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Isolation of recombinant cDNAs encoding chicken erythroid δ-aminolevulinate synthase

(antibody to δ-aminolevulinate synthase/red cell expression library)

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ABSTRACT **We report the isolation of cDNA clones encoding δ-aminolevulinate synthase (ALA synthase; EC 2.3.1.37), the first enzyme in the heme biosynthetic pathway in animal cells. The gene was isolated from a chicken erythroid cDNA library prepared in the bacteriophage λ fusion/expression vector gt11, using rabbit antibody raised against the relatively abundant chicken liver enzyme. The chicken liver and red cell ALA synthase isozymes share substantial crossreactivity to the antibody, thereby allowing isolation of the erythroid-specific gene by using the heterologous antibody in immune screening of the red cell cDNA library. Preliminary analysis documenting the tissue specificity of transcription indicates that the enzyme is encoded by a highly homologous set of messages, which appear to differ in size in various avian tissues. From analysis using strand-specific RNA probes, it appears that the different ALA synthase mRNAs detected may be transcribed from a family of genes that are closely related in nucleotide sequence and are each regulated in a developmentally specific manner.**

δ-Aminolevulinate synthase (ALA synthase; EC 2.3.1.37), the first enzyme in the biosynthetic pathway leading to heme production, catalyzes the condensation of glycine and succinyl-CoA to form δ-aminolevulinic acid (1). The mRNA for the enzyme is encoded in the nucleus, from which it is transported to the cytoplasm, where it is translated into an enzymatically active precursor form. The ALA synthase preenzyme is processed into the mature mitochondrial enzyme (a proteolytic cleavage product of the preenzyme) as it traverses the mitochondrial membrane, where it finally occupies its normal cellular compartment (2).

Various studies have previously shown that both the ALA synthase gene and the enzyme are subject to a complex array of regulatory interactions. Thus, the enzyme form found in the liver is responsive to feedback regulation by the end product (heme) and can be induced by a variety of chemical effectors of hepatic porphyria, notably 3,5-dicarbethoxy-1,4-dihydrocollidine and allylisopropylacetamide. Furthermore, this induction appears to be regulated at both the transcriptional and translational levels (3–11). In the liver, heme also appears to directly interfere with the translocation of the preenzyme from the cytoplasmic to the mitochondrial compartments, causing abnormal increases in the cytoplasmic levels of the preenzyme (12, 13).

In contrast to these observations in hepatic tissue, the erythroid form of the enzyme is unresponsive to chemicals that normally induce porphyria, and this isozyme does not appear to accumulate in the cytoplasm of erythrocytes on treatment of animals with the same porphyrogenic agents (14–18). Instead, the biosynthesis of ALA synthase in erythroid cells appears to be strongly stimulated by chemical agents normally used for induction of anemia [e.g., phenylhydrazine (G. Kikuchi and M. Hasegawa, personal communication)]. Thus the enzyme activity appears to be regulated in a cell-specific manner. In keeping with the findings that there exist differentially regulated ALA synthase enzymes, it has been reported that the liver and erythroid counterparts of the enzyme also differ in size, both as preenzymes and as mature proteins (19).

We are interested in studying the regulatory mechanisms whereby ALA synthase becomes activated in chicken erythroid and liver cells in a developmentally specific manner. Furthermore, one report has claimed that ALA synthase is one of the earliest erythroid genes that is transcriptionally activated after dimethyl sulfoxide treatment of Friend erythroleukemia cells, thus suggesting the intriguing possibility that ALA synthase induction might serve as an early temporal marker for erythrocyte maturation (20). This served as an additional incentive to examine the regulation of this particular erythroid-specific gene in detail.

MATERIALS AND METHODS

Bacteriophage and Host Strains. Bacteriophage λ vectors gt10 and gt11 and lysogenic and lytic *Esherichia coli* host strains (Y1089, Y1090) were obtained from Tom St. John (Department of Pathology, Stanford University Medical School) (21, 22), and strain BSJ37 (23) was obtained from Ed Fritsch (Genetics Institute).

Erythroid cDNA Library Preparation and Screening. cDNA was prepared as described (ref. 24, pp. 230–238), and *Eco*RI linkers (Bethesda Research Laboratories) were ligated to the mixture of erythroid cDNAs. The linkers were then cleaved with *Eco*RI to reveal the cohesive ends, and the cDNAs were fractionated by gel electrophoresis into populations that were greater than and less than 800 base pairs (bp). The two pools were individually collected and separately ligated to λ gt10 DNA treated with *Eco*RI. The ligated "small" (i.e., <800-bp) and "large" (>800-bp) cDNA pools were then packaged *in vitro* (ref. 24, pp. 291–292) and plated on *hfl* host BSJ37. Since the packaged phage that contain recombinant cDNAs will not integrate in the *hfl* strain (the *Eco*RI inserts interrupt the λ *c*I gene, whose expression is essential for lysogeny) only recombinant bacteriophage efficiently produce plaques. The titers of the two libraries were 1.7×10^6 plaque-forming units (pfu) for the large inserts (*ca.* 10^4 pfu of cI^- revertants) and *ca.* 2.8×10^6 pfu for the small fragment library. The large insert library was amplified at 2×10^4 pfu/150-mm dish for 4 hr at 42°C on Y1090; the amplified phage were extracted from top agarose as previously described (25, 26). The phage

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Abbreviations: ALA synthase; δ-aminolevulinate synthase; bp, base pair(s); pfu, plaque-forming units.

were then purified by cesium chloride equilibrium gradient centrifugation; DNA was isolated from the phage by addition of sodium dodecyl sulfate and proteinase K as described (ref. 24, p. 85).

The purified λ gt10 library was transferred into λ gt11 by treatment of the λ gt10 DNA cohesive ends with nuclease *Bal* 31 until *in vitro* packaging efficiency of the gt10 DNA had dropped by a factor of at least 10^4 (T. St. John, J. Rosen, and H. Gershenfeld, personal communication). The exonuclease-treated gt10 phage pool was then digested with *Eco*RI and added to ligated λ gt11 arms that had been digested with *Eco*RI and dephosphorylated by using column-purified calf intestine alkaline phosphatase (27). The phage were ligated at a roughly 2:1 weight ratio (λ gt10 library to λ gt11 arms) and packaged *in vitro*. Packaging efficiency was usually about 10^6 pfu/μg of total DNA added, resulting in 12–40% recombinants as judged by clear plaque formation on 5-chloro-4-bromo-3-indolyl β-D-galactoside (X-Gal) plates. We usually chose ligation ratios that gave about 20% recombinants, to avoid multiple cDNA inserts.

λ gt11 recombinants were plated for screening in top agarose at *ca.* 5×10^4 pfu per 150-mm Petri dish on host strain Y1090 (21). The plates were incubated for 4 hr at 42°C and then overlaid with nitrocellulose filters that had been soaked in 10 mM isopropyl β-D-thiogalactoside and dried. The overlays were then incubated for 4–12 hr more. The filters were then removed into a blocking solution (3% nonfat dry milk plus 0.1% Nonidet P-40 in Tris-buffered saline [20 mM Tris·HCl, pH 7.5/0.5 M NaCl/0.02% sodium azide (3% milk solution)] and placed on a rotator to agitate for 6–12 hr at 4°C in the presence of a sonic lysate of λ gt11-infected Y1090 cells (all subsequent treatment of the filters was also performed at 4°C). Anti-ALA synthase antibody was then added to the filters at a final concentration of 5 μg/ml, and the filters were allowed to bind to the antibody for an additional 6 hr. The filters were then removed from the antibody solution, washed four times for 30 min each in 3% milk solution, and then moved to a second dish, containing ^{125}I-labeled goat antibodies to rabbit IgG (5×10^5 cpm/ml). The filters were bound to the second antibody for 2–3 hr and again washed four times for 30 min in 3% milk solution. The filters were then dried and exposed for autoradiography at −70°C for 12–18 hr. Recombinant plaques were purified as previously described (26).

Antibodies. The antibody recognizing chicken liver ALA synthase was raised in rabbits; the specificity of this antibody has been previously characterized by demonstration that dilute antibody is able to specifically inhibit the enzymatic condensation reaction when presented with partially purified ALA synthase from either chicken liver or red blood cells (19). The antibody was purified by binding to, and elution from, staphylococcal protein A-Sepharose CL-4B (Pharmacia); we found this to be the minimal purification necessary to reduce background adequately for successful antibody screening. Prior to their use in screening, antibodies were treated by preabsorption with a λ gt11-infected Y1090 sonic lysate for a minimum of 12 hr at 4°C (22). Iodinated, affinity-purified, goat antibody to rabbit IgG was the generous gift of Susan K. Pierce (Northwestern University).

Other Methods. Positive hybrid selection and *in vitro* translation of the released mRNA was performed by modification (28) of the original method (29), adapted to the use of single-stranded RNA bound to filters. Rabbit reticulocyte lysate used for *in vitro* translation reactions was from Promega Biotec (Madison, WI) and was used according to the supplier's specifications. Subcloning in Sp6 vector pSp65 (30) was as previously described, as was the preparation of DNA template for the Sp6 *in vitro* transcription reactions; RNA blots were also performed as previously described (31). Isotopically labeled precursor amino acids and nucleotides were purchased from Amersham; labeled and unlabeled protein standards for gel electrophoresis were purchased from Amersham and Sigma, respectively. Protein blotting was performed as specified (32), with the exceptions that the filters were first blocked in 3% milk solution (above) and then washes after the first and second antibody binding reactions were in Tris-buffered saline/0.5% Triton X-100.

RESULTS

Characteristics of the Anti-ALA Synthase Antibody. To ensure that the antibody raised against the chicken liver form of ALA synthase would be successful for isolation of the erythroid ALA synthase gene in antibody screening, two experiments were undertaken to test the affinity of the antibody preparation to erythroid ALA synthase. In the first experiment, ALA synthase from *in vitro* translated anemic chicken red blood cell poly(A)$^+$ RNA was immunoprecipitated by using the antibody and protein A-Sepharose. The immunoprecipitated [^{35}S]methionine-labeled erythroid preenzyme was recovered in high yield from an *in vitro* translation mixture of total reticulocyte poly(A)$^+$ mRNA, as expected (Fig. 1; ref. 19). While it is clear that erythroid ALA synthase is the only protein recognized in immature erythroid cells, a clearly different molecular weight species of ALA synthase is immunoprecipitated from committed erythroid progenitor cells (Fig. 1, lane C). Whether this implies that these virally transformed cells exhibit ambiguous developmental properties in their pattern of gene expression or are developmentally "switched" in the type of ALA synthase isozyme expressed during erythroid maturation is currently unknown (see *Discussion*).

A second, more salient, experiment was performed to ask if ALA synthase would associate with the antibody after the enzyme was immobilized on nitrocellulose filter blots (32). The second experiment successfully demonstrated that the erythroid ALA synthase strongly associates with the antibody raised against the liver isozyme when the antigen is fixed to filters (Fig. 2).

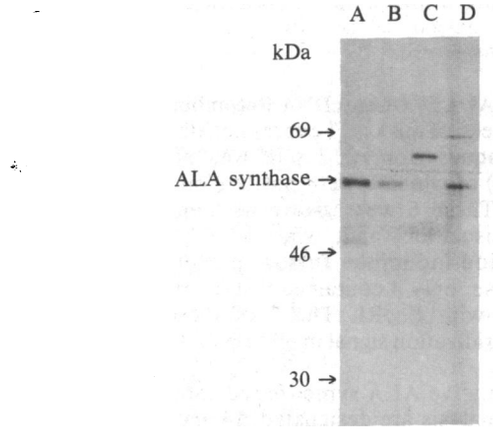

FIG. 1. Immunoprecipitation of ALA synthase from *in vitro* translation of chicken red cell RNA. Chicken red cell polysomes (0.5 A_{260} unit each) isolated from 11-day embryonic (lane A) or anemic adult (lane B) erythroid cells were translated in the presence of [^{35}S]methionine. Poly(A)$^+$ RNA from HD6 cells (tsAEV-transformed early erythroid lineage precursor cells; ref. 33) and from anemic adult chicken red blood cells (lanes C and D, respectively) was translated *in vitro*, also in the presence of radiolabeled methionine. All four samples were then immunoprecipitated from the translation reaction mixtures by using rabbit IgG antibodies to chicken liver ALA synthase (19) and protein A-Sepharose. These precipitates were then electrophoresed on 10% polyacrylamide/0.1% sodium dodecyl sulfate gels (34), fixed and bathed in EN^{3}HANCE (Amersham), dried, and exposed for fluorography. Sizes were derived by comparison of the electrophoretic mobility of ^{14}C-labeled protein standards run in a parallel lane of the same gel.

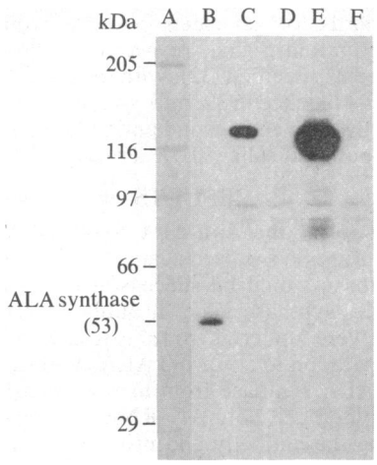

FIG. 2. Immunological detection of ALA synthase β-galactosidase fusion proteins in λ gt11 recombinants. Recombinant bacteriophage that were repeatedly positive on plaque purification were integrated into a lysogenic host strain (Y1089; ref. 21). The lysogenic cells were grown to $OD_{600} = 0.3$ at 32°C, at which time lytic replication was induced by shifting the temperature to 42°C for 15 min; after culturing for an additional 2 hr at 38°C, the cells were harvested and immediately lysed in sodium dodecyl sulfate sample buffer (50 mM Tris·HCl, pH 6.8/1.5% sodium dodecyl sulfate/50 mM dithiothreitol/4 M urea). Identical amounts of protein synthesized by induced λ lysogens [either treated (lanes C and E) or not treated (lanes D and F) by the addition of isopropyl thiogalactoside to 1 mM final concentration at the time of heat induction] were electrophoresed on 7.5% polyacrylamide/0.1% sodium dodecyl sulfate gels (34). The protein was then electrophoretically transferred to nitrocellulose as described (32). The nitrocellulose transfer was then blocked with 3% milk solution, and bound protein was allowed to react with IgG antibody to chicken liver ALA synthase; the filter was washed, exposed to affinity-purified goat ^{125}I-labeled antibody to rabbit IgG, washed, and exposed for autoradiography. Lane A is a parallel lane on the protein blot stained with india ink (35) to determine the position of commercial markers. Lane B contained total mitochondrial matrix proteins from anemic chicken red blood cells, bound to antibody at the same time as were the lysogenic bacterial proteins.

Isolation of ALA Synthase cDNA Recombinants. In our first successful screen of the λ gt11 expression library, we isolated 14 positive plaques (from *ca.* 2×10^6 total pfu screened; 28% recombinants) of which 6 were positive on repeated plaque purification. These 6 were grown as minipreparations for phage DNA isolation, and, while all 6 yielded isopropyl thiogalactoside-inducible fusion proteins larger than β-galactosidase, only 4 contained inserts that were released by digestion with *Eco*RI. The 2 of these that gave the strongest hybridization signal in protein blots were characterized further.

The two putative ALA synthase recombinants chosen for subsequent analysis are designated A4 and A14; the size of the inserts in these two recombinants are *ca.* 530 and 190 bp, respectively. [The fact that these recombinants contain cDNA sequences that are unexpectedly small (since the cDNAs were initially selected to contain inserts >800 bp) suggests that the size fractionation of the cDNA population was not accurate.] As an initial control, we first showed that the putative ALA synthase λ recombinants would produce the antigenically responsive determinant under β-galactosidase control, as expected of an ALA synthase hybrid fusion protein, and that the resultant fusion product was larger than native β-galactosidase. As shown in Fig. 2, the antibody binds proteins that are highly induced after treatment of recombinant A4 and A14 lysogens with isopropyl thiogalactoside (lanes C–F), and both fusion proteins are larger than the native β-galactosidase marker (116 kDa, lane A). Furthermore, as stated in the previous section, the 53-kDa erythroid ALA synthase enzyme (from whole red blood cell mitochondrial matrix) is the only protein recognized in a parallel lane on the same blot (Fig. 2, lane B).

Recombinant A4 Encodes Erythroid ALA Synthase. To prove that the isolated recombinants encode the red cell-specific ALA synthase protein, two complementary experiments were performed. First, if the two recombinants that gave strong antibody signals in protein blots (Fig. 2) both actually code for different or overlapping segments of the same structural gene, three criteria should be met for the corresponding reticulocyte mRNA encoding ALA synthase: recombinants A4 and A14 should hybridize to an RNA of the same size in RNA blotting analysis, hybridization to those red cell mRNAs should be strand specific, and finally, the mRNA that hybridizes to the strand-specific probes must be large enough to encode the erythroid ALA synthase preenzyme of 55-kDa (corresponding to a minimum mRNA size of 1500 nucleotides; ref. 19). The second experiment to verify the identification of these recombinants would be that ALA synthase mRNA should be selectable, in a strand-specific manner, from a population of total red blood cell poly(A)$^+$ RNAs, and that the *in vitro* translation product of that mRNA filter selection should be precipitable with the anti-ALA synthase antibody and should correspond in size to the precursor form of the enzyme found in red cells.

The results of RNA blot analysis are shown in Fig. 3, in which strand-specific radiolabeled probes have been hybridized to identical lanes of anemic adult chicken red blood cell poly(A)$^+$ RNAs. Strand-specific probes were created by subcloning the recombinant *Eco*RI inserts from the A4 and A14 phage in the vector pSp65 (30) in both orientations. The subclones were individually cleaved distal to the Sp6 promoter and recombinant segments of the ALA synthase gene (inserted at the *Eco*RI site of pSp65) and then transcribed by using Sp6 RNA polymerase and radiolabeled nucleotide precursors as previously described (31). The four individual

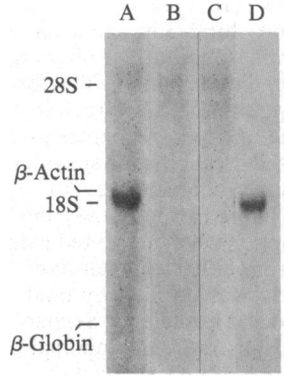

FIG. 3. Blot analysis of erythroid ALA synthase mRNA with strand-specific probes. One microgram of poly(A)$^+$ RNA isolated from the circulating red cells of anemic adult hens was electrophoresed on identical lanes of a vertical 1.2% formaldehyde agarose gels (24); the RNA was transferred to nitrocellulose, baked, and prehybridized as described (31). The inserts from recombinant bacteriophage A4 and A14 were excised with *Eco*RI and subcloned in pSp65 (30) in both fragment orientations. Both orientations of both parental recombinant cDNAs were transcribed in the presence of [α-^{32}P]GTP and Sp6 polymerase (31). These transcripts [called A14e (lane A), A14f (lane B), A4b (lane C), and A41 (lane D)] were then hybridized to individual lanes of red cell poly(A)$^+$ RNA at 55°C, washed in the presence of RNase A (31, 36) at 4 μg/ml, and exposed for autoradiography. Exposure times were 15 hr (lanes A and C) or 5 hr (lanes B and D). Identical lanes were hybridized to λGd1 [a chicken ribosomal gene recombinant (37)], pβ1BR15 [adult chicken β-globin genomic subclone (27)], and a β-actin cDNA clone (38) for internal size standardization (data not shown).

transcripts were then hybridized to separate lanes containing red blood cell mRNA; the blots were then treated with RNase (31) and exposed for autoradiography. As shown in Fig. 3, the two recombinants both recognize an mRNA of the same size (in only one of the two strand-specific probe orientations). The mRNA recognized by the two different recombinants is easily large enough (approximately 2000 nucleotides) to encode a protein of 55 kDa.

Additional proof that A4 encodes erythroid ALA synthase is provided by the hybrid selection/translation experiment shown in Fig. 4. Since we demonstrated above that the transcript produced by Sp6 subclone A41 was complementary to the putative ALA synthase mRNA, this implies that an unlabeled strand-specific transcript of that recombinant, when fixed to filters, should be able to preferentially select an mRNA whose product in an *in vitro* translation reaction should be immunoprecipitable with the anti-chicken liver ALA synthase antibody. Fig. 4 shows the results of such an experiment, in which the mRNA for the preenzyme was indeed selected from total red cell poly(A)$^+$ RNA with the synthetic Sp6 transcript from clone A41 but was not selected with the synthetic Sp6 RNA transcript from the complementary strand (prepared from subclone A4b). Thus, recombinant A4 does contain at least part of the erythroid ALA synthase gene.

Tissue Specificity of ALA Synthase Gene Expression. To determine whether or not we could distinguish between the various tissue-specific forms of ALA synthase at the mRNA level, further RNA blot analyses were performed. It was anticipated that, on the basis of these experiments, we could gain some insight as to whether or not the ALA synthase mRNAs transcribed in various chicken tissue and cell types were the same or different sizes [correlating with the different sizes of the proteins (19)] and that we might be able to make preliminary arguments regarding the possibility that the tissue-specific ALA synthase enzymes were transcribed from the same, or different gene(s). We again made use of the strand-specific Sp6 transcript complementary to erythroid ALA synthase mRNA. One might expect that if the red cell and liver (and perhaps other) forms of ALA synthase were encoded by different genes, they might have diverged sufficiently in nucleic acid sequence that a probe synthesized from one gene would have only partial homology to any or all of the heterologous gene(s). On the other hand, if various tissue-specific forms of ALA synthase mRNA were found to be highly homologous to a single conserved (coding sequence) probe, that would leave open the possibility that the tissue specificity of ALA synthase arises by differential cell-specific processing of transcripts produced from the same genetic locus.

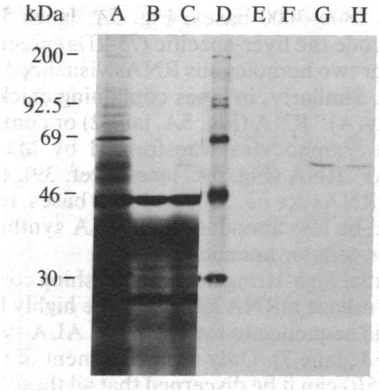

FIG. 4. Hybrid-release *in vitro* translation and immunoprecipitation with anti-ALA synthase antibody. Ten micrograms of red blood cell poly(A)$^+$ RNA (isolated from anemic adult hens) was hybridized to strand-specific Sp6 transcripts of recombinant subclone A4 fixed on nitrocellulose filters. The hybridized mRNA was released from the filters by brief boiling and then translated *in vitro* (28, 29). The *in vitro* translation reactions are shown after the following procedures: lane A, no filter selection (total red blood cell mRNA); lane B, hybridization of total red blood cell mRNA and release from filter-bound Sp6 transcript A4b; and lane C, hybridization and release from Sp6 transcript A41 (lane D shows the mobility of the ^{14}C-labeled protein markers on this 12.5% polyacrylamide/0.1% sodium dodecyl sulfate gel). Final lanes depict the immunoprecipitation reaction of total red cell [^{35}S]methionine-labeled protein after treatment of the translation reaction mixtures with either anti-ALA synthase antibody and protein A-Sepharose (lane H) or protein A-Sepharose alone (lane E). Lanes F and G show the results of immunoprecipitation of the translation products of lanes B and C, respectively, with anti-ALA synthase antibody and protein A-Sepharose.

The results of RNA blot analysis of various poly(A)$^+$ mRNAs isolated from cell lines and various chicken tissues are shown in Fig. 5. Fig. 5*A* shows the hybridization pattern of radiolabeled Sp6 A41 transcript to the mRNAs in a standard "high-stringency" wash solution (equivalent to 10 mM monovalent cation; 52°C) and Fig. 5*B* shows the same blot washed in a solution containing RNase A at 30°C. [We have previously established that the latter condition favors the release of all but specifically bound RNA probes in such blotting experiments (31)]. As is readily apparent from the data presented in Fig. 5, the RNA (probe)·RNA (mRNA) hybrids from different tissues are clearly distinguishable (*A*). Furthermore, the size of the mRNAs homologous to the erythroid-specific probe varies according to tissue and cell type. Thus, the largest cellular RNA that is detectable in

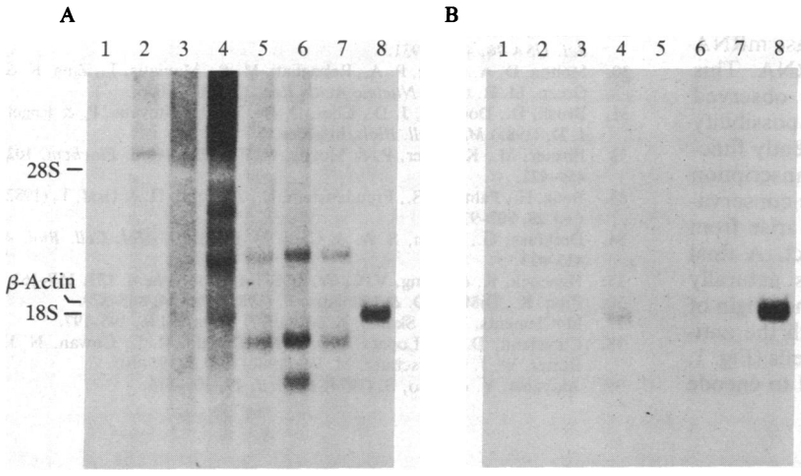

FIG. 5. Developmental specificity of ALA synthase mRNA synthesis. RNA was isolated from various chicken tissues and cell lines by banding of guanidinium thiocyanate-isolated RNA and poly(A)$^+$ RNA was isolated by two cycles of oligo(dT)-cellulose chromatography (31). These RNAs were electrophoresed, blotted, and hybridized to radiolabeled subclone A41 Sp6 transcript (see legend to Fig. 3). Chicken cellular RNA samples were isolated from the following sources: lane 1, MSB-1 cells (39); lane 2, 11-day chicken embryo fibroblasts; lane 3, 4.5-day whole chicken embryos; lane 4, 11-day embryonic brain; lane 5, 17-day embryonic liver; lane 6, 1-mo-old chick liver; lane 7, adult chicken liver; and lane 8, anemic adult hen red blood cells. The blot shown in *A* was washed in 7.5 mM NaCl/0.75 mM sodium citrate/0.1% sodium dodecyl sulfate at 52°C; exposure time was 5 hr. The blot shown in *B* is the same as that shown in *A*, but it had subsequently been washed in 300 mM NaCl buffer plus RNase A at 4 μg/ml (31, 36) after the exposure shown in *A* was taken; exposure time for *B* was 13 hr.

the liver (*ca.* 2800–3000 bases; Fig. 5*A*, lanes 5-7) is large enough to encode the liver-specific (73-kDa) preenzyme (19), while the other two homologous RNAs visualized in the same lanes are not. Similarly, in lanes containing chicken embryo fibroblast poly(A)$^+$ RNA (Fig. 5*A*, lane 2) or cultured MSB-1 cell (chicken lymphocytes transformed by Marek disease virus) poly(A)$^+$ RNA (Fig. 5*A*, lane 1; ref. 39), the homologous cellular RNAs are *ca.* 5500 and 5000 bases, respectively, and appear to be less abundant than ALA synthase mRNAs in either liver cells or anemic red cells.

Under normal high-stringency blot washing conditions, the liver ALA synthase mRNA appears to be highly homologous in nucleic acid sequence to the erythroid ALA synthase gene probe (Fig. 5*A*, lane 7). Only after treatment of the blot with RNase (Fig. 5*B*) can it be discerned that all these RNAs differ in primary nucleotide sequence except in the homologous tissue source. Thus, we conclude from these data that ALA synthase is encoded by a minimum of two (erythroid and liver) separate tissue-specific genes.

DISCUSSION

We report in this paper the isolation of a cDNA sequence corresponding to the erythroid-specific gene encoding the first enzyme contributing to heme biosynthesis in animal cells, ALA synthase. The mRNA encoding this particular isozyme is approximately 2000 nucleotides in length [including poly(A)] and appears, from this preliminary analysis, to be uniquely expressed in erythroid cells. While the presumptive mRNA encoding the liver ALA synthase is highly homologous to the erythroid gene, under very stringent washing conditions the liver ALA synthase mRNA can be distinguished from its erythroid counterpart on the basis of primary nucleotide sequence, thereby implying that the ALA synthase enzymes are encoded by a multigene family whose members are responsive to different developmental stimuli and regulation.

What is the likelihood that we have mistakenly identified a single ALA synthase locus as a family of genes encoding different tissue-specific enzymes, whereas only one gene actually exists and the nuclear RNA transcribed from that locus is differentially regulated, giving rise to multiple isozymes of ALA synthase? We see only one way that the presence of a single ALA synthase gene in chicken cells could be consistent with the results presented in Fig. 5. There is a high probability that the red cell enzyme shares only a subset of the (presumptive) multiple epitopes on the ALA synthase molecule found in the liver, against which the rabbit antiserum was raised. Furthermore, it is at least theoretically possible that while the major antigenic determinant is held in common and is highly conserved within the ALA synthase isozymes of both tissues, this predominant epitope is derived from two (or more) exons of the same transcription unit, one of which is used as part of the red cell ALA synthase mRNA and another as part of liver ALA synthase mRNA. This alternative explanation would also lead to the observed results presented in Fig. 5. However, we view this possibility as very unlikely, since differential use of equivalently functional coding sequence exons within a single transcription unit has not been previously observed. The more conservative conclusion, that the tissue-specific mRNAs arise from different genetic loci, seems likely to be correct. A final resolution to this, and several other questions naturally arising from data presented in this report [e.g., the origin of the large protein that is immunoprecipitable with the anti-ALA synthase antibody in erythroid progenitor cells (Fig. 1, lane C) and the origin of RNA species too small to encode liver ALA synthase in total liver RNA (Fig. 5, lanes 5, 6, and 7)] should be immediately forthcoming from analysis of the chromosomal gene(s).

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