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REGULATION OF THE STABILITY OF CHICKEN EMBRYO LIVER δ-AMINOLEVULINATE SYNTHASE mRNA BY HEMIN

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The effect of hemin on the stability of δ-aminolevulinate (ALA) synthase mRNA was investigated in primary cultures of chicken embryo hepatocytes. Hepatocytes were first incubated with allylisopropylacetamide (AIA) to increase the starting level of ALA synthase mRNA, and then the cells were incubated with α-amanitin (1 μg/ml) to block further transcription of ALA synthase RNA. The rates of depletion of the enzyme's mRNA were then determined by liquid hybridization analyses in cells incubated with or without hemin (10 μM) in the culture medium. The analyses indicated that hemin increased the rate of ALA synthase mRNA degradation. The half-life of the messenger in hepatocytes incubated with hemin was 80 min compared with 220 min in cells incubated without additional hemin in the culture medium. The results thus indicated a novel mechanism by which hemin modulated the biogenesis of ALA synthase in liver.

δ-Aminolevulinate (ALA) synthase is a mitochondrial matrix enzyme (1) that catalyzes the condensation of glycine and succinyl CoA to yield ALA. The reaction is the first and, in liver, the rate limiting step for heme biosynthesis (reviewed in 2). The activity of liver ALA synthase can become elevated significantly when experimental animals are exposed to any of a relatively large number of porphyrogenic agents. The regulation of biogenesis of ALA synthase, especially under the influences of porphyrogenic agents, has been studied most extensively in the chicken embryo (reviewed in 3 and 4).

Exposure of chicken embryo hepatocytes, either in ovo or in culture, to any of a number of porphyrogenic agents can lead to a significant elevation in the cellular concentration of the enzyme's mRNA (5-8), primarily as a result of an increase in the rate of transcription of ALA synthase RNA (6, 7, 9). The resulting elevation in the enzyme's mRNA concentration leads to increased rates of production of the enzyme and to a rise in its mass and activity in the cells (10).

The induction of ALA synthase by a porphyrogenic agent can be blocked by elevations in hemin concentration in the hepatocytes. Hemin, either provided in the culture medium or generated in cells from ALA, lowers the enzyme's mRNA concentration (6-9) and completely blocks the maturation of a precursor form of the enzyme (10-12). This phenomenon presumably reflects the normal end-product regulation of tetrapyrrole synthesis

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in liver, where excess intracellular hemin negatively affects the function of the pace-setting enzyme of the pathway.

The mechanism through which hemin affects the concentration of ALA synthase mRNA has not been determined. Hemin may modulate the level of the messenger either by inhibiting the production of the molecule or by stimulating its degradation. Recently, Srivastava et al. (9) suggested, on the basis of nuclear transcriptional run-off measurements on rat liver nuclei, that hemin is a negative modulator of ALA synthase RNA transcription. In this study, we report that hemin decreases the stability of ALA synthase mRNA in chicken embryo hepatocytes.

EXPERIMENTAL PROCEDURES

Preparation of Hepatocyte Cultures: Primary hepatocyte cultures were prepared from 16-day-old chicken embryos (White Leghorn) as described previously (11). Hepatocytes were initially maintained for 16 h in modified Ham's F-12 medium containing 10% fetal calf serum at 37°C in a humidified CO_2 incubator. The medium was then replaced with modified Ham's F-12 medium containing 0.2 mg insulin and 250 mg of desferoxamine mesylate per liter. Two hours later, the medium was replaced with modified Ham's F-12/insulin medium; α-amanitin, allylisopropylacetamide (AIA), and hemin were added to the medium after that point as indicated in the figure legends. When used, the agents were added to the medium at the following final concentrations: α-amanitin (1 μg/ml), AIA (75 μg/ml), and hemin (10 μM).

Isolation of RNA and Liquid Hybridization Analyses: RNA was isolated from hepatocytes as described by Drew and Ades (6). Liquid hybridization analyses of RNA were performed as described previously (6). Basically, a 24-base oligonucleotide probe complementary to ALA synthase mRNA was end-labelled with ^{32}P (specific activity of 2-4 x 10^6 cpm/pmol) using T4 polynucleotide kinase (6). The hybridizations were performed at 55°C for 18 h in the presence of 4000-8000 cpm (constant for a given determination) of the labelled probe and the amount of RNA indicated. After the hybridization, single stranded nucleic acids were digested with S_1 nuclease, and the acid-insoluble material was collected by filtration and processed for determining its ^{32}P content (6).

Northern Analyses of RNA: RNA was separated on 1.2% agarose gels containing 0.37 M formaldehyde as described by Ausubel et al. (13). Briefly, samples of total RNA were placed in loading buffer [MOPS electrophoresis buffer (13) containing bromphenol blue, 48% formamide (deionized), 2.2 M formaldehyde, and 5% glycerol] and the RNA was denatured at 95°C for 2 min then loaded into wells of the gel. Following electrophoresis, the RNA was transferred by capillary action onto a nitrocellulose membrane (13) and affixed to the membrane by baking for 2 h at 80°C under vacuum. Pre-hybridization and hybridization of RNA to the ^{32}P-labelled probe (prepared as outlined above) were performed as described by Davis et al. (14). Following pre-hybridization, the membrane was incubated for 20 h at 42°C in hybridization buffer containing 10^6 cpm/ml of ^{32}P-labelled oligonucleotide. The membrane was then washed four times at 42°C (30 min per wash) in 300 mM NaCl, 30 mM sodium citrate, pH 7.0, and placed against X-ray film.

RESULTS AND DISCUSSION

The purpose of this study was to determine the effect of hemin on ALA synthase mRNA stability. In previous work, the half-life of the messenger was estimated to be 5.2 h (15). DNA probes complementary to ALA synthase mRNA sequence had not been developed at that time, however, so it was necessary to determine the half-life of the messenger indirectly from the rates of change of ALA synthase activity in hepatocytes incubated with actinomycin D. We did not apply that approach to measure the effect of

hemin on ALA synthase mRNA stability because hemin affects the activity of the enzyme by blocking its maturation, leading to a rapid degradation of its precursor (11).

The specificity of the probe used in the studies described here was tested by Northern-blot analyses. As seen in Figure 1, the probe bound to a 2.3 KB RNA, consistent with the known size of the messenger of liver ALA synthase (16). The concentration of the messenger became significantly elevated in livers of embryos treated with AIA plus diethyl 1,4-dihydro-2,4,6-trimethyl,3,5-pyridine dicarboxylate (DDC) as has been established previously (5, 6), and the rise was less dramatic in embryos given the relatively weak porphyrogenic agent testosterone. Consistent with the results of previous studies (6, 7),

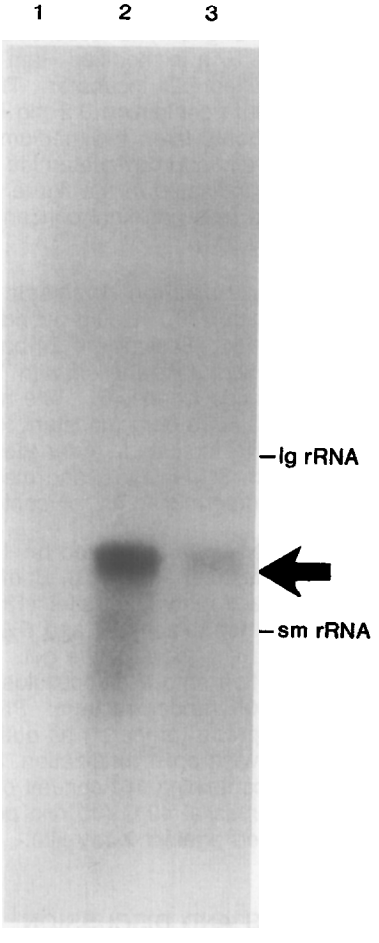

Figure 1. Northern analysis of liver RNA. Eighteen-day-old chicken embryos were each given 2 mg of AIA plus 4 mg of DDC or given 5 mg of testosterone and then incubated for 14 h at 38°C. In each case, the drugs were dissolved in 0.1 ml of dimethylsulfoxide and injected into the fluid surrounding the embryo. Control animals received the vehicle only. At the end of the incubation, the livers were removed and used for RNA isolation. At least three livers were pooled for each determination. The analyses were conducted as described in Experimental Procedures, with 10 µg of RNA being used for each of the following conditions: lane 1 = control; lane 2 = AIA + DDC; lane 3 = testosterone. The position of ribosomal RNA markers are indicated on the figure, and the arrow points to the position of a 2.3 KB RNA.

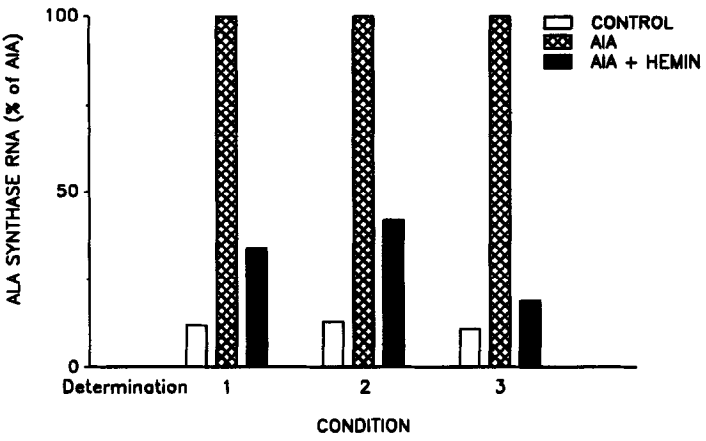

Figure 2. Effect of hemin on ALA synthase RNA. Primary cultures of hepatocytes were prepared from 30-36 embryos. The cells were incubated for 5 h with AIA in the presence or absence of hemin in the culture medium under the conditions described in Experimental Procedures. Control cultures were incubated for the 5 h without added hemin or AIA. At the end of the incubation, total RNA was extracted from the cells and analyzed for ALA synthase-specific sequences by liquid hybridization. The values represent in each case the average cpm of acid-insoluble, ^{32}P-labeled, cDNA obtained after treatment of the hybridization reaction with S_1 nuclease. The values are expressed as the percent of those obtained with RNA from cultures treated with AIA alone. The hybridizations were performed in triplicates for each condition, with 30 µg of RNA per reaction. The results of three independent determinations are shown. The values obtained with RNA from AIA treated cells were 1900 cpm, 1000 cpm, and 2600 cpm for determinations 1-3, respectively. The differences were due primarily to the specific activities of the probes used in the analyses.

hemin limited the elevation in ALA synthase RNA in hepatocytes incubated in primary cultures with AIA, a potent porphyrogenic agent (Fig. 2).

In the studies designed to investigate the effect of hemin on ALA synthase mRNA stability, hepatocytes were first treated with AIA to raise the starting concentrations of ALA synthase mRNA to more easily measurable levels. Further transcription was then blocked by α-amanitin and the levels of ALA synthase mRNA were measured over time in cells incubated with and without hemin. As seen in Figure 3, the concentration of α-amanitin used efficiently blocked the increase in ALA synthase mRNA brought about by the inducer.

To determine whether hemin affected the stability of ALA synthase mRNA, hepatocytes were incubated with α-amanitin then hemin was added to some of the cells. RNA was isolated from cells at various points after an incubation with or without hemin. The RNA was analyzed for ALA synthase-specific sequences by liquid hybridization. As seen in Figure 4, the rate of decay of ALA synthase mRNA was significantly higher in cells incubated with hemin. The half-life of the messenger was 80 min in the presence of hemin compared to 220 min in its absence. Thus, excess hemin increased the rate of degradation of the messenger. This is a novel phenomenon in the regulation of ALA synthase production.

Chicken liver ALA synthase turns over with a half-life of 120 minutes (10), a rate considerably faster than those of most mitochondrial proteins (17). This suggests that the activity of the enzyme could be regulated quite effectively at the level of production. While porphyrogenic agents increase the production of ALA synthase primarily, if not solely, by

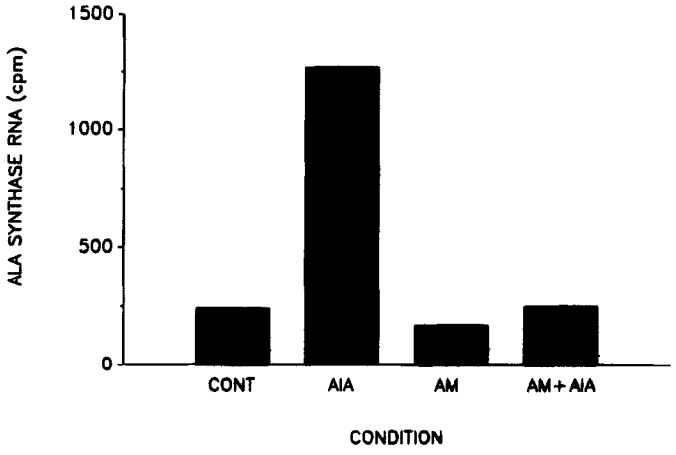

Figure 3. Effects of transcriptional inhibition on the induction of ALA synthase RNA. Hepatocytes in culture, prepared from livers pooled from 20 embryos, were incubated for one hour with α-amanitin (AM) and then AIA was added to some of the cultures. Control cells were not treated with α-amanitin or AIA. The cells were incubated for 6 h, and then total RNA was isolated and analyzed by liquid hybridization. Hybridization assays were performed in triplicates, with 20 μg of RNA used in each reaction. The values indicate the cpm of acid-insoluble, ^{32}P-labeled, nucleic acids remaining after treatment of the hybridization reactions with S_1 nuclease.

increasing the rate of transcription of the enzyme's messenger, elevated intracellular hemin concentration blocks the production of the enzyme at a number of levels: by inhibiting the transcription of the enzyme's RNA (9), by preventing the maturation of pre-ALA synthase (10, 11), and -- as reported here -- by stimulating the degradation of the enzyme's mRNA.

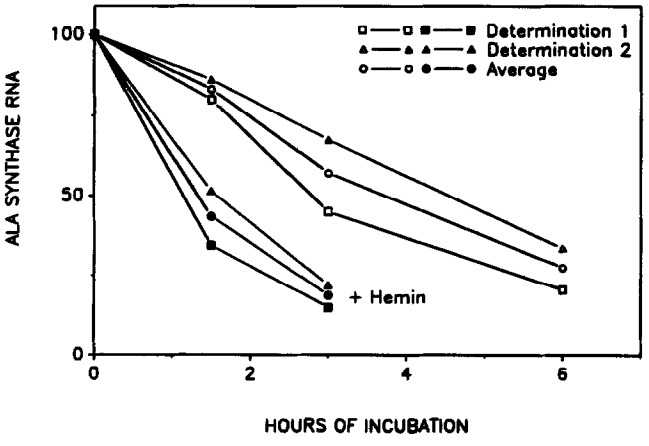

Figure 4. Effects of hemin on the stability of ALA synthase RNA. Hepatocyte cultures were incubated for 4 h with AIA. The medium was then changed to Ham's F-12/insulin containing α-amanitin. One hour later, hemin was added to some of the cultures. The cells were incubated for the indicated periods then processed for RNA isolation. The RNA was analyzed for ALA synthase sequences by liquid hybridization. The time point at which hemin was added is indicated as 0 h on the figure. Hybridization assays were performed in triplicates with 20 μg of RNA used per reaction. The values are expressed relative to those obtained at the time of hemin addition (0 h). The results of two independent determinations are presented in the figure. Hepatocytes from 35 embryos were pooled for each determination. 100% ALA synthase RNA represents 2000 cpm in Determination 1 and 2200 cpm in Determination 2.

The half-life of the messenger in hapatocytes, whether in the presence or absence of excess hemin, is shorter than the average half-life for mRNA molecules in eukaryotic cells (18 and 19). AU-rich sequences at the 3'ends of eukaryotic mRNA molecules have been identified as important in increasing the rates of decay of the messengers (20, 21). Interestingly, AU-rich sequences are present also at the 3' untranslated end of ALA synthase mRNA and may be responsible for the relative instability of that messenger.

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