DEVELOPMENTAL PROGRAM OF MURINE ERYTHROLEUKEMIA CELLS

Effect of the Inhibition of Protein Synthesis

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ABSTRACT

The relationship between protein synthesis and commitment to terminal erythroid differentiation by dimethylsulfoxide-treated murine erythroleukemia (MEL) cells has been studied. Treatment with cycloheximide blocks the commitment of MEL cells. The effects of cycloheximide are completely reversible, however. Treatment of MEL cells before commitment delays commitment for a period of time equal to the length of inhibitor treatment. Puromycin exerts a similar effect on the commitment of MEL cells. These results indicate that there is a continuous requirement for protein synthesis before the commitment event.

KEY WORDS murine erythroleukemia cells . differentiation . commitment . protein synthesis

The analysis of developmental programs in eukaryotic organisms has been limited by two considerations: (a) the difficulty in identifying discrete steps in a specific program, and (b) the difficulty in establishing the molecular basis for such steps when they can be identified. The murine erythroleukemia (MEL) cell system has attracted much interest because it offers a model system in which both these issues can be approached.

Erythroid differentiation in the mouse is one of the few developmental systems in which the behavior of individual cells during a differentiation program has been analyzed. This analysis has been possible because individual erythroid precursor cells can be cloned and induced to differentiate in vitro in semi-solid media (11). On the basis of this approach, Axelrad and co-workers were able to show that erythroid precursors with varying proliferative capacity could be identified and characterized within a single organ. A limitation of this analysis has been that normal precursor cells cannot be obtained as a homogeneous population. The presence of heterogeneous cell types in the population under analysis makes the identification of discrete developmental events difficult at both the molecular and cellular levels. Use of the MEL cell system alleviates this difficulty. Permanent lines of cells resembling nonhemoglobinized pro-erythroblasts can be obtained from mice infected with the Friend leukemia virus (1, 18, 19). These cell lines can be cloned with an efficiency approaching 100% using plasma clot cultures (6–8). When exposed to dimethylsulfoxide (DMSO) or a variety of other chemical agents, these cells will undergo a differentiation program which in many aspects resembles very closely the normal erythroid differentiation program (7, 8). In particular, heme and globin are synthesized in quantity (1, 18); spectrin, an erythrocyte membrane protein, is synthesized in increased amounts (4); and a variety
of changes in enzyme levels characteristic of normal erythroid differentiation is observed as well (19).

Analysis of the differentiation of MEL cells at the clonal level has suggested the following conclusions: (a) A coordinated differentiation program is initiated in each cell that becomes committed to erythroid differentiation. The characteristics of this program include expression of high levels of heme and globin as well as the limitation of proliferative capacity to four additional cell generations (7, 8). (b) A latent period of at least 9 h after addition of inducer is usually observed before a significant number of cells becomes committed. Cells that have become committed no longer require the presence of inducer to execute the differentiation program. The kinetics of commitment of individual cells are consistent with a stochastic model for the commitment event. The proportion of cells that become committed in each cell generation is a function of the concentration of inducer: exposure of cells to a higher concentration of inducer results in a higher proportion of committed cells in each cell generation (7, 8).

The identification of a specific reprogramming step that initiates a coordinated biochemical program raises the issue "What is the molecular basis for the commitment process?" One approach to this issue has been the use of metabolic inhibitors to identify the macromolecular processes necessary for the execution of a specific step in a developmental process (13, 21). Previous studies in the MEL cell system that have employed metabolic inhibitors have failed to clearly define the requirements for specific macromolecular synthesis at different stages of the differentiation program (9, 10, 12). The major technical limitation of these studies has been that only the final end products of the differentiation program have been quantitated rather than the reprogramming decision itself. In the present study, we have approached this problem in a direct manner. As a prototype for further inhibitor studies, we present a detailed analysis of the effects of the inhibitors cycloheximide and puromycin on the commitment process. On the basis of these experiments, specific conclusions about the macromolecular synthesis requirements for commitment have been made.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Cell line 745 was originally obtained from Dr. C. Friend. 745-PC-4 is a subclone of this cell line. All cultures were maintained in a medium (22) lacking nucleosides and supplemented with 13% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Cell density was kept between 1 \times 10^4 and 5 \times 10^5/ml to maintain continuous logarithmic growth. Cell counts were performed with an automatic cell counter (Coulter Counter model ZBI, Coulter Electronics, Hialeah, Fla.).

Plasma culture was performed as described by McCleod et al. (11) and Gusella et al. (7). Briefly, 100-200 cells were plated in 0.1-ml clots in microtiter wells (Linbro Scientific Co., Hamden, Conn.) that had been sterilized by ultraviolet radiation. The clots were incubated at 37°C in a humidified 5% CO₂ atmosphere. Clots were transferred to microscope slides after 90–100 h of culture, dehydrated with filter paper, fixed in glutaraldehyde, stained in benzidine, counterstained with hematoxylin, and covered with permount (Fisher Scientific Co., Pittsburgh, Pa.) and a cover slip. Scoring of colony type was performed at \times 100.

Radioactive Labeling of Protein

5 \times 10^6 MEL cells/ml were seeded in liquid culture in the presence of 1.5% DMSO. Cells were grown for 22 h, at which time they were centrifuged and resuspended in a medium lacking leucine at a density of 2.0 \times 10^5 cells/ml. The culture was then divided into several subcultures, to each of which was added [³H]leucine (sp act = 110 Ci/mM) to a final concentration of 25 μCi/ml. At the appropriate times, a 100-μl aliquot was taken from each culture and added to 1 ml of 0.1 N NaOH. The samples were heated at 37°C for 20 min, then were precipitated by the addition of 1 ml of cold 10% TCA. Samples were chilled on ice for 15 min, then collected by filtration under vacuum onto 24-mm glass fiber filters (Whatman GF/C) and washed with 5% TCA. The filters were dried and counted in a Beckman LS-330 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Chemicals

Cycloheximide and emetine were obtained from Sigma Chemical Co. (St. Louis, Mo.). Puromycin was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio). Puromycin and emetine were kindly provided by Dr. Sheldon Penman. [³H]leucine was obtained from New England Nuclear (Boston, Mass.). All inhibitors were made as 1 mg/ml stocks in a medium supplemented with 13% fetal calf serum, and filter sterilized using a millex disposable filter unit (Millipore Corp., Bedford, Mass.) with a 0.45-μm filter.

RESULTS

Rationale

In this manuscript we have focused on the requirement for protein synthesis in the MEL cell system both before the commitment event and at the time of the commitment event itself. The basic
protocol that we have employed involves the induction of differentiation in MEL cells with DMSO followed by exposure of the cells to an inhibitor of protein synthesis for a portion of the induction period. If events leading to commitment do not require protein synthesis, then an inhibitor-treated culture and its control should show identical kinetics of commitment to the differentiation program. If, however, protein synthesis is essential for any stage of the differentiation program, then treatment of the cells with a reversible inhibitor will delay their progress through the program by an amount of time equal to or greater than the period of treatment with inhibitor.

**Cycloheximide is an Effective and Reversible Inhibitor of Protein Synthesis in DMSO-Treated MEL Cells**

Before initiating experiments on the effects of cycloheximide on the commitment to differentiation, we determined the extent of the inhibition of protein synthesis by cycloheximide on DMSO-treated MEL cells and the degree to which this inhibition was reversible. MEL cells were grown in the presence of 1.5% DMSO for 22 h, and then a series of parallel cultures were treated with cycloheximide at various doses while the DMSO concentration was maintained at 1.5%. Incorporation of \(^{1}\text{H}\)leucine into TCA-precipitable material was followed over a 6-h period. At all doses of cycloheximide, a linear-incorporation rate was observed (correlation coefficients ranged from 0.819 to 0.996). The relative rate of incorporation at each dose of cycloheximide is shown in Table 1. The highest concentrations of cycloheximide used, 5 and 0.5 \(\mu\)g/ml, inhibited the rate of protein synthesis by \(-95\) and \(-87\)% respectively, of the value for an untreated control culture. A cycloheximide dose of 0.05 \(\mu\)g/ml, on the other hand, inhibited the rate of protein synthesis by \(-50\)%.

The lowest cycloheximide concentration employed, 0.005 \(\mu\)g/ml, was found to inhibit the rate of protein synthesis by \(-13\)% of the control culture. Removal of DMSO-treated cultures from the drug resulted in a rapid and significant reversal of the inhibition of protein synthesis. Cultures treated with cycloheximide for 6 h, then washed free of the drug, recover very rapidly in their ability to incorporate \(^{1}\text{H}\)leucine. As shown in Table 1, cultures released from cycloheximide block ranged in their rate of protein synthesis from 71 to 100% of the control value.

| Cycloheximide concn during pulse period | Rate of incorporation of \(^{1}\text{H}\)leucine during cycloheximide treatment | Rate of incorporation of \(^{1}\text{H}\)leucine after removal of cycloheximide |
|----------------------------------------|----------------------------------|----------------------------------|
| 0 \(\mu\)g/ml                         | 100.0 (0.983)*                    | 100.0 (0.900)                     |
| 5 \(\mu\)g/ml                         | 5.5 (0.980)                       | 71.6 (0.962)                      |
| 0.5 \(\mu\)g/ml                       | 13.2 (0.982)                      | 80.5 (0.978)                      |
| 0.05 \(\mu\)g/ml                      | 47.2 (0.996)                      | 92.0 (0.891)                      |
| 0.005 \(\mu\)g/ml                     | 87.0 (0.986)                      | 100.0 (0.819)                     |

\(1 \times 10^9\) MEL cells/ml were grown in liquid culture in the presence of 1.5% DMSO for 22 h. Cells were resuspended at a density of \(2 \times 10^9\) cells/ml in a 0.5% DMSO medium containing 13\% fetal calf serum. DMSO was added to each culture to a final concentration of 1.5%, and 50 \(\mu\)l of a serial dilution of cycloheximide in a 0.5% DMSO medium containing 13\% fetal calf serum was added to give the appropriate final concentration of the drug. Finally, all cultures were pulsed with 25 \(\mu\)Ci/ml of \(^{1}\text{H}\)leucine. Cultures were maintained in cycloheximide for 6 h, at which time cells were centrifuged, washed once in fresh DMSO medium lacking leucine, and resuspended in leucine-free DMSO medium at \(2 \times 10^6\) cells/ml. DMSO was readded to each culture to a final concentration of 1.5%, and all cultures were resuspended with 25 \(\mu\)Ci/ml of \(^{1}\text{H}\)leucine. Cultures were maintained in DMSO for 6 h, at which time cells were centrifuged, washed once in fresh DMSO medium lacking leucine, then resuspended in leucine-free DMSO medium at \(2 \times 10^6\) cells/ml. DMSO was readded to each culture to a final concentration of 1.5%, and all cultures were resuspended with 25 \(\mu\)Ci/ml of \(^{1}\text{H}\)leucine. Cultures were maintained in cycloheximide for 6 h, at which time cells were centrifuged, washed once in fresh DMSO medium lacking leucine, then resuspended in leucine-free DMSO medium at \(2 \times 10^6\) cells/ml. DMSO was readded to each culture to a final concentration of 1.5%, and all cultures were resuspended with 25 \(\mu\)Ci/ml of \(^{1}\text{H}\)leucine. Cultures were maintained in cycloheximide for 6 h, at which time cells were centrifuged, washed once in fresh DMSO medium lacking leucine, then resuspended in leucine-free DMSO medium at \(2 \times 10^6\) cells/ml. DMSO was readded to each culture to a final concentration of 1.5%, and all cultures were resuspended with 25 \(\mu\)Ci/ml of \(^{1}\text{H}\)leucine. Cultures were maintained in cycloheximide for 6 h, at which time cells were centrifuged, washed once in fresh DMSO medium lacking leucine, then resuspended in leucine-free DMSO medium at \(2 \times 10^6\) cells/ml. DMSO was readded to each culture to a final concentr-
FIGURE 1 Effect of cycloheximide treatment on the rate of growth of MEL cells. Panel A: MEL cells were grown in liquid culture in the presence of 1.5% DMSO for 12 h. The cells were then subcultured and growth continued in the presence of 1.5% DMSO alone (○); 1.5% DMSO + 0.005 μg/ml cycloheximide (●); 1.5% DMSO + 0.05 μg/ml cycloheximide (□); 1.5% DMSO + 0.5 μg/ml cycloheximide (■); or 1.5% DMSO + 5 μg/ml of cycloheximide (▲). At the times indicated, a 1-ml aliquot of cells was removed from each culture and the cell density determined using an automatic cell counter. Panel B: MEL cells were grown in liquid culture for 12 h in the presence of 1.5% DMSO. The cells were then subcultured and growth continued in the presence of either 1.5% DMSO alone or 1.5% DMSO + various doses of cycloheximide. After 12 h of treatment with inhibitor, cycloheximide was removed and cell growth was continued in the presence of 1.5% DMSO. At the times indicated, cell density was determined as described above.

served over a 12-h period. Treatment with 5 μg/ml of cycloheximide also inhibited cell growth completely, and in fact a slight decrease in cell number was observed over a 12-h period. This suggests that exposure to 5 μg/ml of cycloheximide for this period produces some cytotoxic effects. The growth rates of cultures treated with these concentrations of cycloheximide for a 12-h period were compared immediately after removal of cycloheximide, as shown in panel B. When cycloheximide was removed, all cultures (except the culture treated with 5 μg/ml of cycloheximide) immediately returned to a normal doubling time of 11 h. These results are consistent with the view that treatment of MEL cells with cycloheximide for a period of 12 h at all doses up to and including 0.5 μg/ml does not directly affect cell viability. A dose of 5 μg/ml of cycloheximide does lead to a reduction in cell division rate when the inhibitor is removed, suggesting that a proportion of the cells lose viability during the drug treatment period. Subsequent experiments involving a measurement of the plating efficiency of cycloheximide-treated cells confirmed this point. As shown in Table II, plating efficiencies of 87% or greater were observed for cultures treated with 1.5% DMSO plus 0.05 or 0.5 μg/ml of cycloheximide during a 12-h exposure period. Cells treated with 5 μg/ml of cycloheximide, on the other hand, showed a significant reduction in plating efficiency. After 12 h of treatment with 5 μg/ml of cycloheximide, the plating efficiency was reduced to 72%. These results suggest that, at cycloheximide concentrations up to and including 0.5 μg/ml, cells did not suffer irreversible cytotoxic damage as a result of inhibitor treatment, and plate with an efficiency essentially equivalent to that of a control culture. Cells treated with 5 μg/ml of inhibitor, on the other hand, showed a progressive loss of plating efficiency, hence cell viability, as the exposure time to the drug was lengthened.

Table II

| Treatment | Exposure to cycloheximide | Plating efficiency |
|-----------|---------------------------|--------------------|
|           | h                         | %                  |
| 1.5% DMSO |                           | 94                 |
| 1.5% DMSO + 0.05 μg/ml cycloheximide | 3 | 96          |
|          |                           | 6                  | 100          |
|          |                           | 12                 | 89           |
| 1.5% DMSO + 0.5 μg/ml cycloheximide | 3 | 100         |
|          |                           | 6                  | 94           |
|          |                           | 12                 | 87           |
| 1.5% DMSO + 5 μg/ml cycloheximide | 3 | 83          |
|          |                           | 6                  | 76           |
|          |                           | 12                 | 72           |

MEL cells were grown in liquid culture in the presence of 1.5% DMSO for 21 h. The cells were then subcultured and growth continued in the presence of 1.5% DMSO alone or 1.5% DMSO plus either 0.05 μg/ml, 0.5 μg/ml, or 5 μg/ml of cycloheximide. At 3, 6, and 12 h after the addition of cycloheximide, an aliquot of cells was removed from each culture and plated in plasma culture in the absence of DMSO or cycloheximide. Approx. 200 cells were plated per clot. After 96 h of incubation, the cells were harvested and the number of colonies was determined as has been described. A minimum of 200 colonies was scored at each time point.
Pretreatment of MEL Cells with Cycloheximide has no Effect on Induction of the Differentiation Program

Commitment of MEL cells is measured by cloning DMSO-treated cells in the absence of either inducer or inhibitor in the plasma culture system. The assay for commitment which we have developed (7) reflects the commitment potential of a cell at the time at which the cell was removed from liquid and plated in plasma clot. The colony phenotype observed after 90–100 h of plasma culture is a measure of the commitment of an individual cell after plating.

An uncommitted cell gives rise to an undifferentiated colony that will be large (>32 cells) and unstained with benzidine. A committed cell gives rise to a differentiated colony. Such a colony is small (<32 cells) and stains bright orange with benzidine, a reagent specific for heme, a characteristic product of differentiating erythroid cells. As we have previously noted (7, 8), benzidine-positive colonies are limited in their proliferative capacity. Sectored colonies (those exhibiting a mixed phenotype) arise if cells become committed after plating in plasma culture (7). In the experiments reported in this study, sectored colonies were never found to represent more than 3% of the total colonies observed in plasma culture. The proportion of sectored colonies that occur under a variety of experimental conditions is shown in Table III. It is clear from these results that cycloheximide treatment does not lead to a significant increase in the proportion of sectored colonies.

The effect of cycloheximide on the induction of the development program of MEL cells with DMSO has been examined by preexposing a culture to cycloheximide and comparing the kinetics of commitment to differentiation of the drug-treated cells with that of a control culture. The results of such an experiment are shown in Fig. 2. When a culture of MEL cells was treated with either 5 μg/ml (panel A) or 0.5 μg/ml (panel B) of cycloheximide before induction with DMSO, no delay in the appearance of committed cells was observed after cycloheximide removal and subsequent treatment with 1.5% DMSO. These results indicate that the effect of cycloheximide treatment on commitment may be rapidly reversed after removal of the inhibitor.

Is Protein Synthesis Necessary before the Time of Commitment?

Under the conditions used in these experiments, treatment with DMSO must continue for a period of at least nine h before a significant portion of the population becomes committed to differentiation.

To examine whether the commitment of MEL cells to erythroid differentiation is dependent on protein synthesis before the commitment event, i.e., during the latent period, the following experimental protocol was adopted: MEL cells were seeded in liquid culture in the presence of 1.5% DMSO plus 0.5 μg/ml of cycloheximide and incubated in the presence of the drug for most or all of the latent period. After cycloheximide removal, continued growth of the cultures was carried out in the presence of 1.5% DMSO. The accumulation of committed cells in the cycloheximide-treated cultures was then compared to that of a control culture that was exposed to 1.5% DMSO for the entire course of the experiments.

The results of two separate experiments employing such a protocol are shown in Fig. 3. MEL cultures were treated with 0.5 μg/ml of cycloheximide for either 9 h (panel A) or 12 h (panel B), before removal of the drug and culture in DMSO alone. In both experiments, the accumulation of committed cells in the population was blocked during the cycloheximide treatment period. In the case of a 9-h exposure to the inhibitor (panel A), the appearance of committed cells, as determined by the percentage of benzidine-reactive colonies,

| Treatment          | Time (h) | Sectored colonies (%) |
|--------------------|----------|-----------------------|
| 1.5% DMSO          | 21       | 3                     |
| 1.5% DMSO          | 24       | 3                     |
| 1.5% DMSO + Cyclo. | 24       | 2                     |
| 1.5% DMSO + Cyclo. | 30       | 1                     |

Table III. Effect of 0.5 μg/ml Cycloheximide on the Proportion of Sectored Colonies

MEL cells were grown in liquid culture in the presence of 1.5% DMSO for 21 h. The cells were then subcultured and growth continued in either 1.5% DMSO, or 1.5% DMSO plus 0.5 μg/ml of cycloheximide. At the times indicated, an aliquot of cells was removed from each culture and plated in plasma clots in the absence of DMSO and cycloheximide for a period of 96 h. Cells were harvested as has been described, and the proportion of sectored colonies was determined at × 400 under oil immersion. A minimum of 200 colonies was scored at each time point.
Effect of a preexposure of cycloheximide on the differentiation of MEL cells. 1 × 10⁵ MEL cells/ml were seeded in liquid culture in the presence (●) or absence (○) of cycloheximide for a period of 12 h. Cells grown in the presence of cycloheximide were centrifuged and resuspended in fresh culture medium lacking the inhibitor. Both this culture and the culture which was initially grown in the absence of cycloheximide were adjusted to a cell density of 1 × 10⁵/ml, then induced to differentiate by the addition of 1.5% DMSO. Panel A: Preexposure to 5 µg/ml cycloheximide. Panel B: Preexposure to 0.5 µg/ml cycloheximide. An aliquot was removed from the cultures at the times indicated, and ~200 cells from each culture were plated in plasma clots in the absence of DMSO. Plasma cultures were harvested 96 h later, and the proportion of benzidine-reactive colonies was determined as described in Materials and Methods.

Is Protein Synthesis Required Subsequent to the Latent Period?

To examine the requirement for protein synthesis at the time of commitment, we employed the following experimental design: MEL cells were treated with 1.5% DMSO for a period longer than the latent period, then exposed to cycloheximide for the remainder of the experiment. If cycloheximide inhibits the commitment event itself, then the proportion of committed cells in the population should be significantly lower than that of a control culture grown in the continuous presence of 1.5% DMSO alone.

The results of such an experiment are shown in Fig. 4. MEL cells were grown in liquid culture in the presence of 1.5% DMSO for either 18 or 25 h. The proportion of benzidine-positive colonies is delayed by ~9 h subsequent to removal of cycloheximide from the culture and treatment with DMSO alone. After a 12-h inhibitor treatment period (panel B), commitment of MEL cells to differentiation was also delayed by ~9 h.

These results support the view that commitment of MEL cells to the program of erythroid differen-
FIGURE 4  Effect of cycloheximide on the commitment to differentiation of MEL cells. MEL cells were grown in liquid culture in the presence of 1.5% DMSO for either 18 or 25 h. At the times indicated by the arrows, the cells were subcultured and growth was continued in the presence of 1.5% DMSO alone ( ), or in the presence of 1.5% DMSO plus 0.5 μg/ml of cycloheximide ( , ). At the times shown, a 1-ml aliquot of cells was removed from each culture and treated as described in the legend to Fig. 3.

At these times, the cells were subcultured and growth continued in the presence of either 1.5% DMSO or 1.5% DMSO plus 0.5 μg/ml of cycloheximide. As demonstrated in Fig. 4, it was observed that commitment was blocked in both cycloheximide-treated cultures. This and other similarly designed experiments (data not shown) suggest that cycloheximide acts to block commitment regardless of when the inhibitor is added subsequent to the end of the latent period. One potential difficulty in assessing the specificity of inhibitor treatment on commitment is the possibility that the block to commitment is due not to a specific effect of cycloheximide on commitment, but rather to a general inhibition of cellular function unrelated to commitment. To show more directly that cycloheximide specifically blocks commitment, we have compared the average size of both committed and uncommitted colonies in plasma clots after cycloheximide treatment. If cycloheximide directly blocks commitment, then the average size of both committed and uncommitted colonies should be equivalent to that of a control culture. If, on the other hand, cycloheximide serves to inhibit cell metabolism in general, and commitment indirectly, then the average size of committed and uncommitted colonies in a drug-treated culture might be expected to be significantly smaller than in an untreated control culture. As shown in Fig. 5, the average size of both committed and uncommitted colonies in a cycloheximide-treated culture and untreated control culture was found to be identical. In this experiment, we have compared the average size of uncommitted colonies in a culture treated for 19 h with 1.5% DMSO, then for 8 h with 1.5% DMSO plus 0.5 μg/ml cycloheximide (panel B), with that in a control culture treated for 27 h with 1.5% DMSO (panel A). The average cell number of benzidine-negative colonies in both cultures was found to be essentially equivalent. We have also compared the size of committed colonies (panel D) in the culture treated with cycloheximide (as described in panel B) with that in a control culture treated for 19 h with 1.5% DMSO (panel C). If cycloheximide does in fact specifically block commitment, then the average size of benzidine-positive colonies in a cycloheximide-treated culture should be the same as the average size of benzidine-positive colonies in a culture treated with inducer up to the point at which inhibitor is added. This point is verified by

FIGURE 5  Effect of cycloheximide treatment on the size of uncommitted and committed colonies. MEL cells were grown in liquid culture in the presence of 1.5% DMSO for 19 h. The cells were then subcultured and growth continued in the presence of 1.5% DMSO alone (panels A and C) or in the presence of 1.5% DMSO + 0.5 μg/ml of cycloheximide (panels B and D). At 19 and 27 h, an aliquot was removed from each culture and plated in plasma clots in the absence of DMSO and cycloheximide for 96 h, after which cells were harvested, fixed, and stained as has been described. The size of pure red or pure blue colonies was then determined at × 400 under oil immersion. A minimum of 200 colonies was scored for each time point. Panels A, B, and D represent colony size distribution at 27 h after the start of the experiment. Panel C represents a culture treated with 1.5% DMSO for 19 h.
the results shown in panels C and D. These results are consistent with the view that the block to commitment exerted by cycloheximide treatment is the result of a direct action of the drug on cellular reprogramming and is not the result of a more general cytotoxic effect of cycloheximide treatment which leads indirectly to the inhibition of commitment.

Varying the dose of cycloheximide has a significant effect on the commitment of MEL cells to erythroid differentiation. A culture was treated with 1.5% DMSO for 21 h, then divided into several subcultures that were either maintained in 1.5% DMSO or treated with 1.5% DMSO plus a dose of cycloheximide ranging in concentration from 5 µg/ml to 0.005 µg/ml. As shown in Fig. 6, the increase in the proportion of committed cells in the cultures treated with either 5 µg/ml or 0.5 µg/ml of cycloheximide was rapidly blocked after treatment with the drug, whereas a culture treated with 0.05 µg/ml cycloheximide showed an intermediate level of inhibition. MEL cells treated with 0.005 µg/ml of cycloheximide, however, exhibited kinetics of accumulation of committed cells which very nearly approximate that of a control culture, while cells treated with 0.0005 µg/ml cycloheximide (data not shown) were indistinguishable from the control culture.

Does Another Protein Synthesis Inhibitor also Block Commitment?

The block to commitment which results from exposure of MEL cells to cycloheximide may be due to a direct effect of the drug on protein synthesis or, alternatively, may be due to a side effect of the drug. One approach to this problem is to employ inhibitors of protein synthesis that operate via other mechanisms such as puromycin. Puromycin causes premature termination of polypeptide chains (15), whereas cycloheximide prevents the formation of peptide bonds (16). The results of an experiment with use of puromycin are presented in Fig. 7. A culture of MEL cells was treated with 1.5% DMSO for 19 h, then subcultured and grown in 1.5% DMSO plus either 0.5 µg/ml cycloheximide or 1 µg/ml of puromycin. These cultures behaved alike with respect to the inhibition of commitment after administration of the drugs. In both cases, the increase in the proportion of committed cells was rapidly inhibited. A culture treated with 0.1 µg/ml of puromycin, on the other hand, showed an intermediate level of commitment when compared to a control culture.

Kinetics of Appearance of Committed Cells after a Pulse with Cycloheximide

The results shown in Fig. 2 and Tables I and II indicate that the effects of cycloheximide on MEL cells are rapidly reversed after removal of the drug. We therefore wished to examine the effect of cycloheximide treatment on the kinetics of reappearance of committed cells after a pulse exposure of MEL cells to cycloheximide subsequent to the latent period. Results of such an experiment are shown in Fig. 8. The increase in the proportion of committed cells in a culture grown in the presence of 1.5% DMSO for 22 h was quickly blocked after treatment with 0.5 µg/ml of cycloheximide. After a 6-h pulse exposure to cycloheximide, removal of the drug, and culture in DMSO alone, an immediate increase in the proportion of committed cells was observed. No significant lag in the rate of increase in number of committed cells was detected. When the cycloheximide-treated culture was normalized in time (by plotting all points 6 h earlier to account for the cycloheximide pulse period) to the control, the two curves are indistin-
FIGURE 7 Effect of puromycin and cycloheximide on the commitment to differentiation of MEL cells. 1 x 10⁶ MEL cells/ml were grown in the presence of 1.5% DMSO for 19 h. The culture was then divided into five subcultures which were treated as follows: A control culture (○) was allowed to continue growth in the presence of 1.5% DMSO, while a second culture (■) was removed from DMSO and grown in fresh media. Of the remaining cultures, all were grown in the presence of 1.5% DMSO, but to one was added puromycin at 1 µg/ml (▲), to a second, puromycin at 0.1 µg/ml (□), and to a third, 0.5 µg/ml of cycloheximide (▲). At the times indicated, a 1-ml aliquot was removed from each culture, centrifuged, and resuspended in fresh media at a density of 2 x 10⁵ cells/ml. Approx. 200 cells were plated in plasma culture in the absence of all agents for 96 h. Cells were then harvested onto microscope slides, fixed, stained, and the proportion of committed cells was determined as previously described.

DISCUSSION

The inhibition of both protein synthesis and commitment resulting from treatment with cycloheximide is rapidly reversed after removal of MEL cells from the inhibitor. The reversibility of cycloheximide-mediated effects has permitted the use of the drug to assess the requirement for protein synthesis for the maintenance of the program of differentiation of MEL cells. The ability to achieve close to 100% plating efficiency in all experimental situations has provided a reliable and accurate means to determine the effect of inhibitor treatment on all cells in the population.

The resumption of the developmental program of MEL cells occurs immediately after removal of cycloheximide. The duration of the block in the program appears to be equivalent in length to the inhibitor treatment period. When MEL cells are treated with DMSO and cycloheximide for a period of time equal to or greater than the length of the latent period, no progress through the latent period is observed. MEL cells treated in this way behave like cells unexposed to DMSO. Commitment is initiated in such cultures ~9 h after removal of cycloheximide.

The administration of cycloheximide to DMSO-treated MEL cells in a pulse-chase type protocol indicates that the rate-limiting step in the differentiation program is not the build-up of one or more proteins with a relatively short half-life. No delay in the kinetics of commitment to differentiation is observed after removal of cycloheximide after a 6-h pulse and subsequent growth in DMSO alone. This observation is consistent with the con-

FIGURE 8 Kinetics of appearance of committed cells after a pulse with cycloheximide. 1 x 10⁶ MEL cells/ml were seeded in liquid culture in the presence of 1.5% DMSO and grown in the presence of inducer for 22 h. The culture was then divided into three subcultures and growth was continued either in the presence of 1.5% DMSO (○), 1.5% DMSO plus 0.5 µg/ml cycloheximide (■), or in the absence of DMSO and cycloheximide (□) for an additional 6 h. The control culture was maintained in 1.5% DMSO, whereas the culture treated with cycloheximide (■) was removed from the presence of inhibitor and growth then continued in the presence of 1.5% DMSO. The culture grown in media without additions during the 6-h pulse period was then made 1.5% by the addition of DMSO (□). At the time indicated, aliquots were taken from each culture, and ~200 cells were plated in plasma culture in the absence of DMSO and cycloheximide. Cultures were harvested at ~96 h, and the proportion of committed cells was determined as has been described. The inset represents a plot of the control culture (○) grown continuously in 1.5% DMSO and the culture pulsed for 6 h with 0.5 µg/ml cycloheximide (■) where the time points for the cycloheximide-treated culture have been normalized in time to the control (by shifting the points 6 h towards the origin).
clusion that no cycloheximide-sensitive process, critical for the differentiation program, decays with a half-life of \(<6\ h\).

A study of the proteins synthesized by induced and uninduced MEL cells has revealed no significant pattern differences when compared on two-dimensional polyacrylamide gels (17). Several new species of proteins were detected in the nucleus, though it is possible that these proteins play no part in the reprogramming of cellular function. One interpretation of these results is that protein synthesis is not a requirement for some steps in the differentiation of MEL cells. The results presented in this manuscript strongly suggest, however, that continuous protein synthesis is required during the course of the differentiation program.

Whether qualitatively new sets of proteins must be synthesized for commitment to occur cannot be determined from the current series of experiments.

The relationship between DNA synthesis and differentiation in MEL cells has been investigated in several studies (5, 10, 12). Although these experiments suggested a potential relationship between differentiation and DNA synthesis, it is difficult to assess the requirement for DNA replication for commitment, since these studies did not focus on the early biochemical changes which occur after induction. The data of Leder et al. (9), on the other hand, argue against a direct relationship between DNA synthesis and the differentiation program in MEL cells. In a study to be reported elsewhere (Levenson et al., manuscript submitted for publication), we have focused directly on the relationship between DNA synthesis and the commitment to differentiation of MEL cells. We have found that inhibition of DNA replication does not block the commitment process. This finding supports the idea that the block to commitment resulting from treatment of MEL cells with cycloheximide is due to the specific inhibition of a component critical for the reprogramming of MEL cells which is independent of DNA synthesis. Similar results have recently been reported by Nadal-Ginard (14) who found that the commitment of myoblasts to the differentiation program occurs in the absence of DNA synthesis.

The potential effects of cycloheximide treatment on RNA metabolism in cultured MEL cells have not been directly examined in the studies reported here. Willems et al. (23) and Craig and Perry (3) have studied the effects of cycloheximide treatment on RNA synthesis. Both studies showed that cycloheximide affects RNA synthesis indirectly by reducing the rate of processing of ribosomal RNA at concentrations of 50 \(\mu\text{g} / \text{ml}\) of cycloheximide. It has also been reported by Ross et al. (20) that globin mRNA accumulation in MEL cells is inhibited by cycloheximide treatment. The significance of this observation is obscured, however, by the fact that neither commitment nor the terminal differentiation status of these cycloheximide-treated cells was determined. It clearly will be of interest to examine the requirement for RNA, particularly mRNA synthesis, for the commitment of MEL cells.

The results of experiments carried out in a variety of other systems also support the view that the initiation of a developmental program exhibits a requirement for protein synthesis. The commitment of yeast (21) and 3T3 cells (2) for entry into the cell division cycle is sensitive to cycloheximide treatment and appears to involve the synthesis of a protein component with a relatively short half-life. In addition, the concanavalin-A induced transformation of cultured lymphocytes is sensitive to the inhibition of protein synthesis but does not appear to require concomitant DNA replication (13).

We have shown in this study that the inhibition of protein synthesis in cultured MEL cells, both before and at the time of commitment, blocks the expression of the differentiation program. The high plating efficiencies obtained throughout these studies rule out the possibility that the block to differentiation that we observe is due to a cytotoxic effect of the inhibitors employed. The fact that both puromycin and cycloheximide, two protein synthesis inhibitors with different mechanisms of action, affect commitment in a similar fashion supports the idea that protein synthesis is a necessary component of the early differentiation program and that the drug-induced block is not the result of a specific side effect of the drug itself.

Our understanding of the biochemical and molecular events underlying the control of developmental programs will depend on our ability to dissect the various components involved in such programs. The basic approach outlined in this paper can be applied in a variety of situations to identify the requirement for specific macromolecular events involved in the reprogramming of cellular function.

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