Iron Protoporphyrin IX (Hemin) But Not Tin or Zinc Protoporphyrin IX Can Stimulate Gene Expression in K562 Cells From Enhancer Elements Containing Binding Sites for NF-E2

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Many genes whose transcription is erythroid-specific contain enhancer or promoter elements that bind the transcription factor NF-E2. Hemin induction increases the expression of globin genes in the human erythroleukemia cell line K562, and increases the expression of reporter genes regulated by enhancer elements containing binding sites for NF-E2. We sought to determine whether metalloporphyrins other than hemin can stimulate the transcriptional regulatory activity of enhancer elements containing binding sites for NF-E2. Aside from hemin, only cobalt protoporphyrin IX, to a limited extent, could increase the expression of reporter genes regulated by an enhancer element containing binding sites for NF-E2. The failure of metalloporphyrins other than hemin to stimulate the transient expression of a CAT reporter gene linked to an enhancer element containing a binding site for NF-E2 was correlated with their failure to induce benzidine-positive K562 cells and increase the steady-state level of γ-globin mRNA. This study suggests that elevated levels of zinc protoporphyrin IX found in the anemia of chronic disease, iron deficiency, and lead poisoning may contribute to a decrease in globin gene expression by interfering with the transcriptional activity of enhancer elements containing binding sites for NF-E2.

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HEMIN, WHICH IS THE iron-containing protoporphyrin IX, is an essential cell constituent. It is required for the oxygen-carrying capacity of hemoglobin and is a requisite prosthetic group of enzymes, such as the cytochromes and peroxidases. Beyond its role as an essential constituent of many proteins, hemin has been shown to induce cellular differentiation. Hemin has been shown to increase the expression of erythroid-specific genes in the human leukemic cell lines K562 and HEL.

Hemin can increase gene expression by regulating many steps along the path from transcription of template DNA into mRNA and its subsequent translation into protein. The DNA sequence TCCGCTAGTCA/T appears to be a hemin-responsive element, and it binds the transcription factor NF-E2. Many erythroid-specific promoter or enhancer elements contain binding sites for the transcription factor NF-E2. How hemin increases the transcriptional activity of NF-E2 protein is not clear. Some investigators have demonstrated that hemin induction may alter the binding of NF-E2 to its cognate DNA binding sequences. Other groups, including this group, have concluded that there is no difference in the binding activity of NF-E2 to its recognition sequence in the genome in uninduced or hemin-induced K562 cells. Nevertheless, hemin does act as an effector of the activity of NF-E2. Whether hemin binds directly to NF-E2 and acts as an allosteric effector of the activity of NF-E2 or whether hemin acts indirectly via the activation of a kinase or other effector of the trans-activation ability of NF-E2 is not yet clear.

Hemin has been shown to affect the stability of mRNAs that contain iron-responsive elements at the 3' end and the translational efficiency of mRNAs that contain an iron-responsive element at the 5' end. Finally, hemin-deficient reticulocytes have been shown to have a general decrease in their rate of initiation of protein translation because of the activation of the heme-regulated inhibitor with the resultant phosphorylation of eukaryotic initiation factor 2a.

Therefore, it is no surprise that disorders of heme synthesis, whether genetic or acquired, have profound effects on cell function. Inherited disorders of heme biosynthesis such as congenital erythropoietic porphyria can be lethal. However, most disorders of heme biosynthesis are acquired. Pyridoxine deficiency results in the loss of enzymatic activity of ALA-synthase, the first enzyme in the heme synthetic pathway. Lead poisoning results in the inhibition of many steps in the heme synthetic pathway, including the last step, which is catalyzed by the enzyme ferrochelatase. Inhibition of ferrochelatase by lead results in increased amounts of free protoporphyrin IX and zinc protoporphyrin IX in cells. Since ferrochelatase catalyzes the insertion of iron (Fe3+) into the protoporphyrin IX ring, absolute or relative deficiency of iron also results in decreased amounts of hemin. However, ferrochelatase can use zinc instead of iron. Thus, an increased level of cellular zinc protoporphyrin IX is a very sensitive indicator of iron deficiency. Increased levels of zinc protoporphyrin IX are also found in many patients with the anemia of chronic inflammatory disease. Of eight metalloporphyrins, including zinc protoporphyrin IX, used to induce the mouse erythroleukemia cell line MEL, only hemin, and to a much lesser extent cobalt protoporphyrin IX in conjunction with dimethyl sulfoxide, was able to induce expression of hemoglobin as measured by the percentage of benzidine-positive cells and hemoglobin content per cell.

Many laboratories have demonstrated that the hemin-induced increase in hemoglobin production in human leukemic cell lines requires DNA sequences found within the locus control regions of the β- and α-globin gene domains. It appears that binding sites for the transcription factor NF-E2 within the locus control regions are sufficient to allow an...
increase in transcription upon hemin induction. Since acquired disorders of hemin production are commonly associated with anemia and because the mechanism of hemin stimulation of the transcriptional activation of NF-E2 is not yet understood, we sought to determine whether naturally occurring analogs of hemin such as zinc protoporphyrin IX were capable of stimulating gene expression from enhancer elements containing binding sites for NF-E2.

In the studies reported here, we determined whether induction of K562 cells with metalloporphyrins other than hemin could result in the production of hemoglobin. We determined whether there were differences in the binding activity of NF-E2 to its binding sequence using nuclear extracts obtained from K562 cells induced with various metalloporphyrins. Transient transfection analysis of reporter plasmids containing enhancer elements that have binding sites for NF-E2 was used to determine whether metalloporphyrins other than hemin were capable of increasing gene expression from these plasmids. We determined that metalloporphyrins other than hemin could increase the steady-state level of globin mRNA in K562 cells.

The studies indicate that of all the metalloporphyrins, only hemin can increase globin gene expression in K562 cells. Elevation of the level of zinc protoporphyrin IX in K562 cells results in a failure of stimulation of the transcriptional activity of enhancers that bind the NF-E2 protein, and a decrease in the steady-state level of γ-globin mRNA.

MATERIALS AND METHODS

Cell culture. K562 cells obtained from the American Type Culture Collection were grown in RPMI 1640 + 10% fetal calf serum; 20 μmol/L of either iron, zinc, tin, or cobalt protoporphyrin IX or free protoporphyrin IX was used to induce the K562 cells. The experiments reported here were performed over the course of 2 years and used many different subclones of K562 cells.

Preparation of the metalloporphyrins. Metalloporphyrins used in our initial studies were the kind gift of Dr. Nader Abraham (New York Medical College). Metalloporphyrins used in latter studies were obtained from Porphyrin Products (Logan, UT).

Metalloporphyrins were dissolved in 0.1N KOH, and the volume was increased with 40 mmol/L Tris, pH 7.8; the final pH concentration was adjusted to 7.8 with 0.3N HCl. Each metalloporphyrin solution was then filtered through a 0.22-μm filter. Each solution was stirred in a Falcon tube wrapped in aluminum foil and kept in the dark at −20°C until use. With this protocol, there appeared to be differences in the solubility of each of the metalloporphyrins—particularly, protoporphyrin IX did not appear to be soluble in this buffer.

To control for differences in solubility, we repeated our experiments using a second method for dissolving the metalloporphyrins. A weight of metalloporphyrin sufficient to yield a 1.8-mmol/L solution in a final volume of 50 mL was first dissolved in 0.5 mL ethanolamine; 7 mL distilled water was then added. The pH of the solution was then adjusted to 7.8 with 1N HCl. Sufficient distilled water was added to make the final volume 50 mL. The solution was then filtered through a 0.22-μm filter and stored in the dark until use. With this protocol, our results showed that this protocol produced equal solubility of all of the metalloporphyrins, including protoporphyrin IX. Dissolving the metalloporphyrins in this manner did not appear to alter their activity—hemin dissolved by either of these two methods was equally active in increasing reporter gene expression.

Benzidine staining of K562 cells. The stock solution of benzidine (Sigma Chemical, St. Louis, MO) was 0.2% (wt/vol) in 0.5 mol/L acetic acid. This solution was stored in the dark in a brown glass bottle. Before use, 100 μL 3% hydrogen peroxide was added to 2.5 mL of the stock solution.

To stain the K562 cells, 1 mL of a cell suspension was centrifuged and then washed twice with phosphate-buffered saline. The washed cells were resuspended in 100 μL phosphate-buffered saline, 10 μL fresh benzidine solution was added, and after a 15- to 30-minute incubation at room temperature, the percentage of benzidine-positive cells was determined.

Electrophoretic mobility shift analysis. After preincubation of nuclear extract with 1 μg poly dIdC on ice for 20 minutes, the protein-DNA binding reaction was performed at 20°C for 15 minutes. DNA was end-labeled with 32P. Reaction products were separated by electrophoresis on 4% acrylamide gels made in 0.25 mol/L Tris-borate EDTA and 5% glycerol. Electrophoresis was performed at 200 V for 4 hours. The oligonucleotide was TNF-E2 and the sequence was 5’CAATGCCTAGCTAGTCATGCTAGAGG3’ for the NF-E2/tandem AP-1 binding site.

Transient expression experiments. Each test plasmid was constructed by insertion of a transcriptionally active sequence into the Bgl II site of the vector pAlOCAT2, which contains the SV40 early promoter and the CAT reporter gene. The following test plasmids and transcriptionally active, sequences were used: TNF-E2 CAT, 5’GATCGCTAGCTAGTCATGCTAGGCTAG3’, NF-E2/tandem AP-1 site; and TAP-1 CAT, 5’GATCTCAGCTTATGAGTGGTATGCTT3’, NF-E2/tandem AP-1 site with G to T transversions (in boldface) at all guanines potentially required for NF-E2 binding.

Uninduced K562 cells (4×10⁵) were electroporated with 30 μg of a test plasmid and 6 μg of the Rous sarcoma virus (RSV) luciferase plasmid at 280 V and 960 μF of capacitance using a Bio-Rad electroporator with a capacitance extender (Richmond, CA). Following electroporation, each cuvette of cells was transferred into a dish of media. After an 8-hour recovery period, each ‘parental’ dish of cells was split into four (or more) equal dishes. Of the ‘progeny’ dishes of cells, one remained uninduced, and each of the remaining dishes of cells underwent induction with hemin or other inducing agents. This procedure ensures equal electroporation efficiency between dishes of cells containing the same test plasmid. Following 60 to 96 hours of induction, whole-cell lysates were made. The protein concentration of each whole-cell lysate was measured by the method of Bradford. Equal amounts of protein from each whole-cell lysate were tested for CAT and luciferase activity using standard protocols.

Northern blot analysis. Twenty micrograms, total RNA obtained from K562 cells treated with a metalloporphyrin was loaded into a lane of a 1% agarose gel containing formaldehyde with 3(N-morpholino)propanesulfonic acid buffer, electrophoresed, and then transferred to nylon filters and UV-fixed. Aγ-globin cDNA was labeled with 32P by random priming and then hybridized to the nylon filter. To control for differences in loading, the probed filter was stripped of the Aγ-globin cDNA probe and then rehybridized to a 32P-labeled glyceraldehyde-3-phosphate dehydrogenase probe.

RESULTS

Hemin and protoporphyrin IX but not cobalt, tin, or zinc protoporphyrin IX produce benzidine-positive K562 cells. We determined whether metalloporphyrins other than hemin can increase hemoglobin production in K562 cells. The initial experiments used the percentage of benzidine-positive cells as an estimate of hemoglobin production in K562 cells. Each dish of K562 cells was induced with a metalloporphyrin...
Effect of 96 hours of induction with various metalloporphyrins on induction of benzidine-positive K562 cells. K562 cells were exposed for 96 hours to 20 μmol/L concentrations of metalloporphyrins. K562 cells were washed twice in PBS and then stained with benzidine. Each bar represents an average of the percentage of benzidine-positive cells from three independent inductions of K562 cells with each metalloporphyrin. Induction of K562 cells with Cobalt, Tin, or Zinc protoporphyrin IX did not produce an increase of benzidine-positive K562 cells above uninduced levels at 24, 48, 72, or 96 hours of induction. Values represent means ± standard deviation from three independent experiments. Induction of K562 cells with any of the metalloporphyrins did not change cell viability or the 24-hour cell-doubling time, except for induction with protoporphyrin IX that led to almost complete cell death by 48 hours of induction (data not shown).

for 24 to 96 hours. After 96 hours of cell growth, there were on average approximately 8% benzidine-positive uninduced K562 cells. After 24 hours of induction, approximately 50% of the K562 cells induced with protoporphyrin IX were strikingly benzidine-positive. Indeed, at 24 hours protoporphyrin IX was more effective at inducing benzidine-positive K562 cells than hemin. However, by 48 hours after initiation of induction of K562 cells with protoporphyrin IX, there was almost complete cell death. Figure 1 shows the average percentage of benzidine-positive K562 cells induced with metalloporphyrins for 96 hours; this average is an arithmetic mean of three independent induction experiments.

The data are in agreement with the study by Chang and Sassa of the MEL cell line, except for induction with cobalt protoporphyrin IX. They reported that 100 μmol/L cobalt protoporphyrin IX in conjunction with 0.2% dimethyl sulfoxide resulted in an increase in the percentage of benzidine-positive MEL cells compared with uninduced MEL cells. However, the percentage of benzidine-positive MEL cells produced by cobalt protoporphyrin IX was not greater than 8% after 10 days of exposure. In our study, 20 μmol/L cobalt protoporphyrin IX was used and produced on average 8% benzidine-positive K562 cells after 4 days of exposure. However, the percentage of benzidine-positive K562 cells in the uninduced state was also on average 8%. Therefore, it is possible that cobalt protoporphyrin IX may have some activity that was obscured by the high basal level of benzidine positivity, as well as the difference in the concentration of cobalt protoporphyrin IX used.

Effect of metalloporphyrin induction on expression of endogenous globin genes of K562 cells. To verify that the level of production of benzidine-positive K562 cells by each of the metalloporphyrins reflected the steady-state level of globin mRNA, Northern blot analysis using a cDNA probe specific for γ-globin gene expression was performed on RNA obtained from K562 cells induced with the various metalloporphyrins. As demonstrated in Fig 2A, only hemin and free protoporphyrin IX could increase the steady-state level of γ-globin gene expression as compared with uninduced K562 cells. Measurement of induction was obtained by normalizing the signal obtained from the γ-globin probe to the signal obtained from hybridization of the same blot to the probe for the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (G3PDH).
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3-phosphate dehydrogenase gene (Fig 2B; results are the mean of three independent experiments). Therefore, there is a correlation between the ability of a metalloporphyrin (hemin and protoporphyrin IX) to induce an increase in the steady-state level of γ-globin mRNA and the induction of benzidine-positive K562 cells.

The inability of cobalt, tin, and zinc protoporphyrin IX to increase the steady-state level of γ-globin mRNA could be secondary to a failure of these metalloporphyrins to activate trans-acting factor/s required for globin gene transcription. Indeed, K562 cells induced with cobalt, tin, or zinc protoporphyrin IX demonstrate a modest decrease in the steady-state level of γ-globin mRNA in comparison to uninduced K562 cells. However, the half-life of globin mRNA in K562 cells is quite long, perhaps greater than 48 hours; even complete abolition of initiation of globin gene transcription by metalloporphyrins would not result in the absence of globin mRNA following 72 hours of induction.

No difference in binding of NF-E2 to its recognition sequence in vitro between nuclear extracts obtained from K562 cells treated with any of the metalloporphyrins. Recent studies have indicated that the sequence TCGCTGAGTCAC/T contains a hemin-responsive element.\textsuperscript{10} This putative hemin-responsive element binds the transcription factor NF-E2. There is no clear model for how hemin increases the transcription-activating ability of NF-E2. One possibility is that hemin induction could facilitate the binding of NF-E2 to DNA.

Since cobalt, tin, and zinc protoporphyrin IX did not increase production of benzidine-positive K562 cells, we determined whether we could detect differences in the binding of NF-E2 in vitro to its recognition sequence, using nuclear extracts obtained from K562 cells induced with the various metalloporphyrins. Nuclear extracts obtained from K562 cells treated with hemin, cobalt, or tin protoporphyrin IX produced a rapidly migrating band that corresponded to the NF-E2 bandshift (Fig 3, data not shown for zinc protoporphyrin IX). Nuclear extracts obtained from K562 cells treated with free porphyrin IX demonstrated decreases in the formation of all four specifically shifted bands of DNA. This is likely a manifestation of the toxicity of free protoporphyrin IX to the K562 cells. Within the limits of sensitivity of the electrophoretic mobility shift assay, we conclude that induction of K562 cells with hemin or with cobalt, tin, and zinc protoporphyrin IX has no effect on the binding of NF-E2 to its recognition sequence. If the transcription factor NF-E2 is indeed the transcription factor through which hemin induction can increase globin gene expression, then the in vitro binding data would suggest that the hemin-induced increase in the transcriptional regulatory activity of NF-E2 occurs following its binding to DNA. However, we cannot rule out the possibility that the endogenous production of hemin in K562 cells is sufficient to permit binding of NF-E2 to its recognition sequence.

Effect of metalloporphyrins on expression of reporter plasmids containing NF-E2 binding sites transiently transfected into K562 cells. To determine whether there was a correlation between the increased transcriptional activ-

Fig 3. Electrophoretic mobility shift assay of binding activity of nuclear extracts, obtained from K562 cells induced with various metalloporphyrins, to the core NF-E2/AP-1 element of the 5′HS2 β-globin gene domain locus control region enhancer. Nuclear extracts were obtained from K562 cells induced with each metalloporphyrin for 72 hours. Nuclear extracts were incubated with an oligonucleotide probe corresponding to the core transcriptional activating element of 5′HS2 of the β-globin gene domain locus control region. This 31-bp sequence contains a tandem AP-1/NF-E2 binding site. Incubation of the 31-bp probe with nuclear extract produces at least four specifically shifted bands. Using specific cold competitors as well as antibodies to c-Jun protein (see Solomon et al\textsuperscript{16}), we have found that the most rapidly migrating specifically shifted band is produced by the binding of NF-E2, whereas the more slowly migrating bands are produced by occupancy of the AP-1 family of transcription factors.
Fig 4. Effects of hemin and zinc protoporphyrin IX (Znpp) induction on transient expression of reporter genes in K562 cells. To control for differences in electroporation efficiency, a single electroporation cuvette of K562 cells was electroporated with the test plasmid, and then split into four (or more) aliquots; each of which was induced with a different metalloporphyrin. In addition, to control for general effects of each of the inducing agents on K562 cells, we coelectroporated the RSV luciferase reporter plasmid with each of the test plasmids. Therefore, we can calculate the absolute fold induction of the test CAT reporter plasmid above its uninduced basal level (because our electroporation efficiency is identical between induced and uninduced K562 cells) and we can calculate the preferential effect of an inducing agent on activation of NF-E2 binding sites by dividing the absolute fold induction of the test CAT reporter plasmid by the fold induction of the coelectroporated RSV luciferase plasmid (which is the control for a general effect of the inducing agent on K562 cells). Each panel represents a single cuvette of K562 cells electroporated with the test plasmid and then split into equal aliquots before induction with each agent. K562 cells were harvested 72 hours after electroporation. Plasmid pAI0CAT is an enhancerless plasmid. Plasmid pTNF-E2 CAT contains an enhancer that binds NF-E2 and AP-1. Plasmid pTAP-1 CAT contains an enhancer that binds AP-1 but not NF-E2. This is a representative CAT assay; the data were reproducible in independent transient expression studies.

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Gene expression and hemin inducibility of the CAT reporter gene (Fig 4). However, induction of this same pTNF-E2 CAT plasmid with zinc protoporphyrin IX (Fig 4) did not yield increased CAT expression in comparison to the basal uninduced level. In three independent experiments (using zinc protoporphyrin IX dissolved in Tris buffer), induction of K562 cells with zinc protoporphyrin IX did not increase CAT gene expression from the plasmid pTNF-E2 CAT above that of uninduced K562 cells (1.1 ± 0.15).

The plasmid pTAP-1 CAT containing a mutant version of the core NF-E2/AP-1 element of 5'HS2, which does not bind the transcription factor NF-E2, retains binding sites for AP-1 and remained inducible by hemin but not by zinc protoporphyrin IX (or other metalloporphyrins). However, the level of hemin inducibility was significantly decreased in comparison to hemin inducibility of pTNF-E2 CAT (Figs 4 and 5B).

The failure of hemin to induce CAT gene expression activity from the mutant plasmid pTAP-1 CAT to a level equal to that of the wild-type pTNF-E2 CAT plasmid is not secondary to a general decrease in the transcriptional activity of the mutant AP-1—only enhancer versus the wild-type AP-1/ NF-E2 enhancer. Induction of K562 cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which increases the transcriptional activity of AP-1 factors, results in equal stimulation of CAT gene expression from both the wild-type (AP-1/NF-E2) and mutant (AP-1) enhancers.

We controlled for a general effect of each of the inducing agents on K562 cells by normalizing the results of the CAT
assays to the results of luciferase assays. There was a reproducible fivefold to sevenfold preferential increase in hemin-induced expression of pTNF-E2 CAT in comparison to any general effect of hemin on expression of the coelectroporated RSV luciferase plasmid. However, normalization of the CAT/luciferase assays performed following induction of the coelectroporation of TAP-1 CAT/RSV luciferase plasmids resulted in a ratio of 1, which indicates that there was not a preferential effect of hemin induction on reporter gene expression in the absence of binding sites for NF-E2.

We were concerned that the failure of zinc protoporphyrin IX and other metalloporphyrins to induce expression of the highly hemin-inducible pTNF-E2 CAT construct was secondary to failure to dissolve adequately the metalloporphyrins. In subsequent experiments, we used a protocol for dissolving metalloporphyrins (see Materials and Methods for details) that has been used for production of metalloporphyrin solutions used for intravenous delivery to prevent neonatal kernicterus.29,30

Figure 5A demonstrates that there was no difference in the activity of hemin prepared by either of the protocols. The lane marked H1 represents K562 cells electroporated with TNF-E2 CAT and induced with the “standard” hemin preparation, and the lane marked H2 represents the same electroporation of K562 cells induced by the hemin prepared in ethanolamine. Either of the hemin preparations produced greater than a 30-fold induction of CAT activity compared with uninduced K562 cells. Indeed, in more than five independent experiments hemin (dissolved in ethanolamine) induction of K562 cells transiently expressing the TNF-E2 CAT plasmid always resulted in at least a 30-fold induction of CAT activity in comparison to uninduced K562 cells expressing the same TNF-E2 CAT plasmid. However, in four independent experiments K562 cells derived from electroporations of TNF-E2 induced with either tin or zinc protoporphyrin IX (dissolved in ethanolamine) did not show increases in CAT gene expression in comparison to uninduced K562 cells derived from the same electroporation.

Interestingly, induction of K562 cells expressing the TNF-E2 CAT plasmid with cobalt protoporphyrin IX resulted in a twofold to fivefold induction of TNF-E2 CAT activity compared with the CAT activity obtained from uninduced K562 cells. Furthermore, when the cobalt protoporphyrin...
IX–induced CAT activity was normalized to induction of coelectroporated RSV luciferase activity, there was at least a twofold preferential effect of cobalt protoporphyrin IX induction on the TNF-E2 CAT plasmid. It is possible that cobalt protoporphyrin IX can specifically increase gene expression when binding sites for NF-E2 are present in the transcriptional enhancing element. However, both the absolute CAT (twofold to fivefold) and preferential CAT/luciferase (twofold) increases in reporter gene expression produced by cobalt protoporphyrin IX induction are much less than the hemin-induced increases—30–to 50-fold and fivefold to sevenfold, respectively.

The transient transfection analysis indicates that all of the metalloporphyrins used as inducing agents, only hemin and to a lesser extent cobalt protoporphyrin IX (we cannot draw conclusions about protoporphyrin IX because of its toxic effects) are capable of increasing gene expression of transiently transfected CAT reporter plasmids containing binding sites for NF-E2 (Fig. 5C).

Since we measured CAT protein levels and not CAT mRNA levels, our results are confounded by the possibility that some of the effects of hemin, as well as other metalloporphyrins, can be exerted at the level of mRNA translation.

**DISCUSSION**

*Hemin and free protoporphyrin IX can increase expression of both endogenous and introduced genes in K562 cells.*

The experiments demonstrate that of the metalloporphyrins tested, only iron protoporphyrin IX (hemin) can increase endogenous globin gene expression in K562 cells and significantly increase expression of transiently transfected reporter plasmids containing the core NF-E2/AP-1 element of the 5′HS2 enhancer of the locus control region for β-like globin gene domain. Cobalt protoporphyrin IX induction did lead to some increase in the transcriptional regulatory activity of enhancers containing binding sites for NF-E2, albeit at a much lower level than did hemin. Protoporphyrin IX containing tin or zinc did not increase endogenous globin expression or expression of transiently transfected reporter genes.

K562 cells exposed to free protoporphyrin IX were induced to increase the number of benzidine-positive K562 cells, suggesting that free protoporphyrin IX could increase expression of endogenous globin genes. Although we exposed K562 cells to free protoporphyrin IX, it is possible that either in the media or upon entry into K562 cells free protoporphyrin IX was converted to hemin, because free protoporphyrin IX could come into contact with molecules of iron that could be inserted into protoporphyrin IX, creating molecules of hemin. We could not ascertain whether protoporphyrin IX was as active as hemin in increasing the expression of transiently transfected reporter genes, because free protoporphyrin IX is lethal to K562 cells within 48 hours of exposure.

One explanation for the inability of metalloporphyrins other than hemin to activate the hemin-responsive DNA element (T/CAGCTAGTCAC/T) via the transcription factor NF-E2 is the failure of metalloporphyrins to enter K562 cells. This explanation is not likely for a number of reasons. Metalloporphyrins are lipophilic molecules, i.e., they can enter the cell by an active diffusion process across the cell membrane. In addition, both cobalt and tin protoporphyrin IX have been shown to bind to hemopexin in vitro and are subsequently internalized by the hemopexin receptor via receptor-mediated endocytosis. Since 20-μmol/L concentrations of the metalloporphyrins were used for inducing the K562 cells, it is likely that all of the available hemopexin receptors were saturated, so that much of the metalloporphyrin uptake was due to its lipophilic properties.

*Hemin induction may increase the activity of transcription factors other than NF-E2 that bind to the 5′HS2 enhancer core in K562 cells.*

Whether the target of hemin induction at the core element of 5′HS2 of the locus control region for β-like globin gene domain expression is solely the transcription factor NF-E2 is not certain. Indeed, the expression of a reporter plasmid containing a mutant version of this core element that did not bind NF-E2 but did bind AP-1 was also inducible by hemin, but not by the other metalloporphyrins. However, our transient expression experiments measured the amount of CAT protein; it is therefore possible that some of the increase in CAT reporter plasmid expression was due to an effect of hemin on translation and not on transcription initiation or mRNA stability. Hemin is known to be a regulator of gene expression at the level of protein translation. The heme-regulated inhibitor (HRI) in its active form is a cyclic adenosine monophosphate (cAMP)-independent protein kinase that specifically phosphorylates eukaryotic initiation factor 2α, which leads to the shut-off of initiation of mRNA translation on ribosomes. In vitro, hemin appears to regulate the kinase activity of HRI by binding to a heme-binding site on the HRI molecule that promotes the formation of intramolecular disulfide bonds, resulting in the dimerization and inactivation of HRI. Cobalt protoporphyrin IX is at least as active as hemin in promoting the dimerization and inactivation of HRI and restoring protein synthesis in heme-deficient reticulocyte lysates.

Indeed, cobalt protoporphyrin IX induction of K562 cells transiently transfected with CAT reporter plasmids driven by enhancers activated by NF-E2/AP-1 (TNF-E2 CAT) or AP-1 only (TAP-1 CAT) resulted in a twofold to fivefold increase in the amount of CAT protein as compared with uninduced K562 cells derived from the same transfection. In hemin-replete reticulocyte lysates, addition of more hemin or cobalt protoporphyrin IX results in a twofold stimulation of translation. It is possible then that some of the increases in CAT protein levels in the cobalt protoporphyrin IX– and hemin-induced K562 cells are due to the action of cobalt protoporphyrin IX or hemin on increasing the translational initiation activity on ribosomes in K562 cells.

The hemin-induced 10-fold increase in gene expression of the TAP-1 CAT reporter plasmid containing an enhancer that binds AP-1 factors but not NF-E2 is not likely to be entirely due to boosting of the efficiency of initiation of mRNA translation by hemin. It is possible that a factor other than NF-E2 that binds to the core element of the 5′HS2 enhancer is also stimulated directly or indirectly to increase...
its transcriptional activity upon induction of K562 cells with hemin.

*Activation of transcription factors by hemin.* The transcription factor HAP1 found in the yeast *Saccharomyces cerevisiae* is a transcriptional activator that does not bind to DNA in the absence of hemin. Hemin is a direct allosteric effector of the activation of HAP1 binding to DNA, because there is a heme-binding domain within the HAP1 protein. Binding of hemin to its domain within HAP1 promotes dimerization of HAP1 proteins and consequent binding to DNA.55

The transcription factor that appears to be preferentially activated in our test system is not a zinc-finger factor like HAP1; instead, NF-E2 is an erythroid-specific member of the basic leucine zipper family of transcriptional regulatory factors. NF-E2 appears to bind to DNA as a heterodimer after association with a ubiquitous basic leucine zipper factor. We did not see an increase in binding activity of NF-E2 to DNA in gel shift assays using nuclear extracts obtained from hemin-induced K562 cells in comparison to nuclear extracts obtained from uninduced K562 cells.

Indeed, as noted by Peters et al.,61 a mutant and presumably transcriptionally inactive form of NF-E2 found in the microcytosis mouse binds to DNA as well as wild-type NF-E2. Interestingly, the point mutation in this inactive form of NF-E2 changes codon 173 valine to alanine. This amino acid change lies near a serine, which could be a target for phosphorylation perhaps by protein kinase A. If the increase in the transcription-activating ability of NF-E2 is not due to a change in its binding by hemin but to a protein kinase A–induced phosphorylation event occurring after binding to DNA, is this then consistent with our data demonstrating that of all the metalloporphyrins only hemin can induce the activation of NF-E2 as well as other transcription-activating proteins? Peripheral blood mononuclear cells exposed to hemin demonstrated a rapid increase in cAMP levels; peripheral blood mononuclear cells exposed to tin, cobalt, or zinc protoporphyrin IX did not show an increase of cAMP.57 cAMP is an allosteric effector of protein kinase A. Whether hemin can stimulate cAMP production and therefore protein kinase A in K562 cells and whether activation of protein kinase A causes a posttranslational modification of NF-E2 that stimulates its trans-activation ability remain to be clarified.56

*Significance of elevated levels of zinc protoporphyrin IX in the anemia of chronic inflammatory disease.* Anemias that result in decreased hemoglobinization of the erythrocyte are often secondary to a failure of production of hemin, either due to true iron deficiency or to failure of delivery of iron to siderocytes in the bone marrow. Whether there is an absence of or a relative deficiency in iron delivery to the siderocytes, the end result can be detected as an increase in the level of zinc protoporphyrin IX in erythrocytes and presumably their bone marrow precursor cells. Increases in erythrocyte zinc protoporphyrin IX are also found in individuals who suffer from lead poisoning. Our data would suggest that the failure to produce hemin results in a failure to activate NF-E2. Aside from being an indicator of the failure of heme synthesis, elevated levels of zinc protoporphyrin IX may also contribute to the decrease in globin synthesis in erythroid cells by failing to increase the trans-activation ability of NF-E2.

*Differential effects of the metalloporphyrins on the specificity of enhancer elements with binding sites for NF-E2 and accumulation of hemoglobin.* Since it is likely that NF-E2 is required for α- and β-globin gene expression, its restricted activation by hemin may reflect the unique ability of hemin to serve as a high-affinity oxygen-carrying moiety of hemoglobin. Indeed, cobalt protoporphyrin IX has been substituted for hemin in the hemoglobin molecule to produce coboglobin. The coboglobin tetramer can bind molecular oxygen in a cooperative and reversible fashion; however, its affinity for oxygen is approximately two orders of magnitude less than that of hemoglobin.36,39 Thus, it would not be efficient for cells to permit activation of transcription of the globin genes by cobalt protoporphyrin IX, whose analog cobalamin is found in all cells. It is also possible that hemin is an efficient activator of the transcriptional regulatory ability of NF-E2, because of all the metalloporphyrins, hemin is most effective at binding molecular oxygen, altering the redox state of the cell, and promoting the formation of intermolecular disulfide bonds. Indeed, the transcription-activating abilities of a number of transcription factors such as Jun and Fos have been shown to be contingent upon the redox state of the cell.40,41

The difference between the abilities of hemin and cobalt protoporphyrin IX and the failure of tin and zinc protoporphyrin IX to stimulate the transcriptional regulatory activity of enhancers containing binding sites for NF-E2 lead to a tentative model for the regulation of the trans-activating ability of NF-E2. We believe it is possible that hemin, and to some extent cobalt protoporphyrin IX, may directly interact with NF-E2 to increase the formation of disulfide bonds between the trans-activation domains of NF-E2 and other transcription-activating proteins, thereby stabilizing a transcription-activating complex. That hemin induction can also increase the activity of enhancer elements that bind only AP-1 factors could also be secondary to the formation of disulfide bonds stabilizing a transcriptional complex; however, the preferential effect of hemin on NF-E2 could be secondary to a specific interaction of hemin with a binding site within the trans-activation domain of NF-E2. Furthermore, as noted above, the effect of hemin may also be indirectly mediated through an increase in cAMP levels and consequent activation of protein kinase A.

We did not detect any preferential activation of the TNF-E2/AP-1 enhancer versus the TAP-1 enhancer upon induction of K562 cells with TPA, and this is likely to be the consequence of the increased binding of AP-1 factors to either of these enhancer elements. Yet, induction of K562 cells with TPA leads to a shut-off of globin gene expression. However, as demonstrated by Lumelsky and Forget and by our studies (submitted for publication), there is a drastic reduction in the half-lives of both α- and γ-globin mRNAs following TPA induction.42

As noted above, Chang and Sassa demonstrated that he-
min, and to some extent cobalt protoporphyrin IX, could stimulate hemoglobin accumulation in the MEL cell line.\(^6\) Our data extend this observation to the human K562 cell line. Of greatest importance is the finding that there is a correlation between the ability of a metalloporphyrin to stimulate gene expression activity from enhancer elements containing binding sites for NF-E2, the corresponding steady-state level of \(\gamma\)-globin mRNA, and the induction of a positive benzidine stain (which is a measure of endogenous heme accumulation). Taken together, the data indicate that the hemin-induced upregulation of the trans-activation ability of NF-E2 is a critical step in the synthesis of both hemin and globin in K562 cells. Uncovering the pathway of the activation of NF-E2 by hemin is feasible in view of the cloning of NF-E2.\(^5\)\(^6\) This will allow comparison of the effects of the various metalloporphyrins on possible posttranslational modifications of NF-E2 and interactions of NF-E2 with other transcriptional regulatory proteins.

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REFERENCES

Iron protoporphyrin IX (hemin) but not tin or zinc protoporphyrin IX can stimulate gene expression in K562 cells from enhancer elements containing binding sites for NF-E2

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