The Role of the Erythroid-Specific δ-Aminolevulinate Synthase Gene Expression in Erythroid Heme Synthesis

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Using antisense technology, the effects of suppressed gene expression of the erythroid-specific δ-aminolevulinate (ALA) synthase (ALAS-E) on heme synthesis, expression of mRNAs encoding an erythroid-specific transcription factor NF-E2, other heme pathway enzymes, and β-globin were examined in murine erythroleukemia (MEL) cells. In MEL cells in which an antisense ALAS-E RNA was expressed (AS clone), sense ALAS-E mRNA levels in both untreated and dimethylsulfoxide (DMSO)-treated cells were decreased compared with their respective controls. Heme synthesis in AS clones was decreased in proportion to the suppressed levels of ALAS-E mRNA. In addition, mRNAs for ALA dehydratase, porphobilinogen deaminase, ferrochelatase (FeC), and β-globin were also decreased in AS clones. There was a strong correlation between the level of ALAS-E mRNA and most of the mRNAs of the heme pathway enzymes and β-globin. There was a decrease in the mRNA level of p45, but not of mapK, which are the large and the small subunits of NF-E2, respectively, in AS clones. Treatment of AS cells with hemin and ALA in the presence of DMSO partially restored the suppressed mRNA levels for β-globin and FeC and heme content, respectively. These findings thus indicate that heme formation, which is determined by the level of ALAS-E, plays an essential role on gene expression of many proteins necessary for erythroid development.

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These findings suggest that ALAS-E gene expression and resulting heme formation significantly influence the regulation of genes for erythroid-specific proteins.

MATERIALS AND METHODS

Cell culture. A clone of dimethylsulfoxide (DMSO)-sensitive MEL cells (DS-19) was grown in suspension in a modified Ham's F12 medium containing 10% heat-inactivated defined bovine calf serum (HyClone Laboratories, Logan, UT). Cells were routinely split every 3 to 4 days to maintain a logarithmic growth. For each experiment, cells from a 24- to 48-hour-old culture were resuspended in fresh medium at a cell density of 5 × 10³ cells/mL and incubated for 16 hours, before the addition of chemicals. Incubations were then continued for various periods as indicated in each figure.

Plasmid construction and transfection. A 0.8-kb cDNA fragment that contained a sequence spanning from the 5' end to the first HindIII site of the mouse ALAS-E cDNA, which we termed “0.8-kb HindIII fragment,” was inserted in an antisense orientation into the HindIII site of an eukaryotic expression vector, pRC/CMV, which contained a neomycin-resistant gene (Invitrogen Corp, CA) (Fig 1). For transfection, 30 μg of plasmid was linearized by digestion with ScaI and introduced into 1 × 10⁷ DS-19 cells by electroporation using CellJect electroporator CEL-1000 (BIOS Corp, New Haven, CT). Twenty-four hours after transfection, cells were incubated in fresh medium containing G418 (0.6 mg/mL; Geneticin; Gibco-BRL, Gaithersburg, MD) for 48 hours and then plated in fresh medium containing 0.8% (w/vol) methicellulose and G418 (0.6 mg/mL). After several days of growth, individual colonies were picked and resuspended in fresh medium containing G418 (0.6 mg/mL), and incubation was continued for expansion. Cell cloning using methicellulose culture was repeated twice to confirm results.

cDNA probes. An Xho I/Sph I fragment of the mouse ALAS-E, and a rat ALAS-N cDNA plK2EA, were used as ALAS-E and ALAS-N probes, respectively. A rat ALAD cDNA (pALAD-1), a rat PBGD cDNA (p445SB-1), a mouse ferrochelatase (FeC) cDNA and a mouse β-globin cDNA (pCR1/2) were used as probes in Northern blot analysis. Radiolabeled RNA probes specific for mouse mapK and p45 mRNAs were synthesized using T3 RNA polymerase. Chicken β-actin cDNA was used as an internal control.

Northern blot analysis. Total RNA was isolated according to the method of the acid guanidium thiocyanate phenol chloroform method, applied to a 1.2% agarose-formaldehyde gel, electrophoresed, and transferred to a sheet of Zeta-probe filter (Bio-Rad, Richmond, CA). The filters were hybridized with an appropriate probe at 43°C for 24 hours in a solution containing 50% formamide, 0.25 mol/L NaPO₄, 0.25 mol/L NaCl, 1 mmol/L EDTA, 2% sodium
Mouse ALAS-E cDNA

![Diagram of plasmid pRc/CMV ALAS-E AS](image)

**Fig 1.** Construction of a plasmid bearing an antisense ALAS-E RNA transcription unit. A 0.8-kb cDNA fragment, containing a sequence from the 5'-end to the first HindIII site of the mouse ALAS-E cDNA, was inserted into pRc/CMV vector at the unique HindIII site in antisense orientation. pRc/CMV ALAS-E antisense vector contained a human CMV promoter, bovine growth hormone polyadenylation signal, and neomycin phosphotransferase gene (Neo) under the control of SV40 early promoter.

dodecyl sulfate (SDS), 5× Denhardt’s solution and 100 μg/mL sonicated salmon sperm DNA. Filters were washed in 2× sodium citrate solution (SSC), 0.5× SSC, and 0.2× SSC with 0.1% SDS at 50°C for 30 minutes, and then exposed at −80°C to Kodak X-omat XAR-5 films (Eastman Kodak, Rochester, NY). mRNA concentrations were quantified by an LKB Ultroscan XL enhanced laser densitometer (Pharmacia, Piscataway, NJ). There was a linear relationship between the amount of mRNA and the absorbance at 633 nm. Experiments were performed two to three times using separate preparations of RNA and representative results were shown in the figures.

**Immunochromic quantification of ALAS isozymes.** Immunochromic quantification of ALAS isozymes was performed using mitochondria fractions isolated from 1.4 × 10^6 cells, by Western blot analysis with enhanced chemiluminescence detection. An antibody used was a rabbit antiserum against purified rat ALAS-N which recognizes both ALAS-N and ALAS-E.

**Assays on ALAD and PBGD activities and heme content.** ALAD activity was determined using 5 × 10^6 cells/assay by semimicrocolorimetry, and PBGD activity was determined using 5 × 10^6 cells/assay by fluorometry, as described previously. Heme content was determined fluorometrically using 10^6 cells per assay as described previously. All determinations were made in triplicate, and experiments were repeated two to three times.

**RESULTS**

**Suppression of ALAS-E mRNA levels by antisense ALAS RNA.** The antisense construct (Fig 1) was introduced into DS-19 cells by electroporation. Because the ALAS-E cDNA inserted into the antisense RNA expression vector was ~800 bp, it generated a 1.1-kb RNA. Thus, it was possible to distinguish the antisense RNA and the sense ALAS-E mRNA as 1.1-kb and 2.0-kb bands, respectively, in Northern blot analysis (Fig 2A). Of 30 neomycin-resistant clones, 9 clones expressing high levels of antisense ALAS-E RNA were isolated by two courses of clonal selection, using a methicellulose culture. Then 6 of the 9, expressing relatively low (<0.27-fold of the untreated mock control level; clones AS1 and AS2), intermediate (0.45- to 0.7-fold; clones AS3 and AS4), and high levels (>0.7-fold) of ALAS-E mRNA (clones AS5 and AS6) were chosen for further studies (Figs 2B and 3). After treatment with 1.5% (vol/vol) DMSO for 72 hours, these AS clones also showed relatively low (10.1 ± 1.9-fold and 10.7 ± 0.8-fold [mean ± SEM], for AS1 and AS2, respectively), intermediate (16.6 ± 3.3-fold and 18.2 ± 3.2-fold, for AS3 and AS4, respectively), and high (21.1 ± 4.4-fold and 24.0 ± 5.2-fold, for AS5 and AS6, respectively) increases in ALAS-E mRNA levels compared with the untreated mock control cells (Fig 3). DS-19 cells transfected with the plasmid alone (termed “control cells” hereafter) showed a 40.9 ± 9.2-fold increase of ALAS-E mRNA after DMSO treatment (Fig 3).

**Fig 2.** Northern blot analysis of clones expressing antisense ALAS-E RNA. (A) Ten micrograms of total RNA from AS clones selected by the first methicellulose culture were electrophoresed on a 1.2% (wt/vol) agarose gel containing 1.1 mol/L formaldehyde. After blotting onto a Zeta-probe filter, the filter was hybridized with the mouse ALAS-E 0.8-kb HindIII fragment. A band at 2.0 kb (slightly higher than 18S rRNA location) and a band at 1.0 kb (slightly lower than 18S) correspond to ALAS-E mRNA and anti-ALAS-E mRNA, respectively. DS, DS-19 cells (without transfection) treated with 1.5% DMSO for 72 hours, which exclusively express the sense ALAS-E mRNA (as control). (B) Fifteen micrograms of total RNA from 9 AS clones selected by the second methicellulose culture were analyzed by Northern blot analysis. Six clones expressing high levels of antisense RNA were shown. DS, DS-19 cells (without transfection) treated with 1.5% DMSO for 72 hours, as control.
Fig 3. Effect of DMSO on ALAS-E mRNA levels in AS clones. Fifteen micrograms of total RNA from AS cells with or without incubation with 1.5% DMSO for 72 hours was examined by Northern blot analysis using a [32P]-labeled ALAS-E Xho I/Sph I cDNA fragment as a probe. Levels of ALAS-E mRNA were expressed as the ratio to that in the untreated control that had been transfected with the plasmid only. Data represent the mean with SEM of three to four experiments.

Fig 4. Immunoblot analysis of ALAS. Immunoblot analysis was performed as described in Materials and Methods. Each lane corresponds to mitochondria isolated from 1.4 x 10^6 cells. Bands at 59K and 54K correspond to the precursor and the mature form of ALAS-E, respectively. A band at 65K corresponds to ALAS-N, but it was insignificant under the condition of detection. Data represent the mean with SEM of three experiments.

Fig 5. Northern blot analysis of p45 and mafK mRNAs. p45 and mafK mRNA levels were examined by Northern blot analysis as described in Materials and Methods. Twenty micrograms of total RNA was electrophoresed, blotted onto a Zeta-probe filter. Hybridization of the filter with [32P]-labeled RNA probes was performed in the presence of both probes for p45 and mafK, followed by RNase treatment, and washing under the most stringent conditions. Data represent the mean with SEM of three experiments.

**Immunoblot analysis.** Western blot analysis of ALAS was performed in control cells and in AS1 cells. The results showed that the level of ALAS-E was lower in untreated AS1 cells (~54%) than in control cells transfected with the plasmid alone (Fig 4). After DMSO treatment, the level of the enzyme protein increased 5-fold and 2.7-fold in control and AS1 cells, respectively. The induced level of the ALAS-E protein in AS1 cells was ~30% of that in the induced control cells (Fig 4), which was similar to its mRNA level (~25% of that in the induced control).
Suppression of p45 mRNA levels by antisense ALAS RNA. Levels of p45 and majK mRNA, the large and the small subunit of the erythroid-specific transcription factor NF-E2,23,24 respectively, were examined by Northern blot analysis in DS-19, control, and AS cells. Results for DS-19, control, and AS1 cells that expressed the lowest level of ALAS-E mRNA are shown in Fig 5. mRNA levels are expressed as the ratio to that of untreated control cells. p45 mRNA levels were 1.28-, 1.0, and 0.67-fold in untreated DS-19, control, and AS1 cells, respectively. The reason for decreased p45 mRNA in control cells is unclear at present, but the results were reproducible. p45 mRNA levels in DMSO-treated cells were 2.85-, 1.49-, and 1.07-fold in DS-19, control, and AS1 cells, respectively (Fig 5). In contrast with p45 mRNA, majK mRNA expression was much lower, and there was little difference in its level among these clones, and it was not influenced by DMSO treatment (Fig 5).

Effects of ALAS-E suppression on the levels of mRNAs encoding other heme pathway enzymes and β-globin. Effects of the suppression of ALAS-E expression on the levels of mRNAs encoding other heme pathway enzymes and β-globin were examined. ALAS-N mRNA levels decreased after DMSO treatment in all clones (Fig 6), consistent with the downregulation of ALAS-N in MEL cells that was reported earlier in the DS-19 clone.18,20 It is also interesting to note that untreated AS clones showed a higher level of ALAS-N mRNA than untreated control cells (Fig 6), suggesting that the suppression of ALAS-E mRNA may augment the expression of the nonerythroid transcript. The suppression of ALAS-E mRNA in untreated AS clones decreased the levels mRNAs encoding ALAD, PBGD, FeC, and β-globin, but not β-actin, which was used as control (Fig 6). These findings indicate that decreased expression of the ALAS-E gene is correlated with decreased expression of other heme pathway enzyme genes and that of the β-globin gene.

Relationships of ALAS-E mRNA and mRNA levels for PBGD, FeC, and β-globin are shown in Fig 7. These mRNAs showed a significant correlation with ALAS-E mRNA levels (Fig 7A, 7B, and 7C). In contrast, ALAD mRNA levels, which were decreased in AS cells to 20% to 50% of those that, in the control, did not show a significant correlation with ALAS-E mRNA levels (data not shown).

Effects of ALAS-E suppression on the levels of ALAD, PBGD activities, and heme content. To examine whether a decrease in ALAS-E mRNA is reflected in other heme pathway enzymes and products, we determined ALAD and PBGD activities and heme content. Both ALAD (Fig 8A) and PBGD activities (Fig 8B) in AS clones were found correlated with the level of ALAS-E mRNA (r = .679, P < .05 for ALAD, and r = .930, P < .01 for PBGD, respectively). Similar to the significant correlation between heme pathway
Effects of DMSO, hemin, and succinylacetone (SA) on the level of FeC and β-globin mRNAs in AS clones. Our earlier studies have shown that heme is necessary for accumulation of mRNAs encoding FeC and β-globin during erythroid differentiation of DS-19 cells, and thus, effects of DMSO (an inducer of heme synthesis in this cell type), hemin, and SA (a potent inhibitor of heme synthesis) on the level of FeC and β-globin mRNAs were examined in AS clones. When cells were treated with DMSO and hemin (100 µmol/L), the level of FeC mRNA was similar to, or slightly higher than, DMSO treatment alone in AS clones, whereas it was decreased when cells were treated with DMSO and SA (0.5 mmol/L) (Fig 9A). Thus, the average increase in FeC mRNA levels in the four AS clones was 122% ± 7% (mean ± SEM) for cells treated with DMSO and hemin compared with cells treated with DMSO alone, whereas its level was 59% ± 10% (mean ± SEM) for cells treated with DMSO and SA. Similar findings were observed with β-globin mRNA, which was higher in all AS clones after DMSO and hemin treatment (208% ± 47%, mean ± SEM) compared with DMSO treatment alone, whereas SA treatment inhibited the DMSO-mediated induction of β-globin mRNA (51% ± 12%, mean ± SEM) (Fig 9B). Although Beaumont et al. reported that SA treatment does not affect DMSO-mediated induction of FeC activity, other groups showed that an inhibitor of heme synthesis such as desferrioxamine or SA decreases FeC mRNA, or a lack of expression of ALAS-E decreases FeC mRNA. Thus, the majority of the findings on this point confirm our earlier observation that heme is positively involved in the increase in FeC and β-globin mRNA in differentiating cells.

Effects of ALA on the level of heme synthesis. Because ALA treatment should bypass the suppressed level of ALAS expression, we examined the effect of ALA on heme concentrations in AS clones. Heme content in untreated cells was 65 ± 4, 51 ± 6, and 46 ± 4 pmol/10^6 cells (mean ± SEM) for control, AS1 and AS6 cells, respectively, whereas heme content in cells treated with 1.5% DMSO for 96 hours was 305 ± 3, 74 ± 1, and 146 ± 2 pmol/10^6 cells (mean ± SEM) for control, AS1 and AS6 cells, respectively. For cells treated with both DMSO and ALA (600 µmol/L), heme content was 601 ± 30, 287 ± 61, and 351 ± 76 pmol/10^6 cells (mean ± SEM) for control, AS1 and AS6 cells, respectively. These results indicate that the blocked heme synthesis by ALAS-E downregulation can be partially restored by providing ALA to cells.

Terminal differentiation of AS clones. DS-19 cells show terminal cell differentiation after continued incubation with DMSO. To examine whether the downregulation of ALAS-E may have any effect on terminal cell differentiation, we examined the growth curve of AS clones either in the presence, or in the absence of 1.5% DMSO for 13 days. Cells were maintained at a low cell density by diluting the culture daily with fresh medium to yield 1 to 2 × 10^6 cells/mL. This procedure allowed untreated cells to grow at a logarithmic rate. Results showed that all untreated clones grew in a logarithmic manner, whereas DMSO-treated clones showed the cessation of cell growth, starting 4 days in culture, and the cell number of all DMSO-treated cultures
Fig 8. Suppression of ALAS-E mRNA, its effects on enzyme activities of ALAD and PBGD, and heme content. ALAS-E mRNA and β-globin mRNA levels and ALAD and PBGD activities were examined using cells that had been incubated in the presence of 1.5% DMSO for 72 hours, as described in the legend to Fig 7. Heme content, which is known to show a delayed increase compared with ALAS-E, ALAD, and PBGD, was determined using cells incubated with DMSO for 96 hours. Data are the mean of two to three experiments.

on day 13 was $\approx 1/10,000$ of that in untreated cultures. There was no difference in the cell number between control and AS cells (data not shown).

DISCUSSION

The distinctive tissue-specific regulation of heme synthesis between the liver and erythroid cells has been documented in several studies. In the liver, the rate of heme formation is determined by the level of ALAS, the first enzyme of the heme biosynthetic pathway, and the level of ALAS is in turn controlled in a feedback manner by intracellular free heme concentration. The increase in ALAS is implicated in the chemical-induction of heme synthesis, but other heme pathway enzymes are not. In contrast with the liver, there is an upregulation of a host of heme pathway genes when erythroid heme synthesis is increased during cell differentiation. Hemin also stimulates, rather than inhibits, the synthesis of ALAS-E. Hemin is also known to increase the expression of FeC mRNA and globin mRNA in erythroid cells, as well as the synthesis of heme and hemoglobin. Additionally, in vitro erythroid colony culture systems, hemin stimulates the growth and differentiation of erythroid colonies. The transferrin receptor gene is also known to be upregulated during erythroid differentiation, which positively influences the synthesis of ALAS-E at the posttranscriptional level. Thus the mode of regulation of heme synthesis by heme in the liver and in erythroid cells is distinct.

Our findings in this study show that transfection of an antisense ALAS-E RNA transcription unit in DS-19 cells decreased not only levels of ALAS-E mRNA (Figs 3 and 6) and its protein (Fig 4), but also levels of mRNA encoding p45 (Fig 5), some other heme pathway enzymes, and β-globin (Figs 6 and 7). There was a highly significant correlation between the level of ALAS-E mRNA and the levels of mRNAs encoding PBGD (Fig 7A), FeC (Fig 7B), and β-globin (Fig 7C), suggesting that the expression of ALAS-E has significant influences on gene expression of other heme pathway enzymes and β-globin. In contrast, ALAD mRNA levels did not show a significant correlation with ALAS-E mRNA, but its activity showed a weak correlation with ALAS-E mRNA levels ($r = .679, n = 9$) (Fig 8A). The reason for this discrepancy is unclear at present. However, it has been recently reported that there are tissue-specific ALAD mRNAs, ie, the erythroid-specific and the nonspecific forms. Therefore, there may be a possibility that a relationship between ALAD and ALAS-E mRNA levels may be made obscure by the presence of the nonspecific ALAD mRNA, even if the erythroid-specific downregulation of ALAD mRNA had been present in AS clones.

Among various comparisons, the highest correlation coefficient was observed between heme content and β-globin mRNA ($r = .941, P < .001$) (Fig 8D), suggesting that intracellular heme may play a significant role in regulating β-globin mRNA levels. In support of this hypothesis, the effect of ALAS-E suppression on these indices was partially re-
Northern blot analysis was performed as described in Materials and Methods. 

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\text{p-globin mRNA levels are expressed as the ratio to those in untreated control cells. Data are the mean of two experiments. (A) Effects of DMSO, hemin, and SA on FeC mRNA levels. Treatments: none (open column); 1.5\% DMSO (dotted column); 1.5\% DMSO and 100 \mu\text{mol/L hemin} (hatched column); 1.5\% DMSO and 0.5 \text{mmol/L SA} (solid column).}
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Fig 9. Effects of DMSO, hemin, and SA on \(\beta\)-globin and FeC mRNA levels in \(A5\) clones. Cells were incubated with or without chemicals for 72 hours. Northern blot analysis was performed as described in Materials and Methods. \(\beta\)-globin mRNA levels are expressed as the ratio to those in untreated control cells. Data are the mean of two experiments. (A) Effects of DMSO, hemin, and SA on \(\beta\)-globin mRNA levels. Treatments: none (open column); 1.5\% DMSO (dotted column); 1.5\% DMSO and 100 \mu\text{mol/L hemin} (hatched column); 1.5\% DMSO and 0.5 \text{mmol/L SA} (solid column).

stored by the exogenous supply of hemin (Fig 9). It should also be noted that decreased heme synthesis in \(A5\) clones per se did not influence the commitment process. Thus, our findings in \(A5\) clones support and are consistent with the idea that cellular commitment is a separable event from induction of heme synthesis.  

Using a stable MEL cell mutant in which ALAS-E expression is not inducible by DMSO, hexamethylenbisacetamide, or butyric acid, Lake-Bullock and Dailey have recently shown that PBGD and coproporphyrinogen oxidase, but not protoporphyrinogen oxidase, FeC, and \(\beta\)-globin are induced by DMSO treatment. However, after the addition of DMSO and hemin, but not hemin alone, a normal program of erythroid differentiation was observed including the induction of FeC and \(\beta\)-globin. Based on these findings, these authors suggested that the induction of the heme biosynthetic pathway is biphasic and the induction of the terminal enzymes may be mediated by heme. Our findings in this and earlier studies are consistent with their observations, and the findings in this study further indicate that there is a quantitative relationship between ALAS-E mRNA expression and mRNAs for PBGD, FeC, and \(\beta\)-globin, as well as ALAD activity and heme content. These findings confirm that heme plays a positive role in the regulation of heme pathway enzyme expression and \(\beta\)-globin gene expression. Thus, in differentiating erythroid cells, autoregulation of these events may be necessary in maintaining the differentiated state.

The mechanisms for downregulation of other heme pathway genes and that of \(\beta\)-globin by the suppression of ALAS-E expression are unclear at present, but it is likely that heme may play a significant role in this action. Namely, the suppressed expression of \(\beta\)-globin and FeC mRNAs can be partially restored by hemin treatment. It should also be noted that ALA treatment restored heme synthesis in \(A5\) clones. In addition, there are several findings that suggest heme-mediated upregulation of heme pathway enzyme genes and \(\beta\)-globin in erythroid cells (Fig 9).  

If heme is involved in the upregulation of erythroid-specific transcription factors, heme deficiency induced by ALAS-E downregulation in \(A5\) cells may result in the suppression of other erythroid-specific gene expression. There are two major erythroid-specific transcription factors that should be considered in this regard, ie, GATA-1 and NF-E2. GATA-1 expression in \(A5\) clones, however, is not expected to be much different from the wild-type MEL cells, because its expression in a mutant clone, DR-1, which lacks ALAS-E mRNA, was similar to that of DS-19. On the other hand, our findings related to NF-E2 showed that the mRNA level of \(p45\), one of the two subunits of NF-E2, was suppressed in \(A51\) cells similarly to the suppression of ALAS-E level in these cells (Fig 5). Our findings on the concerted downregulation of PBGD, FeC, and \(\beta\)-globin in \(A5\) cells, all of which possess an NF-E2 binding cis-element, and the restoration of these transcript levels by the combined addition of DMSO and hemin (Fig 9) appear to be consistent with the hypothesis that intracellular heme may positively regulate NF-E2 activity. Recently Solomon et al reported that erythroid differentiation of K562 cells can be induced by hemin, but suppressed by 12-O-tetradecanoylphorbol-13-acetate (TPA). It is of interest to note that heme induction of K562 cells acts to increase the binding activity of NF-E2, whereas TPA increases the binding of ubiquitously expressed AP-1 transcription factors to a regulatory element in the transcriptional control element of various erythroid-specific genes, thereby inhibiting the binding of NF-E2 to this element. However, it is unclear at present whether heme upregulates NF-E2, because ALAS-E suppression in \(A5\) cells decreased the level of \(p45\) mRNA, but not that of \(\text{magnF}\), the small subunit of NF-E2. Thus, the effect of intracellular heme on the functional activity of NF-E2 is yet to be determined, and such studies are currently under way in our laboratory.

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The role of the erythroid-specific delta-aminolevulinate synthase gene expression in erythroid heme synthesis

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