in pattern of chromatin changes with cell cycle between cells cultured with and without Me₂SO.

MATERIALS AND METHODS

Cells. Strain 745A, which is infected with Friend virus complex, was kindly provided by Charlotte Friend and has been maintained in culture in our laboratory as described elsewhere (14). Isolation of the Me₂SO-sensitive cell line (DS19) and resistant cell line (DR10) was described by Ohta et al. (15). Cell concentration and proportion of hemoglobin-containing (benzidine-reactive) cells were determined as previously described (14).

Pulse Labeling with Thymidine. DNA synthesis was determined as incorporation of [³H]thymidine (20 Ci/mmol, New England Nuclear Corp.) into cells (2 × 10⁵ cells/per ml) incubated for 20 min at 37° at a concentration of 20 μCi/ml of culture medium. Thymidine pulse labeling in this manner was performed on aliquots removed from culture at the times indicated for each experiment. Following incubation with radioisotope, cells were washed with phosphate-buffered saline, pH 7.4, containing 2 mM thymidine, and precipitated with 10% trichloroacetic acid. Precipitates were collected on Millipore filters and their radioactivity was measured in Aquasol. A separate aliquot of cell suspension was removed from the culture and, washed with the same phosphate-buffered saline, and smears were prepared employing the cytocentrifuge. These slides were processed for radioautography (16).

Flow Microfluorometry (FMF). Cells were recovered from aliquots of culture media by centrifugation at 1000 rpm in the PRJ model IEC centrifuge for 10 min; 5 ml of propidium iodide solution, 0.05 mg/ml in 0.1% sodium citrate, was added to make a final cell concentration of 10⁵ cells per ml and the relative cellular fluorescence was measured by the method of Krishan (17, 18). To determine the pattern distribution of DNA content per cell, cells were cultured for different durations but harvested at the same time to ensure reproducible conditions for microfluorometric determinations. The channel number is a relative measure of the fluorescence intensity and the values corresponding to peaks in the DNA distribution histograms obtained on such cultures were reproducible within ± 1 channel. To perform FMF on isolated nuclei, cells were lysed with a buffered solution containing 0.5% Triton X-100, 4 mM MgCl₂, 0.6 M sucrose, and 10 mM Tris·HCl, pH 7.5. The nuclei were recovered by centrifugation at 7000 rpm for 30 sec in an RC-2 Sorvall centrifuge, washed once and stained with propidium iodide, and relative fluorescence was determined.
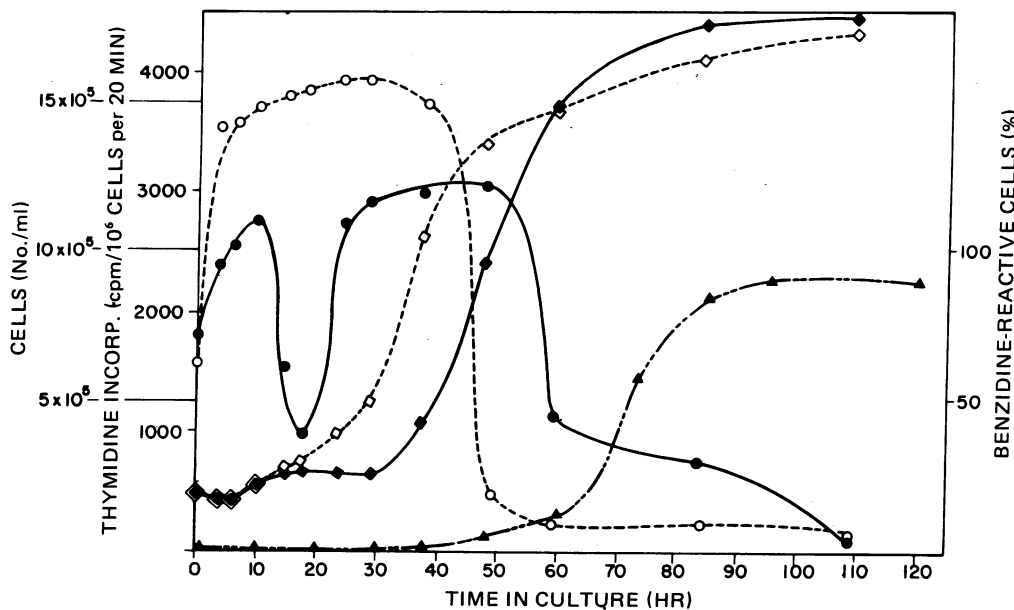
FIG. 1. Cell growth and entry into S-phase of control and Me₂SO-induced MELC. DS19 cells after 60 hr culture were transferred to medium with and without Me₂SO at the initial cell concentration of 2 × 10⁵/ml, and at each time indicated, an aliquot of cell suspension was removed to determine cell number (◊----◊, control; ◆——◆, Me₂SO), benzidine-reactive cells (▲——▲, Me₂SO) and [³H]thymidine incorporation (20 min incubations) into trichloroacetic-acid-insoluble material (O---O, control; ●——●, Me₂SO) as indicated in the *text*.

DNA Measurement by the Fluorescent Feulgen Technique.

Approximately 10^4 cells were collected on a glass slide by cytocentrifugation and fixed with 85% methyl alcohol, 10% formalin, and 5% acetic acid. After 45 min in 5 M HCl at room temperature, the cells were stained with Schiff's reagent (19) and bleached with potassium metabisulfite, 0.5% in 0.05 M HCl. Slides were stored in the dark at 4°. Fluorescence measurements were performed within 2 weeks, using a MPV 2 microscope Ploem Photometer equipped with an HBO-100 mercury arc lamp and Ploem illuminator for fluorometry with a filter module for narrow band green (20) excitation. The objective lens was a 40×, oil immersion NA-1.30 (Ernst Leitz Co.).

RESULTS

Cell growth and Me₂SO-induced hemoglobin formation

Both in the presence and absence of 280 mM Me₂SO, MELC achieve a density of 1.5 to 2.0 × 10⁶ cells per ml after 80 hr of culture (Fig. 1). As previously reported (2), there is a 15- to 20-hr lag in the onset of cell growth in cultures with Me₂SO, compared to cultures without inducer. Without inducer, fewer than 1% of the cells become benzidine reactive. In cultures with 280 mM Me₂SO, an increase in the proportion of the cells that are benzidine reactive is detected between 40 and 50 hr, and the proportion increases to 80–90% by 90–100 hr.

DNA synthesis

In control cultures, there is an initial rise in the rate of thymidine incorporation (pulse labeled) and a maximum value is achieved by about 10 hr; between 10 and 40 hr this rate remains relatively constant, decreasing thereafter to less than 10% of the peak level by 60 hr (Fig. 1). The initial increase in rate of thymidine incorporation probably reflects entry into S-phase of cells partially synchronized in the post-logarithmic growth phase of the previous passage. The plateau level, observed between 10 and 40 hr, suggests a relatively constant proportion of cells in S-phase (loss of the partial synchronization). The fall in DNA synthesis

after 60 hr coincides with the onset of stationary growth phase.

Although there is an initial rise in the rate of thymidine incorporation in cells cultured with Me₂SO, a difference between cultures with and without 280 mM Me₂SO is observed as early as 4–6 hr. In the population of cells cultured with Me₂SO, a decrease in the rate of thymidine incorporation is observed between 10 and 20 hr. At 20 hr the rate of thymidine incorporation is at its lowest, about 25% of the rate in control cultures (Fig. 1). The initial rise in the rate of [³H]thymidine incorporation in cells cultured with inducer can be explained if some cells are not as rapidly affected as others. As cells become affected by the agent, the proportion that enters S-phase decreases. In the induced cultures, the rate of thymidine incorporation rises between 20 and 30 hr to reach a peak value (about 75% of the highest rate in control cultures), where it remains until 50–60 hr and then decreases (Fig. 1).

Cells in S-phase

In control cultures, the proportion of cells that are labeled, assayed by radioautography after a 20 min pulse of [³H]thymidine, rises from the initial level of 30–35% to greater than 80% by 20 hr, and then falls to below 10% by 60 hr (Fig. 2). A difference in the proportion of labeled cells in control cultures and cultures with 280 mM Me₂SO is detected at 6 hr (Fig. 2). In cultures with inducer, the proportion of labeled cells falls after 6 hr to reach a low level of 10% by 20 hr, then rises to a peak value of 70–75% by 30–50 hr (Fig. 2).

Distribution of DNA content per cell by FMF

The distribution of DNA content per cell in MELC cultured with Me₂SO, measured by propidium iodide staining and FMF, shows a decrease in the proportion of cells in S, G₂, and M phases of the cell cycle after 19 hr, compared with control cells (Fig. 3). By 19 hr of culture, in the presence of inducer, more than 85% of the cells were in G₁, compared with 50% in the control cultures (Fig. 3D and A). In the induced cultures, an increasing proportion of cells enter S, G₂, and M between 20 and 40 hr,
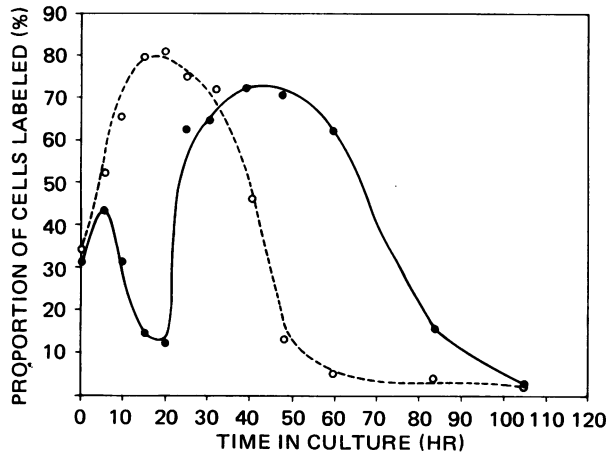
FIG. 2. Thymidine labeling index during erythroid cell differentiation of MELC induced by Me₂SO. DS19 cells were transferred to medium with and without Me₂SO as described in the legend for Fig. 1. At each time indicated, an aliquot of cell suspension was removed for incubation with [³H]thymidine for 20 min to obtain the thymidine labeling index by radioautography (O---O, control; ●—●, Me₂SO), according to the methods described in the text.

reaching a peak value of 45% of cells in the S-phase at 38 hr. By comparison, 55% of the control cells are in S-phase at 19 hr and by 38 hr the proportion of cells in S begins to decline (Fig. 4). After 60 hr in culture with or without Me₂SO, most of the cells have accumulated in G₁.

In FMF recording, the channel number is primarily a measure of the relative amount of propidium iodide intercalated in DNA (17, 18). MELC cultured without inducer display an increase in the channel number corresponding to the peak for cells in G₁ by 10 hr, which persists for at least 40–50 hr (Table 1). This shift in G₁ peak channel is not observed in cells cultured with Me₂SO. The Me₂SO-resistent cell line, DR10, behaves like control DS19 cells, even when cultured with Me₂SO. The channel number corresponding to the peak value for cells in G₁, in repeated determinations of a series of samples in a given experiment, did not vary by more than ±1. In order to determine if a cytoplasmic component contributes to this difference in propidium iodide binding, isolated nuclei were studied by
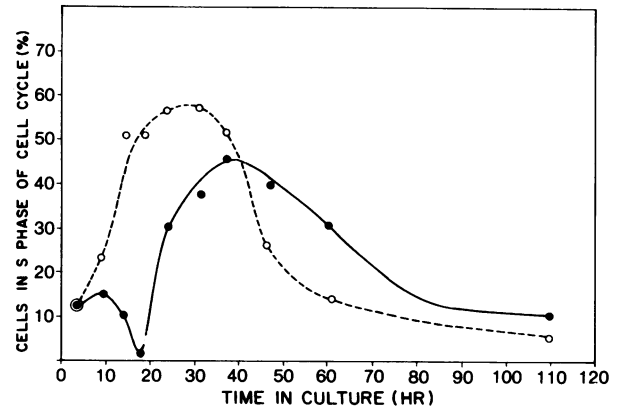
FIG. 4. FMF analysis of percent of cells in S-phase of MELC cultured with and without Me₂SO. MELC were cultured with and without Me₂SO as described in the legend of Fig. 1. At each time point indicated, the cells were removed to analyze the percent of cells in S-phase (O----O,.control; ●—●, Me₂SO). The data presented in this figure come from the same experiment presented in Table 1, Exp. 1.

FMF. Nuclei prepared from control and Me₂SO-treated DS19 cells show a difference in the G₁ peak channel number, similar to that observed with intact cells. Furthermore, addition of 280 mM Me₂SO to control cells just prior to mixing with propidium iodide does not alter the value for the channel number for the peak of cells in G₁.

DNA content per cell by the fluorescent Feulgen reaction

By the fluorescent Feulgen reaction and fluorescence microscopy, an increase in proportion of Me₂SO-treated cells in G₁, relative to the control population, can be detected by 19 hr (Fig. 5B). By 66 hr, and thereafter, cells in both control and induced cultures show a trend toward accumulation in G₁ (Fig. 5D, E, and F). Owing to the lower number of cells scored with this technique compared to the FMF method, these studies are less sensitive in demonstrating changes in a cell-cycle-related phenomenon than the experiments with propidium iodide.

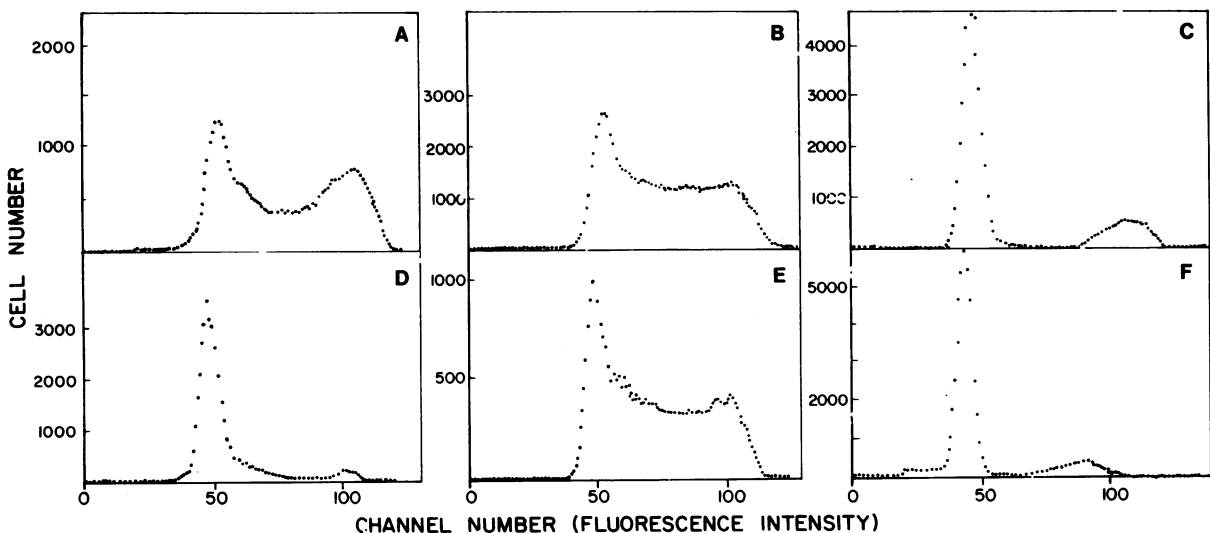The distributions of DNA per cell in the control and induced

FIG. 3. Distributions of DNA content per cell measured by propidium iodide staining and FMF. Peak corresponding to G₁ cells was adjusted to be about channel 50. At the indicated times after beginning of culture with and without Me₂SO, an aliquot of cell suspension was removed to determine the DNA distribution histogram by FMF according to the method described in the text. (A to C, cells cultured without Me₂SO; and D to F, cells cultured with Me₂SO, for the following periods of time: A and D, 19 hr; B and E, 38 hr; C and F, 88 hr.)

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Table 1. Fluorescence intensity of cells in G_1 after treatment with propidium iodide

| Cell line | Time in culture (hr) | G_1 peak channel number* | | | | | | |
| | | Exp. 1 | | Exp. 2 | | Exp. 3 | | |
		C	Me$_2$SO	C	Me$_2$SO	C	Me$_2$SO	HOBt
DS19	5	48	48	48	48	48	48	48
	9	51	49					
	14	53	48					
	19	53	48	54	48	54	48	52
	24	53	49					
	32	53	48					
	38	52	48	50	47	52	48	46
	46	49	47					
	62	48	47	50	47	48	47	46
	88			48	45	48	44	
	108	47	44					
	144			46	43			
	168			46	43			
DS19† Nuclei	24	44	39					
	168			42	39			
DR10	5					44	44	
	19					49	48	47
	88			45	45	44	45	

C refers to control cells cultured without Me$_2$SO. Me$_2$SO refers to cells cultured with 280 mM Me$_2$SO. HOBt refers to cells cultured with 1.8 mM butyric acid.
* G_1 peak channel number is a relative measure of the fluorescence intensity due to bound propidium iodide.
† Nuclei prepared as described in the text.

cultures were compared with that in normal liver cells of DBA mice (Fig. 5A). The G_1 population of MELC have more DNA per cell, on the average, than the G_1 population of liver cells (compare Fig. 5A and B). During culture of MELC with Me$_2$SO, the fluorescent Feulgen reaction of the G_1 population decreases toward that of liver (compare Fig. 5A and F). This decrease is not seen in control cells, nor in Me$_2$SO-resistant (DR10) cells cultured with Me$_2$SO (these latter data are not illustrated).

DISCUSSION

MELC cultured with Me$_2$SO to induce erythroid differentiation develop a transient block in initiation of DNA synthesis or entry into the S-phase of the cell cycle, most marked at about 20 hr in nonsynchronous cultures. This has been demonstrated by four different assays for DNA synthesis and DNA content per cell, namely, measurement of the rate of DNA synthesis by pulse labeling with [^{3}H]thymidine, determination of proportion of cells in S-phase by measuring the proportion of cells that become labeled during 20-min exposure to isotopically labeled thymidine, and determination of the relative DNA content per cell using both the fluorescent Feulgen assay and propidium iodide binding measured with flow microfluorometry. The block in initiation of DNA synthesis or prolongation of G_1 is transient in that MELC cultured with Me$_2$SO eventually enter S-phase, but only after an approximately 15- to 20-hr lag, compared with MELC cultured without inducer. These observations suggest that prolongation of the G_1 phase of the cell cycle may be related to the induction of MELC to express the program of erythroid differentiation.

In previous studies, it has been demonstrated that the earliest commitment to differentiation in MELC (cell line DS19) cultured with Me$_2$SO occurs between 24 and 30 hr, as measured by studies in which cells cultured with inducer are transferred to fresh medium without inducer and permitted to proliferate and differentiate (20). Furthermore, under conditions similar to those used in the present experiments, the first detectable accumulation of mRNA for globin and of globin synthesis is at 24–30 hr (6). Taken together, these observation indicate that the transient block in initiation of DNA synthesis recognized between 6 and 20 hr precedes detectable expression of the program of erythroid differentiation.

Darzynkiewicz and co-workers (22) have suggested that a change in chromatin structure occurs in MELC after 6 days of culture with Me$_2$SO, based on finding a lower staining of DNA with acridine orange, in comparison with cells grown in the absence of inducer. These late changes are probably related to the final stages of erythroid cell maturation. In the present studies, an alteration in chromatin is detected by as early as 10 hr of culture with Me$_2$SO, manifested by a lower level of propidium iodide binding by G_1 cells, compared to control MELC. The present data suggest that a change in chromatin structure is an early phenomenon in the course of Me$_2$SO-induced erythroid differentiation. This conclusion is supported by the observation that other inducers, such as dimethylacetamide and butyric acid, cause a lower level of propidium iodide binding, similar to that observed with Me$_2$SO. On the other hand, Me$_2$SO does not alter the pattern of propidium iodide binding in a MELC strain (DR10) resistant to induction by Me$_2$SO.

Previous data from this and other laboratories (21, 23) indicate that Me$_2$SO must be present during at least one round of DNA synthesis for expression of the program of erythroid differentiation. More recently, Fibach et al. (10) have provided evidence that the presence of inducer during DNA synthesis is not sufficient for the stable expression of the differentiated program in all progeny of a cell exposed to inducer. As a working hypothesis, it is suggested that induction to erythroid differentiation involves a series of events that includes (a) a
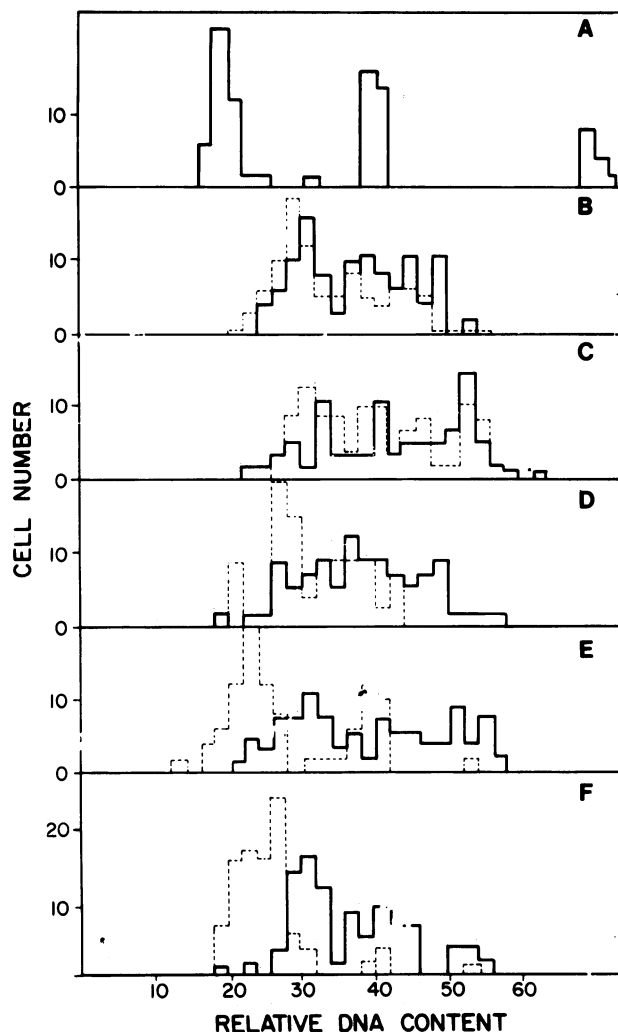
FIG. 5. Fluorescence microscopic measurement of relative DNA content per cell by Feulgen staining (as described in the *text*). B, C, D, E, and F are the Feulgen DNA distribution histograms of the cells cultured for 19, 27, 36, 66, and 108 hr, respectively.———, control; ---, Me₂SO cultured cells. A is a DNA histogram of liver cells from a DBA mouse.

transcription. The period of DNA synthesis (S-phase) that follows the prolonged G_1 is then essential to the reconstitution of chromatin and expression of the new program for differentiation.

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triggering event related to the prolongation of G_1, which is associated with restricted binding of intercalating dyes to chromatin, (*b*) the subsequent S-phase of the cell cycle, and (*c*) additional stabilizing events occurring following this S which are required to render differentiation irreversible. It has been suggested (24) that the activity of genes is dependent on the association of regulatory proteins with particular regions of the chromosomes. It is presumed that these proteins are at least partially dissociated from chromosomes during the process of DNA replication. These regulatory proteins may be synthesized during the previous interphase. Prolongation of the duration of G_1 may permit accumulation or degradation of a regulatory substance to a concentration that can effect an alteration in the pattern of chromosomal proteins and the program of gene