Expression of ferrochelatase mRNA in erythroid and non-erythroid cells

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Ferrochelatase, which catalyses the last step in haem biosynthesis, i.e. the insertion of Fe(II) into protoporphyrin IX, is present in all cells, but is particularly abundant in erythroid cells during haemoglobinization. Using mouse ferrochelatase cDNA as a probe two ferrochelatase transcripts, having lengths of 2.9 kb and 2.2 kb, were found in extracts of mouse liver, kidney, brain, muscle and spleen, the 2.9 kb transcript being more abundant in the non-erythroid tissues and the 2.2 kb transcript more predominant in spleen. In mouse erythroleukemia cells the 2.9 kb ferrochelatase transcript is also more abundant; however, following induction of erythroid differentiation by dimethyl sulphoxide there is a preferential increase in the 2.2 kb transcript, which eventually predominates. With mouse reticulocytes, the purest immature erythroid cell population available, over 90% of the

total ferrochelatase mRNA is present as the 2.2 kb transcript. Since there is probably only one mouse ferrochelatase gene, the occurrence of two ferrochelatase transcripts could arise from the use of two putative polyadenylation signals in the 3' region of ferrochelatase DNA. This possibility was explored by using a 389 bp DNA fragment produced by PCR with synthetic oligo-primers having sequence similarity with a region between the polyadenylation sites. This fragment hybridized only to the 2.9 kb ferrochelatase transcript, indicating that the two transcripts differ at their 3' ends and suggesting that the 2.2 kb transcript results from the utilization of the upstream polyadenylation signal. The preferential utilization of the upstream polyadenylation signal may be an erythroid-specific characteristic of ferrochelatase gene expression.

INTRODUCTION

Haem synthesis, which occurs in almost all types of cells, is a multi-step process. In higher organisms it involves four cytosolic and four mitochondrial enzymes where the steps are identical but the rates differ dramatically from cell to cell. In immature erythroid cells, which have the highest rates of haem formation, several erythroid-specific mRNAs for haem-pathway enzymes have been found. However, there does not appear to be a general pattern defining erythroid as compared with non-erythroid enzymes at the gene, mRNA or regulatory levels.

In spite of earlier evidence of erythroid-specific isoenzymes (Bishop et al., 1981; Yamamoto et al., 1986) of δ -aminolevulinic acid synthase (ALAS; EC 2.3.1.37), the first enzyme in the haem pathway, it was originally claimed (Elferink et al., 1987) that there is a single ALAS mRNA species in all tissues. However, it is now established that there are erythroid and ubiquitous, or 'housekeeping', forms of ALAS encoded by distinct genes (Riddle et al., 1989; Schoenhaut and Curtis, 1989; Dierks, 1990). No tissue-specific isoenzyme is known for δ -aminolevulinic acid dehydratase (ALAD; EC 4.2.1.24, the second enzyme in the haem pathway) (Bishop et al., 1989). Until recently it was believed that there is only one kind of ALAD transcript; however, Bishop et al. (1989) recently cloned ALAD cDNAs from mouse liver and erythroid cells and showed that although the coding regions of mRNAs from both tissues are identical, there are subtle differences in 5' untranslated regions of erythroid and 'housekeeping' ALAD mRNAs, the production of which is controlled by different promoters (Bishop et al., 1991).

Porphobilinogen deaminase (EC 4.3.1.8), the third enzyme of the haem pathway, exists in two isoforms, one present in all cells and the other only in erythroid cells. They are translated from two mRNAs that differ solely in their 5' end but are encoded by a single structural gene (Grandchamp et al., 1987; Chretien et al.,

1988). These two mRNAs arise from two overlapping transcription units. The first (upstream) is active in all tissues and its promoter has some structural features of a housekeeping promoter. The second, active only in erythroid cells, contains two *cis*-acting sequences that bind erythroid factors GATA-1 and nuclear factor-erythroid 2 (NF-E2) that are also known to interact with the β -globin promoter (Miguotte et al., 1989). Importantly, erythroid ALAS (eALAS) (Cox et al., 1991) and ALAD (Bishop et al., 1991) promoters also show sequence similarities with the β -globin gene promoters.

These differences in genes and/or mRNAs may partly explain the differences in regulation and rates of haem synthesis in erythroid and non-erythroid cells. In non-erythroid cells the rate-limiting step of haem production is ALAS, the synthesis and perhaps also activity of which is feedback-inhibited by haem (Granick and Urata, 1963; Granick et al., 1975). On the other hand, in erythroid cells haem does not inhibit either activity (Neuwirt et al., 1969; Woods, 1974) or synthesis (Granick and Sassa, 1978) of ALAS, but does inhibit cellular iron acquisition from transferrin (Ponka and Neuwirt, 1969; Schulman et al., 1974) without affecting its utilization for haem synthesis (Ponka and Schulman, 1985a). Transferrin is the only source of iron for haemoglobin and its availability limits haem- (Ponka and Schulman, 1985b; Laskey et al., 1986) and consequently haemoglobin-synthesis rates, since haem is required for globin mRNA translation (Bruns and London, 1965; Zucker and Schulman, 1968). Moreover, since the 5' untranslated region (UTR) of eALAS mRNA contains an iron-responsive element (Dierks, 1990; Cox et al., 1991; Dandekar et al., 1991), a *cis*-acting sequence responsible for translational induction of ferritin by iron (Kühn et al., 1990; Munro et al., 1990), the availability of the metal may control ALAS expression in haemoglobin-synthesizing cells. In contrast, in non-erythroid cells the removal of iron from transferrin is not regulated by intracellular haem

Abbreviations used: MEL, mouse erythroleukemia; FeSIH, iron salicylaldehyde isonicotinoylhydrazone; UTR, untranslated region; ALAS, δ -aminolevulinic synthase; eALAS, erythroid ALAS; ALAD, δ -aminolevulinic acid dehydratase; DMSO, dimethyl sulphoxide; SSPE, 0.15 M NaCl, 0.01 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 0.001 M sodium EDTA.

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(Schulman et al., 1981), iron availability does not seem to control the overall haem-synthesis rate, and ubiquitous ALAS mRNA does not contain the iron-responsive element.

With regard to the further steps of haem synthesis, cDNAs for both uroporphyrinogen synthase (EC 4.2.1.75) (Tsai et al., 1988) and uroporphyrinogen decarboxylase (EC 4.1.1.37) (Romeo et al., 1986), the fourth and fifth haem-pathway enzymes, have been isolated, but there is no evidence that the hepatic and erythroid enzymes are different. Neither coproporphyrinogen oxidase (the sixth enzyme) nor protoporphyrinogen oxidase (the seventh enzyme) have yet been cloned, but both enzymes were proposed to have a unique pattern of regulation during erythroid differentiation (Conder et al., 1991).

cDNAs encoding mouse (Taketani et al., 1990; Brenner and Frasier, 1991) and human (Nakahashi et al., 1990) ferrochelatase, the last enzyme of the haem pathway, that inserts Fe^{2+} into protoporphyrin IX were recently cloned. The aim of the present study was to explore the possibility that there may be an erythroid-specific pattern for ferrochelatase mRNA expression *in vivo* and to examine the regulation of ferrochelatase gene expression during erythroid differentiation *in vitro* of mouse erythroleukemia (MEL) cells. MEL cells are a useful model since, upon addition of an inducer such as dimethyl sulphoxide (DMSO), the cells differentiate to synthesize haemoglobin with a concomitant loss of proliferative capacity; such changes are reminiscent of terminal differentiation of erythroblasts (Friend et al., 1971). The differentiation programme is characterized by an increase in the levels of enzymes of the haem biosynthetic pathway (Sassa, 1976), an increase in transferrin-receptor expression (Hu et al., 1977; Wilczynska et al., 1984) and iron uptake (Laskey et al., 1986), and an induction of globin-gene expression (Ross and Sautner, 1976).

MATERIALS AND METHODS

Materials

Molecular-biology-grade chemicals were purchased from Sigma and additional chemicals were purchased from BDH. ^{32}P -labelled nucleotides were obtained from Dupont-New England Nuclear. BALB/c mice used in this study were retired breeders. Plasmids containing β -globin cDNA were from Dr. W. French Anderson (NIH, Bethesda, MD, U.S.A.), mouse transferrin receptor cDNA from Dr. L. M. Neckers (NIH, Bethesda, MD, U.S.A.), ferrochelatase cDNA from Dr. S. Taketani (Kansai Medical University, Osaka, Japan) and eALAS cDNA from Dr. P. Curtis (The Wistar Institute, Philadelphia, PA, U.S.A.).

Reticulocyte preparation

Enhanced production of mouse reticulocytes was induced by phenylhydrazine. BALB/c mice (20 g body wt.) were subcutaneously injected daily with 0.12 ml of 1% (w/v) phenylhydrazine (pH 7.4) for three consecutive days. On the fourth day, blood was drawn, centrifuged to remove plasma and buffy coat leucocytes, and the cells were washed exhaustively with saline.

Cell culture

MEL cells (clone 745A) were grown in minimal essential medium supplemented with 10% (v/v) fetal-calf serum. To induce erythroid differentiation, cells at an initial cell density of $1 \times 10^6/\text{ml}$ were grown for 4 days in medium containing 1.5% (v/v) DMSO. For uninduced controls, cells were seeded at a density of $0.75 \times 10^6/\text{ml}$ in culture medium without DMSO. To determine mRNA stability, 5 μM actinomycin D was added on

the third day of cell culture. Mouse reticulocytes were kept in RPMI medium supplemented with 20% (v/v) fetal-calf serum at a final haematocrit reading of 20–30%, conditions under which no cell lysis occurred within 8 h.

RNA isolation

Total RNA was isolated according to Chromczynski and Sacchi (1987). Fresh mouse tissues were rinsed with normal saline and then quickly frozen in liquid nitrogen for subsequent mincing on ice. Cultured cells were washed once with sterile PBS. Acid guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol) was then added to the minced tissues for homogenization, or to the cell pellets for lysis. To the tissue homogenates or cell lysates, 0.1 $\times$ vol. of 2 M sodium acetate (pH 4) was added followed by an equal volume of water-saturated phenol and then 0.2 vol. of chloroform/isoamyl alcohol (49:1). After 30 min on ice, the mixture was spun for 30 min at 4 °C in a microfuge. The aqueous layer was transferred to a fresh tube, mixed with an equal volume of propan-2-ol and placed at -20 °C for from 1 h to overnight for RNA precipitation. RNA was collected by centrifugation for 20 min at 4 °C and was dissolved in the acid guanidinium thiocyanate solution and mixed with an equal volume of propan-2-ol for the second precipitation at -20 °C for at least 1 h. The final RNA pellet was collected by centrifugation at 4 °C for 10 min, washed once with 75% (v/v) ethanol and dried under vacuum. For Northern-blot analyses, the pellets were dissolved in 0.5% SDS at 65 °C for 10 min.

Northern-blot analysis

Identical amounts (15 μg) of RNA samples were denatured by heating at 95 °C for 2 min in 7–8 μl of RNA loading buffer containing 50% (v/v) deionized formamide, 6.5% (v/v) formaldehyde, 5% (w/v) Bromophenol Blue and 5% (v/v) glycerol in $1 \times$ Mops buffer [0.02 M Mops (pH 7), 5 mM sodium acetate and 1 mM EDTA]. Denatured RNA samples were mixed with 0.5 μl (2 $\mu\text{g}/\text{ml}$) of ethidium bromide before electrophoresis in an agarose gel (1% in $1 \times$ Mops buffer) containing 2% (v/v) formaldehyde. The amounts of RNA in each sample were the same as assessed by ethidium bromide staining. After electrophoresis, RNA was transferred to nitrocellulose paper by a vacuum or capillary-blotting method. The filters were then air-dried, and baked in a vacuum oven at 80 °C for 2 h. Hybridizations were carried out as described by Meinkoth and Wahl (1984) at 42 °C in hybridization solutions [50% (v/v) formamide, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, 0.1% BSA, 0.1% SDS, $5 \times$ SSPE ($1 \times$ SSPE is 0.15 M NaCl, 0.01 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 0.001 M EDTA, sodium salt), 10% (w/v) dextran sulphate, and 200 $\mu\text{g}/\text{ml}$ of denatured herring sperm DNA] containing ^{32}P -labelled probes prepared by nick-translation or a random-primed reaction. The filters were washed according to the method of Maniatis et al. (1982).

Preparation of DNA fragment by PCR

cDNA libraries for mouse reticulocytes in $\lambda\text{gt}11$ vector obtained from Dr. Terry Rogers Bishop and for mouse spleen λzap vector were amplified and extracted as described (Maniatis et al., 1982). Purified DNAs were used as templates for PCR.

Two 24-oligonucleotide primers were synthesized based on the ferrochelatase cDNA sequence reported by Taketani et al. (1990). The sequence of the 5' primer is 5'-AGTTGACCTGTGTCT-

GATAGCCTT-3' which corresponds to positions 2263–2287 of ferrochelatase cDNA and that of the 3' primer is 3'-GGG-AGTATTGATTAAGAACGGACT-5' which corresponds to positions 2628–2652.

The reaction mixture for PCR contained 0.5 μ M each of the four deoxynucleoside triphosphates, 50 pmol each of the primers, 2 μ g of template DNA and 1.5 units of TAQ polymerase (Bio/Can Scientific Inc.) in 100 μ l of TAQ buffer. PCR was performed in a DNA thermal cycler beginning with denaturation at 90 °C for 3 min and then for 30 to 40 cycles of denaturation at 94 °C for 1 min, renaturation at 52 °C for 1 min and polymerization at 72 °C for 1 min. The PCR-amplified product was electrophoresed in 1% (w/v) agarose gel, visualized by ethidium bromide staining, and extracted for random-primed labelling.

RESULTS

Northern-blot analyses of mouse tissue and MEL-cell RNA

Total RNA was isolated from mouse liver, spleen, brain, muscle, kidney and reticulocytes and uninduced and induced MEL cells, for Northern-blot analyses using two probes; ferrochelatase cDNA and a 389 bp DNA fragment generated by PCR and corresponding to a region at the 3' end of the 2.9 kb cDNA sequence described by Taketani et al. (1990). The results of these analyses are presented in Figure 1.

Figure 1 Ferrochelatase mRNA in mouse tissues and MEL cells

RNA samples were collected from different mouse tissues (liver, spleen, brain, muscle, kidney and reticulocytes) and uninduced and induced MEL cells, 15 μ g of total RNA was electrophoresed on 1% (w/v) agarose-formaldehyde gel, transferred to a nitrocellulose filter, and probed with 32 P-labelled ferrochelatase cDNA (a and b) and 32 P-labelled PCR fragment (c).

Two transcripts of 2.9 kb and 2.2 kb were detected by using ferrochelatase cDNA as a probe. In spite of the presence of both transcripts in all tissues studied, spleen contained more of the 2.2 kb transcript than the 2.9 kb transcript (Figure 1a). Since spleen is one of the erythropoietic tissues in mice, this observation suggests that the smaller transcript may be related to erythroid differentiation.

To explore this possibility the RNA from reticulocytes and from uninduced and induced MEL cells was analysed. Figure 1(b) shows the autoradiograph after probing with the radio-labelled ferrochelatase cDNA. The results could be summarized as follows: (i) In uninduced MEL cells, the 2.9 kb transcript was more abundantly expressed than the 2.2 kb transcript whereas an opposite pattern was found in the spleen. (ii) The band pattern in haemoglobin-synthesizing induced MEL cells was completely different from the uninduced cells. About 50% of the total ferrochelatase mRNA was contributed by the 2.2 kb transcript. (iii) Most striking of all, mouse reticulocytes contained predominantly the 2.2 kb transcript. Although there was a band which corresponded to the 2.9 kb transcript, the signal was so weak that it might actually have come from remnants of leucocytes in the washed red blood cells. On the other hand, if it represented authentic transcript from reticulocytes, the percentage of it was so low compared with the 2.2 kb transcript that its relative importance could be considered minimal.

The reported sequence for ferrochelatase cDNA has two putative polyadenylation signals, which may explain the presence of the two transcripts (Taketani et al., 1990). We addressed this tissue directly by determining whether the two transcripts differ at their 3' ends. Filters from the previous set of experiments were reprobbed with a 389 bp DNA fragment generated by PCR which corresponds to a region between the putative upstream and downstream polyadenylation signals of the full-length ferrochelatase cDNA. As shown in the autoradiograph in Figure 1(c), this probe hybridized only with the 2.9 kb transcript, demonstrating clearly that some part at the 3' end of the 2.9 kb transcript was not present in the shorter 2.2 kb transcript.

Ferrochelatase, eALAS, transferrin receptor and β -globin mRNA levels during MEL-cell differentiation

MEL cells were induced to differentiate using 1.5% (v/v) DMSO. The cells gradually lose their capacity to proliferate as they start synthesizing large amounts of haemoglobin by 3 days after induction. In 4 days, over 90% of the cells synthesize large quantities of haemoglobin. Total cellular RNA was isolated from MEL cells at various times after induction and examined by Northern-blot analyses using cDNA probes for ferrochelatase, eALAS, transferrin receptor and β -globin. The results in Figure 2 show that the levels of all the mRNAs examined increase during MEL-cell differentiation. Transferrin-receptor mRNA levels increase dramatically between 48 h and 72 h after induction and similar trends were observed with eALAS (Elferink et al., 1988) and β -globin RNA (Ross and Sautner, 1976).

In both uninduced and induced MEL cells, we detected two species of ferrochelatase transcripts (Figure 2a) which correspond to 2.2 kb and 2.9 kb, as estimated by their positions relative to 18 S (2 kb) and 28 S (5 kb) ribosomal RNA. Before induction, MEL cells contain mostly the 2.9 kb transcript. However, upon DMSO induction, the 2.2 kb transcript increases by more than 5-fold while the 2.9 kb transcript increases by only about 2-fold. As measured by scanning laser densitometry, both transcripts in fully induced MEL cells were equally abundant.

Figure 2 Changes in eALAS, ferrochelatase, transferrin receptor and β -globin mRNA levels during MEL-cell differentiation

MEL cells were induced with 1.5% (v/v) DMSO for the indicated time periods. Samples of 15 μ g of total RNA, from each time interval, were electrophoresed in 1% (w/v) agarose-formaldehyde gel and transferred to nitrocellulose paper. Identical amounts of samples were assured by the same actin mRNA levels and ethidium bromide staining. The nitrocellulose papers were hybridized with 32 P-labelled cDNAs for (a) ferrochelatase, (b) transferrin receptor, (c) β -globin, and (d) eALAS, washed and exposed to X-ray film as described in the Materials and methods section.

Figure 3 Ferrochelatase mRNA stability in uninduced and induced MEL cells

MEL cells were grown in the absence or presence of 1.5% (w/v) DMSO for 3 days when the cultures were split into halves for further incubation with or without 5 μ M actinomycin D. RNA was extracted at 2 h intervals and Northern-blot analyses were performed with the ferrochelatase 32 P-labelled cDNA probe. (a) Untreated uninduced cells; (b) uninduced cells treated with actinomycin D (N.B. the intensities of signals at zero time in samples (a) and (b) were identical but the sample (b) was overexposed to enable an estimation of RNA decay rates); (c) untreated induced cells; and (d) induced cells treated with actinomycin D.

Ferrochelatase mRNA stability in uninduced and induced MEL cells

To examine whether there is any change in ferrochelatase mRNA stability before and after MEL-cell induction, actinomycin D was added to uninduced and induced cells on day 3 and total RNA was collected at time intervals and probed with ferrochelatase cDNA. Figure 3 shows the autoradiographs of the Northern-blot analyses. Ferrochelatase mRNA levels remained almost unchanged in uninduced MEL cells from day 3 to day 4 (Figure 3a). Induced cells accumulated more of the mRNA, with the 2.2 kb transcript representing at least 50% of the total as differentiation continued (Figure 3c); this is consistent with the results in Figure 2(a). In the presence of actinomycin D, which inhibits the synthesis of new RNA transcripts, ferrochelatase

Figure 4 Graphical representation of densitometric values for ferrochelatase mRNA levels in actinomycin D-treated uninduced and induced MEL cells

The results are expressed as percentage of the zero-time intensity for the 2.9 kb transcript in uninduced cells (□) and induced cells (■); and for the 2.2 kb transcript (○) in induced cells.

Figure 5 Ferrochelatase mRNA stability in mouse reticulocytes

At 2 h intervals, total RNA was extracted from reticulocytes which had been incubated at (a) 4 °C and (b) 37 °C *in vitro*. Each sample (15 μ g) was subjected to Northern-blot analysis using ferrochelatase 32 P-labelled cDNA probe.

mRNA disappeared within 8 h in uninduced MEL cells (Figure 3b). In contrast, both ferrochelatase transcripts were considerably more stable in induced cells (Figure 3d). Arbitrary values for the ferrochelatase mRNA levels were determined by scanning the autoradiographs with a laser densitometer. As shown in Figure 4, the half-life of the 2.9 kb transcript was about 4 h in the uninduced cells, but increased to more than 8 h in the induced cells. The 2.2 kb transcript, which could only be detected in induced MEL cells, exhibited a half-life of more than 10 h.

Ferrochelatase mRNA stability in mouse reticulocytes

To determine how stable the 2.2 kb transcript was in mouse reticulocytes, cells were incubated at 4 °C and 37 °C *in vitro* and their total RNA was extracted at various times for Northern-blot analyses. Since reticulocytes do not contain nuclei, RNA degradation could be monitored by simply keeping the cells at 37 °C without adding actinomycin D, while incubation of the cells at 4 °C would serve as control. As shown in Figure 5, the 2.2 kb erythroid transcript appeared to be very stable at both temperatures. Using densitometer scanning, we estimated that the half-life of the message in reticulocytes was longer than 8 h, which agreed with our findings with induced MEL cells.

Figure 6 Effects of haemin, FeSIH, and desferrioxamine on ferrochelatase mRNA in uninduced and induced MEL cells

Cells grown with or without 1.5% (w/v) DMSO were treated with different agents. RNA was collected for Northern-blot analysis as described in the text. (a) Lane 1, untreated uninduced control; lane 2, uninduced cells treated with 200 μ M FeSIH for 4 days; lane 3, uninduced cells (72 h culture) treated with 100 μ M desferrioxamine for 18 h; lane 4, untreated induced control; lane 5, induced cells treated with 200 μ M FeSIH for 4 days; lane 6, induced cells (72 h culture) treated with 100 μ M desferrioxamine for 18 h. (b) Lane 1, untreated uninduced cells; lane 2, untreated induced cells; lane 3, uninduced cells (72 h culture) treated with 50 μ M haemin for 18 h; lane 4, induced cells (72 h culture) treated with 50 μ M haemin for 18 h.

Effects of iron, desferrioxamine, and haemin on ferrochelatase mRNA expression in MEL cells

We attempted to examine the effects of iron on ferrochelatase mRNA expression; however, the results were inconclusive. As shown in Figure 6, four days of incubation of cells with iron salicylaldehyde isonicotinoylhydrazone (FeSIH), which is an effective donor of iron for haemoglobin synthesis in MEL cells (Laskey et al., 1986), increased ferrochelatase mRNA levels in uninduced cells but had little effect on induced cells. Desferrioxamine, a strong iron chelator, is cytotoxic, so cells could be incubated with it for only 18 h and as shown in Figure 6(a) it had only a slight inhibitory effect on ferrochelatase mRNA levels in both induced and uninduced cells. Haemin increased the levels of ferrochelatase mRNA in uninduced cells, which is not surprising since haemin is known to be an inducer of MEL-cell differentiation. Since induced MEL cells probably show maximum expression of components of erythroid differentiation, addition of haem would not be expected to cause further increases. Taken together the results suggest that induction of MEL-cell differentiation *per se* causes an increase in ferrochelatase mRNA. Whether this is mediated by iron or haem remains problematic since long-term exposure of MEL cells to FeSIH causes a measurable increase in cellular haem content (P. Ponka and H. M. Schulman, unpublished work) and long-term effects of desferrioxamine could not be determined because of its cytotoxicity.

DISCUSSION

Haem synthesis, which involves eight different enzymes, is ubiquitous in mammalian cells with the exception of mature erythrocytes. However, the rates of synthesis differ greatly in different tissues. Rapid rates of haem biosynthesis occur in liver and erythroid cells, where large amounts of haem are needed not only for mitochondrial cytochromes but as prosthetic groups for cytochrome *P*-450 and haemoglobin respectively. In spite of involving identical enzyme activities, the rate-limiting steps for haem biosynthesis in these two tissues are different. ALAS, which is the first enzyme in the pathway, has been shown to limit haem formation in non-erythroid tissues (Kappas et al., 1982), while we have shown previously that ALAS does not control haem synthesis in erythroid cells (Ponka and Schulman, 1985a). Instead, the supply of iron, which eventually gets inserted into protoporphyrin IX by the activity of ferrochelatase, the terminal enzyme in the haem pathway, is the controlling step for erythroid haem biosynthesis (Ponka and Schulman, 1985b; Laskey et al.,

1986). Recent molecular biology studies have demonstrated that there are also differences in the gene products for some haem-pathway enzymes in erythroid and non-erythroid tissues and a variety of mechanisms may account for these differences.

The first example of such diversity is ALAS. At least two genes have been found for the enzyme (Dierks, 1990), one of which is only expressed in erythroid tissues while the other is expressed in all cells. Interestingly, recent characterization of the mRNA derived from the eALAS gene shows that it contains an iron-responsive element (Dierks, 1990) at its 5' UTR similar to that in the 5' UTR of ferritin mRNA and 3' UTR of transferrin-receptor mRNA (Kühn et al., 1990; Munro et al., 1990). Since this iron-responsive element may be sufficient to mediate iron-dependent translational regulation of eALAS *in vivo* (Dandekar et al., 1991), it opens a new perspective on understanding how iron may control haem synthesis in erythroid tissue, and suggests that eALAS expression may be translationally controlled by iron. The second example is porphobilinogen deaminase which, unlike ALAS, has only one structural gene but two promoters (Grandchamp et al., 1987; Chretien et al., 1988). The erythroid-specific promoter exhibits several binding sites for erythroid-specific transcription factors and is totally inactive in non-erythroid cells. The third example is ALAD, in which two classes of transcripts have been revealed in mouse tissues: one class was found in both liver and erythroid cells; the other, only in erythroid cells (Bishop et al., 1991). Two promoters have been found which count for this difference in erythroid-specific and ubiquitous gene transcription.

The characterization of a recently cloned cDNA for ferrochelatase, which is the last enzyme in the haem pathway, has revealed two putative polyadenylation signals (Taketani et al., 1990), which led us to speculate that ferrochelatase may possess a third possible mechanism for erythroid-specific gene expression of haem-pathway enzymes. In the present study, we have provided confirmatory results showing that there are two ferrochelatase transcripts. However, only one of them is found predominantly in erythroid tissues *in vivo* and is also preferentially induced during MEL-cell erythroid differentiation *in vitro*.

If more than one functional polyadenylation signal is used during ferrochelatase gene transcription, one would expect its transcription pattern to have the following feature: (i) transcripts of different lengths will exist; and (ii) the transcripts will differ at their 3' ends. The first feature was demonstrated by others (Taketani et al., 1990; Brenner and Frasier, 1991) and confirmed here where we have shown that there are two ferrochelatase transcripts of 2.9 kb and 2.2 kb in various mouse tissues including spleen, liver, brain, kidney, muscle and reticulocytes and in MEL cells. However, to prove the hypothesis, it was necessary to demonstrate a difference in the 3' ends of the two transcripts. We generated a radiolabelled PCR fragment that corresponded to a region between the two putative polyadenylation sites and used it as a probe for Northern-blot analyses of tissue RNA. If our hypothesis is correct, the PCR fragment will hybridize only with the larger transcript, which is the product of transcription beyond the first polyadenylation site, and not with the smaller transcript. As shown in Figure 1(c) the radiolabelled PCR probe detected the 2.9 kb transcript but not the 2.2 kb transcript, thus clearly demonstrating that the two transcripts differ at their 3' ends. Furthermore, it also suggests that the 2.2 kb ferrochelatase transcript is produced by the utilization of the upstream polyadenylation signal. A similar situation has been described recently for transcripts of the human haemoxygenase-2 gene (McCoubrey et al., 1992).

Although the two transcripts do not differ qualitatively from those reported by others (Taketani et al., 1990; Brenner and

Frasier, 1991), we observed dramatic quantitative differences in their relative abundance in non-erythroid and erythroid tissues. We found that the 2.2 kb transcript is expressed highly in erythroid tissues such as spleen and reticulocytes as compared with non-erythroid tissues which contain mostly the 2.9 kb transcript. The most striking finding is that in reticulocytes over 90% of the total ferrochelatase mRNA is represented by the 2.2 kb transcript. In addition, we have recently analysed ferrochelatase mRNA in rat reticulocytes and found the 2.2 kb transcript (P. Ponka and H. M. Schulman, unpublished work). Since no RNA synthesis occurs in reticulocytes, because of the absence of nuclei, but haem synthesis takes place actively, it is reasonable to speculate that the 2.2 kb transcript is related to erythroid differentiation and may be the only mRNA involved in erythroid ferrochelatase production.

Since the 2.2 kb transcript was found to be more abundant in erythroid tissues *in vivo*, we chose to examine the pattern of ferrochelatase mRNA expression in the MEL cell, which is a well-characterized model system for studying erythroid differentiation. During MEL-cell differentiation, haem-synthesis enzymes are induced (Sassa, 1976), enabling the cells to produce large amounts of haemoglobin. We examined ferrochelatase mRNA expression in these cells and found that the level of ferrochelatase transcript increases during the course of differentiation. Corresponding mRNA levels of eALAS (as shown here), porphobilinogen deaminase and uroporphyrinogen deaminase have also been shown to increase during MEL-cell differentiation (Grandchamp et al., 1985). Thus, the genes for ferrochelatase and other haem-pathway enzymes belong to a group of genes whose expression is increased during erythroid differentiation. Although they are expressed in nearly all tissues, they also have an erythroid-specific pattern of inducibility. In the MEL-cell model, the induction of ferrochelatase mRNA may represent one of the characteristic features in the erythroid-differentiation programme.

The examination of ferrochelatase mRNA levels during MEL-cell differentiation also revealed some interesting features with relation to the increase in ferrochelatase enzyme activity and the induction of other mRNA species (eALAS, β -globin and transferrin receptor). First, the induction profile of ferrochelatase mRNA parallels the increase in ferrochelatase enzyme activity (Beaumont et al., 1984), suggesting that the increase in mRNA probably accounts for the increase in enzyme activity during MEL-cell differentiation. Secondly, the induction profile of ferrochelatase mRNA overlaps with that of ALAS and β -globin mRNA, i.e. the mRNA levels for both haem-synthesis enzymes and β -globin increase most dramatically and coordinately between 48 h and 96 h. There is a large body of evidence showing a tight coordination between globin and haem formation during erythroid differentiation. Experimentally, induction of haem accumulation stimulates both globin translation (Bruns and London, 1965; Zucker and Schulman, 1968) and transcription (Fuchs et al., 1981), and simultaneously feedback-inhibits its own synthesis (Karibian and London, 1965; Ponka and Neuwirt, 1969; Ponka and Schulman, 1985a). Our results showing concomitant induction of mRNA for β -globin and haem-synthesis enzymes ALAS and ferrochelatase lend further support to the notion that some of the mechanisms responsible for the coordination of haem and globin synthesis occur at the transcriptional level.

A striking feature about ferrochelatase gene expression during MEL-cell differentiation is the change in the relative abundance of the two transcripts. While the uninduced MEL cells contain mostly the 2.9 kb transcript, MEL-cell differentiation is accompanied by a preferential induction of the 2.2 kb transcript,

resulting in it being over 50% of the total ferrochelatase mRNA in fully induced cells. Thus, not only is the 2.2 kb transcript more abundantly found in erythroid tissues *in vivo*, there is also a close relation between this transcript and erythroid differentiation of MEL cells *in vitro*. It is highly likely that terminal erythroid differentiation probably induces the transcription of the ferrochelatase gene which was recently shown to contain binding sites for NF-E2 (Taketani et al., 1992).

The stability of mRNA is an important factor in the regulation of gene expression. Controlling decay of individual mRNA species can have a significant effect on their levels in the cytoplasm. A typical example of this is β -globin gene expression. In addition to activated transcription of the gene (Fuchs et al., 1981), its mRNA becomes more stable during MEL-cell differentiation (Krowczynska et al., 1985). Our results, showing an increased stability of ferrochelatase mRNA during MEL-cell differentiation and a similar stability in mouse reticulocytes, are interesting because both ferrochelatase and β -globin are involved in haemoglobin synthesis. Although little is known about how the cells control mRNA decay, this coordinated increase in mRNA stabilities may be an important mechanism for concerted regulation of metabolically related proteins.

In conclusion, following induction of erythroid differentiation of MEL cells, there is a preferential increase in the 2.2 kb ferrochelatase transcript that results from the utilization of the upstream polyadenylation signal. The high abundance (over 90%) of the 2.2 kb transcript in reticulocytes, the only pure erythroid cells used in this study, strongly suggests that its expression is related to erythroid differentiation. It is conceivable that the increased survival of 2.2 kb transcripts in reticulocytes is caused by the absence of (A+U)-rich sequences in the 3' UTR of the mRNA, which when present cause destabilization (Caput et al., 1986; Malter, 1989). The persistence of the 2.9 kb fragment in induced MEL cells may be related to the fact that these are transformed cells which are not exclusively erythroid.

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