Specific pattern of gene expression during induction of mouse erythroleukemia cells

Peter J. Fraser and Peter J. Curtis

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104 USA

We have studied the expression of several characterized genes during induction of mouse erythroleukemia (MEL) cells with dimethyl sulfoxide (DMSO) and have observed a specific pattern of changes in transcriptional activity and steady-state RNA levels associated with erythroid differentiation. During induction there is a gradual, steady decrease in total transcriptional activity and RNA content per cell, which by day 3 of DMSO treatment amounts to less than 50% of the level in the uninduced cell. During this time we observe increases in transcriptional activity for 5-aminolevulinic acid synthase, carbonic anhydrase form II, and band 3 coordinate with the large increase in β -globin gene transcription. The results also demonstrate an early decrease in transcription for carbonic anhydrase form I, which precedes decreases in transcription for glyceraldehyde phosphate dehydrogenase and rRNA genes. Changes in steady-state RNA levels reflected changes in transcriptional activity during induction except for carbonic anhydrase II mRNA. These results represent the first report characterizing the regulated expression at transcriptional and posttranscriptional levels of several known genes that are characteristically expressed in the erythrocyte. The results demonstrate that coordinate gene expression in erythroid differentiation occurs primarily at the level of transcription.

[*Key Words:* Coordinated gene regulation; transcriptional activity; erythroid differentiation]

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Adult erythroid differentiation requires activation and/or modulation of many genes whose expression is regulated both temporally and quantitatively. The result is the generation of highly specialized cells whose functions and capabilities are defined by expression of specific gene products. Several of these genes are considered tissue specific, such as globin and glycophorin (Colin et al. 1986), in that their expression is restricted to cells of erythroid lineage. Others, such as the genes for carbonic anhydrase form I (CAI) (Tashian 1977) and erythroid forms of the anion transport protein, band 3 (Cox et al. 1985), and the cytoskeletal protein spectrin (Riederer et al. 1986), are limited in expression to a few tissues, but may still be considered erythroid specific. A third class of genes exist that are expressed in nearly all tissues but maintain erythroid-specific patterns of inducibility or modulation. Examples of this latter group are 5-aminolevulinic acid (ALA) synthase (Sassa 1976), the first enzyme in the heme biosynthetic pathway, and carbonic anhydrase form II (CAII) (Tashian 1977), which is involved in the reversible hydration of CO_2 . Both these genes are expressed in a variety of tissues, but their expression is specifically modulated during erythroid differentiation. Several of the gene products induced during erythroid differentiation, for example, the heme biosynthetic enzymes and α - and β -globin polypeptides, are mutually dependent for function and presumably require coordination of expression. Likewise, peripheral and integral membrane proteins must be expressed coor-

dinately such that they may assemble to form the characteristic erythrocyte cytoskeleton.

One approach to the study of gene expression in erythroid differentiation utilizes the mouse erythroleukemia (MEL) cell system. MEL cells are erythroid precursor cells transformed by the Friend virus complex. Upon addition of dimethyl sulfoxide (DMSO) or a variety of other agents, MEL cells undergo a series of biochemical and morphological changes that closely resemble the events of normal erythroid differentiation. Volloch and Housman (1982) have shown that under the proper culture conditions, subcloned, rapidly inducing MEL cells undergo changes in morphological characteristics similar to those of normal mouse orthochromatophilic erythroblasts and reticulocytes, resulting in terminal differentiation and enucleation. Among the observed changes are progressively smaller cells and increasingly condensed nuclei, leading to a reduction in RNA content and a decreased rate of synthesis of total RNA (Sherton and Kabat 1976). The most dramatic and oft noted change is the marked increase in the rate of transcription of α - and β -globin genes resulting in accumulation of globin mRNA and hemoglobin (Aviv et al. 1976; Curtis and Weissmann 1976; Nudel et al. 1977; Orkin and Swerdlow 1977; Lowenhaupt et al. 1978; Hofer et al. 1982). Also observed is an increase of newly synthesized carbonic anhydrase II mRNA (Curtis 1983) as well as increased synthesis of α - and β -spectrin (Eisen et al. 1977; Pfeffer et al. 1986), band 3 (Sabbat et al.

1980), band 4 (Pfeffer and Redman 1981), and other erythroid-specific polypeptides. Induction of the activities of the heme synthesizing enzymes ALA synthase, ALA dehydrase, uroporphyrinogen synthase, and ferrochelatase have also been reported (Sassa 1976).

In MEL cells, accumulation of globin mRNA and hemoglobin has been shown to result mainly from an increase in the rate of transcription of the globin gene (Ganguly and Skoultschi 1985). However, little or no information is available concerning the regulation of other genes expressed during erythroid differentiation. Our study attempts to identify regulatory steps in the expression of coordinately controlled genes by examining the expression of several genes involved in the specific pattern of gene activation and inactivation during MEL cell induction. Cloned cDNAs for mouse α -spectrin, band 3, CAI, CAII, globin, ALA synthase, and glyceraldehyde phosphate dehydrogenase (GAPD) and genomic sequences for ribosomal RNA were used to determine the changes in transcription rates and steady-state RNA levels of the respective genes. Our results demonstrate changes at the transcriptional level that are accompanied by changes in steady-state levels. ALA synthase, CAII, band 3, and globin show transcriptional increases at approximately the same time, suggesting the possibility of coordinate control of transcription of these genes.

Results

Changes in transcription rate during MEL cell induction

To assess changes in transcription rate for the various genes during induction, we employed in vitro nuclear run-on techniques. Nuclei were isolated from 10^8 cells from each time point: uninduced cells (day 0) and cells treated with 1.8% DMSO for 1, 2, and 3 days (days 1–3). [32 P]UTP was incorporated into nascent RNA via elongation and isolated for hybridization to immobilized plasmids containing cDNA inserts. Figure 1 displays the results of 32 P-labeled nuclear RNA, obtained from MEL cells from days 0–3, hybridized to plasmids containing cDNA inserts.

To obtain data that would indicate the change in transcription rate of a gene during induction, it is necessary to measure the transcriptional activity of a gene for a constant number of cells, rather than as a proportion of total transcriptional activity, which diminishes with time after induction. The amount of labeled RNA used in the hybridizations was equivalent to that obtained from an equal number of cells from each day. Our results are therefore proportional to the number of RNA molecules produced from a given gene per cell over unit time and indicate the change in the rate of synthesis of RNA at that locus for each day.

Previous studies have shown and our results confirm that induction of MEL cells with DMSO is accompanied by a decrease in transcriptional activity (Orkin and Swerdlow 1977; Patel and Lodish 1984). Transcriptional output, measured in terms of total yield of TCA-precipi-

Figure 1. Autoradiogram of in vitro transcription assay with isolated nuclei from MEL cells from days 0–3 of DMSO treatment. Four separate hybridization vessels were used, each of which contained a set of filters (A–H) and an equal proportion of the total yield of 32 P-labeled nuclear RNA from day 0, 1, 2, or 3. Filter I (rRNA) was hybridized separately with 1/22 as much [32 P]RNA as filters A–H. (A) α -Spectrin; (B) band 3; (C) CAI; (D) CAII; (E) globin; (F) ALA synthase; (G) GAPD; (H) rabies virus glycoprotein; (I) rRNA.

table counts, dropped greater than 50% from day 0 to day 3 (Fig. 2A).

As is characteristic of MEL cell induction, we observe a greater than 40-fold increase per cell in globin gene transcription. ALA synthase shows a parallel increase in transcription. For both these genes the largest increase occurred on day 2, with the transcription rate reaching its highest point on day 3. Increases in transcription are also seen for carbonic anhydrase form II (CAII) and band 3. CAII also shows its largest increase in transcription on day 2 before decreasing on day 3.

Transcription of rRNA and GAPD decreased during induction, with the most significant reductions occurring between days 1 and 2, falling 60% and 80%, respectively. CAI transcription was highest in the uninduced MEL cell, followed by a rapid decrease of nearly 50% in the first 24 hr of induction. CAI transcription levels continued to decline to near background levels by day 3. A graphic representation of the actual number of counts bound after correcting for background is shown in Figure 2. In all our transcription experiments with MEL cells, the signals obtained for α -spectrin were not significantly above those of the negative control plasmid pRG, which contained a cDNA for the rabies virus glycoprotein. The

Figure 2. Graphic representation of changes in transcription rate for the various genes, as determined by in vitro transcription assay with isolated nuclei. Each filter from Fig. 1 was counted separately in scintillation fluid before mounting for autoradiography. The values shown above indicate ^{32}P counts per minute bound to each filter after correcting for background hybridization by subtracting the value of the negative control plasmid pRG (rabies glycoprotein) for each day. (A) (○) Total transcription; (●) rRNA. (B) (◆) GAPD; (◇) globin. (C) (▲) CAII; (△) CAI; (□) ALA synthase; (■) band 3.

results obtained for the remaining genes were consistently reproducible.

Changes in steady-state RNA levels during MEL cell induction

Steady-state RNA levels were assayed during MEL cell induction through analysis of total RNA isolated from MEL cells on days 0–3. Since MEL cells undergo sharp

reductions in cell volume and RNA content during induction, results obtained assaying equal amounts of total RNA for days 0–3 would indicate the change in the fraction or percentage of total RNA that contained sequences specific for our probes. To obtain results that would indicate the average change in the level of a mRNA on a per-cell basis, we used an equal number of cells from each day and modified our total-RNA preparation procedure to correct for variable recovery by adding an internal standard. Briefly, 10^8 cells were harvested from each time point, and upon lysis in SDS, 2×10^5 cpm of ^3H RNA was added to each sample. Purification of total RNA proceeded as previously described. The yield of total RNA from each day was corrected for variable recovery by quantitation of ^3H counts, and the amount of RNA in 10^8 cells was determined. The range of recovery was 65–75%. Our results indicate a 50% decrease in the average amount of total RNA per cell from day 0 to day 3.

Total RNA from approximately 7×10^5 cells from each day (i.e., equal amounts of ^3H counts) were electrophoresed in formaldehyde-containing agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P -labeled cDNA probes. A dramatic increase in the steady-state level of globin mRNA, which is characteristic of MEL cell induction, is seen in Figure 3. ALA synthase mRNA levels also increase per cell, in parallel with globin. CAII and GAPD show minor reductions by day 3 of induction, whereas CAI is dramatically reduced from the level in the uninduced cell. Band 3 mRNA was below detectable levels in MEL total RNA but was readily detectable in preparations of mouse anemic spleen total RNA (data not shown). The changes in steady-state levels noted above were consistently observed.

Discussion

We have determined the changes in transcriptional activity and steady-state RNA levels for a number of characterized genes, in addition to globin, on a per-cell-average basis using DMSO-inducible MEL cells as an erythropoietic model system. Induction is most notably characterized by a marked increase in globin gene transcription and accumulation of globin mRNA as has been reported by a number of independent investigators (Aviv et al. 1976; Curtis and Weissmann 1976; Nudel et al. 1977; Orkin and Swerdlow 1977; Lowenhaupt et al. 1978; Hofer et al. 1982). Our results show that even though the total transcriptional output of the cell is drastically reduced during induction, globin gene transcription increases greater than 40-fold. In addition, our findings demonstrate concomitant increases in the transcription rates of CAII, ALA synthase, and band 3 genes with the largest increases occurring on day 2 or 3. There is an early decrease in transcription for CAI within the first 24 hr of induction, and large decreases in transcription for GAPD and rRNA genes on day 2.

Through analysis of total RNA and comparison with our transcription data we were able to correlate changes in the transcription rates of the various genes with

Figure 3. Northern blot analysis of total RNA from MEL cells from days 0–3 of DMSO treatment. Total RNA from $\sim 7 \times 10^5$ cells from the indicated days (0–3) was loaded per lane and probed with 2×10^6 cpm to 7×10^6 cpm of ^{32}P -labeled cDNA and exposed as follows. (A) CAI 28 hr; (B) globin 28 hr; (C) CAII 96 hr; (D) ALA synthase 72 hr; (E) GAPD 4 hr. Markers are 28S and 18S rRNA.

changes in steady-state levels of mRNA during induction. For CAI, globin, ALA synthase, and GAPD, changes in the transcriptional activity of the gene were accompanied by a similar increase or decrease in the steady-state level of mRNA for that gene. The exceptions were CAII and band 3. In the case of CAII, transcriptional activity increases six- to eightfold during induction; however, CAII steady-state RNA levels decrease somewhat over the same period. We did detect a small increase in band 3 gene transcription during induction, which correlates with the appearance of the band 3 polypeptide (Sabban et al. 1980; Pfeffer and Redman 1981; Patel and Lodish 1984), but band 3 mRNA was not detectable through analysis of total MEL RNA. Band 3 gene transcription and mRNA were readily detectable in transcription assays and total RNA preparations from mouse anemic spleen (data not shown). Although comparison is difficult due to the heterogeneous and changing cell population of the anemic spleen, this result may reflect a quantitative difference in band 3 expression between the MEL cell system and the mouse anemic spleen (Sabban et al. 1981).

Much is known about the transcription and kinetics of globin mRNA and polypeptide accumulation during MEL cell induction. Much less is known about the regulated expression of nonglobin gene products. ALA synthase activity has been shown to increase in MEL cells during induction with DMSO. Ebert and Ikawa (1974) and Sassa (1976) reported a steady increase in ALA synthase activity during induction. The results of Rutherford et al. (1979) indicated that ALA synthase activity increases on the first day of DMSO treatment and then decreases and levels out. Care must be taken in comparing these results since different MEL cell lines were used. Our studies show that ALA synthase gene transcription, in parallel with that of globin, increases during induction, with the most significant increase occurring between days 1 and 2. ALA synthase steady-state

RNA levels increase accordingly. These data would support the results of Ebert and Ikawa (1974) and Sassa (1976) and suggest that ALA synthase expression is controlled primarily at the level of transcription, as is globin. However, we cannot rule out the possibility of translational or posttranslational steps in control of expression, especially in light of the results of Rutherford et al. (1979).

CAI gene transcription decreases during MEL cell induction as does the level of CAI steady-state mRNA. Stern et al. (1977) showed that when measurements are expressed as a percentage of soluble protein, CAI protein levels appear unchanged during MEL cell induction and CAII levels increase. A previous study (Sherton and Kabat 1976) demonstrated that induction of MEL cells is characterized by a 30–40% decrease in protein content per cell by day 4. Taken together, these results suggest that the amount of CAI protein per cell decreases. Our results for CAI would support this interpretation of reduced CAI expression per cell during induction due to decreased rate of transcription and steady-state RNA level. Studies on human CAI indicate that it is a specific marker of early normal human erythroid differentiation (Villevall et al. 1985). Our results suggest that CAI may also be a marker for early erythroid differentiation in the mouse, since it is transcribed primarily in the uninduced cell and appears to be downregulated early during induction. The results for CAII are more difficult to interpret. As mentioned above, during induction CAII gene transcription increases six- to eightfold per cell, the CAII steady-state RNA level decreases slightly, whereas the percentage of CAII in soluble protein increases approximately twofold by the fourth day of DMSO treatment (Stern et al. 1977). Different posttranscriptional events could be invoked to explain these observations, e.g., changes in mRNA translatability and mRNA half-life during induction (Yenofsky et al. 1983; Krowczynska et al. 1985). However, these changes are unique to CAII ex-

pression; they do not appear to be involved in the coordinate regulation of globin, band 3, and ALA synthase genes, which occurs primarily at the level of transcription.

GAPD and rRNA gene transcription decrease during MEL cell induction, as does the level of steady-state GAPD mRNA. Both these genes have been shown to be constitutively expressed in all tissues. The decreased expression of these genes reflects the terminally differentiating state of the MEL cells. Previous studies have shown, and our studies confirm, that induced MEL cells contain only 50–60% as much RNA as control cells (Sherton and Kabat 1976). In addition, the total transcriptional output, measured in terms of yield of nascent ³²P-labeled RNA, decreases steadily during induction, approaching 50–60% of the level of the uninduced cell. Studies of erythroid cells have shown that RNA synthesis declines throughout erythropoiesis as the cells become progressively smaller and their nuclei increasingly condensed. Denton et al. (1975) have shown that the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, adenosine deaminase, and nucleoside phosphorylase exhibit a steep decline as erythroid cells complete their final cell division. The reduced transcription for GAPD and rRNA may be part of the overall program of reduced expression of these and other genes not directly involved in the terminal stages of erythroid differentiation.

We have demonstrated that, in addition to the transcriptional activation of the β -globin gene that occurs upon induction of MEL cells, increases in the rate of transcription occur on the CAII, ALA synthase, and band 3 genes. The CAI gene is presumably activated transcriptionally at an earlier stage of erythropoiesis than is represented by the MEL cell and is downregulated upon induction. These results demonstrate the role of transcriptional regulation during erythroid differentiation and pose two questions central to understanding the molecular basis of differentiation: Is the coordinated activation of these genes determined by common *cis*-acting and/or *trans*-acting factors, and is there a cascade of steps which ensures that expression of the CAI gene precedes that of globin? Our studies of the elements and factors responsible for the regulation of transcription of these genes are aimed at answering these very important questions.

Methods

Recombinant plasmids

The following plasmids containing cDNA inserts were used in this study: pMaSp, mouse α -spectrin (pB129) (Cioe and Curtis 1985); pMEB3-18, mouse erythrocyte band 3 (Demuth et al. 1986); pMCAI, mouse carbonic anhydrase form I (Fraser and Curtis 1986); pMCAII, mouse carbonic anhydrase form II (Curtis 1983); pM β G, mouse β -major globin; pMS20, mouse ALA synthase (Schoenhaut and Curtis 1986); pGAPD, mouse glyceraldehyde phosphate dehydrogenase (unpubl.); pRG, rabies virus glycoprotein (Anilionis et al. 1981). The plasmid pABE was a gift from Dr. R.D. Schmickel and contains genomic sequences for human 28S rRNA (Erickson et al. 1981).

Cells and culture conditions

The MEL cell line C-3 was the generous gift of Dr. D. Parker and was subcloned and maintained as previously described (Parker and Housman 1985). Cells were induced by addition of 1.8% DMSO to the culture medium.

Isolation of nuclei

Nuclei were prepared based on the method of Weintraub and Groudine (1976). Briefly, 10^8 cells were pelleted and resuspended in 4 ml of ice-cold reticulocyte standard buffer (10 mM Tris pH 7.5; 10 mM NaCl; 3 mM MgCl₂) containing 0.1% Triton mM dithiothreitol, and 0.1 M sucrose. The cells were lysed with 12 strokes of an all-glass B type dounce (Kontes) and diluted with an equal volume of the same solution containing 0.25 M sucrose. The crude homogenate was then layered over 0.5 volumes of 0.33 M sucrose, 5 mM MgCl₂, 10 mM Tris pH 8.0, 0.5 mM dithiothreitol, and the nuclei were pelleted at 800g for 5 min at 4°C.

In vitro nuclear transcription assay

Transcription in isolated nuclei and isolation of ³²P-labeled nuclear RNA was performed essentially as described by Groudine et al. (1981) with the exception that [³²P]UTP of 3000 Ci/mmol was used. Spot blotting of plasmid DNA onto nitrocellulose was performed as follows. Five micrograms of linearized plasmid DNA was denatured for 5–10 min in 0.2 N NaOH on ice and then neutralized with an equal volume of 1.4 M Tris pH 3.5, 6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M Na citrate) and spotted immediately with a Schleicher and Schuell spot blotter. After baking the spotted nitrocellulose at 80°C under vacuum for 2 hr, the spots were cut out with a paper punch and prehybridized for 3–5 hr at 65°C in 0.3 ml prehybridization buffer according to Hofer and Darnell (1981). Hybridizations were performed in 0.3 ml of the same buffer for 36–48 hr at 65°C. The number of counts used in the hybridizations was proportional to that obtained from the nuclei from 10^8 cells from days 0–3 and ranged from 6×10^6 cpm to 2.4×10^6 cpm, respectively. Each step of the purification procedure was monitored to insure no significant loss of labeled RNA. RNA was partially degraded by treatment with 0.2 N NaOH for 80 min on ice prior to hybridization (Jelinek et al. 1974). After hybridization, the filters were rinsed three times in 0.5 M NaCl, 10 mM Tris pH 7.5 at room temperature, and single-stranded RNA was digested according to the method of Wall and Darnell (1971) in 1 ml of the same solution containing 120 μ g/ml RNase A at 37°C for 30 min. Filters were then washed twice in 2 $\times$ SSC, 0.1% sodium dodecyl sulfate (SDS) for 20 min and once in 0.5 $\times$ SSC, 0.1% SDS for 30 min at 52°C. Filters could then be counted in scintillation fluid and mounted for exposure on Kodak XAR-5 film with intensifying screens at –70°C.

Analysis of steady-state RNA

Total RNA was extracted from uninduced and induced MEL cells as described by Curtis and Weissmann (1976) and electrophoresed on formaldehyde-containing 1% agarose gels (Maniatis et al. 1982). RNA was then transferred to nitrocellulose, baked under vacuum for 2 hr at 80°C, prehybridized for 2 hr, and hybridized for 20 hr in roller bottles at 50°C in 5 $\times$ SSC, 0.08% Ficoll, 0.08% polyvinylpyrrolidone, 0.08% BSA, 8 μ g/ml poly(A), 100 μ g/ml sheared and denatured salmon sperm DNA. After hybridization, the filters were washed twice in 2 $\times$ SSC, 0.1% SDS for 20 min and once in 0.1 $\times$ SSC, 0.1% SDS for 30 min at 65°C and exposed to Kodak XAR-5 film with intensifying screens at –70°C.

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