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Novel Regulation of δ-Aminolevulinate Synthase in the Rat Harderian Gland

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ABSTRACT. The mode of expression of δ-aminolevulinate synthase (ALAS), as well as that of mRNAs for other heme pathway enzymes, was examined in the rat Harderian gland. Northern blot and *in situ* hybridization analyses demonstrated that the non-specific ALAS (ALAS-N) mRNA is highly expressed in this tissue, whereas the erythroid-specific ALAS (ALAS-E) mRNA is not. Immunoblot analysis of ALAS also confirmed this finding at the protein level. ALAS-N mRNA was maximally induced in the Harderian gland and was not increased further by treatment of animals with 2-allyl-2-isopropylacetamide (AIA). The levels of mRNAs for other heme pathway enzymes, i.e., δ-aminolevulinate dehydratase, porphobilinogen deaminase, uroporphyrinogen decarboxylase, and coproporphyrinogen oxidase, also were increased markedly in the Harderian gland and not influenced by AIA treatment. The level of ferrochelatase (FeC) mRNA in the gland was, however, lower than that in the liver. The gland contained an extremely high level of protoporphyrin, while heme was undetectable. Microsomal heme oxygenase-1 (HO-1) mRNA levels were significantly higher in the Harderian gland than in the liver. When isolated glands were incubated with hemin *in vitro* in organ cultures, the level of HO-1 mRNA was increased, whereas the ALAS-N mRNA level was not. These findings indicate that markedly elevated levels of protoporphyrin and extremely low levels of heme in the Harderian gland are the results of both decreased expression of FeC and markedly increased expression of ALAS-N and HO-1. The constitutive expression of the ALAS-N gene in the Harderian gland suggests a novel transcriptional control mechanism of this gene. BIOCHEM PHARMACOL 53;5:643–650, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Harderian gland; δ-aminolevulinate synthase; ferrochelatase; heme; heme oxygenase

It is known that heme biosynthesis in the liver and in erythroid cells is differently regulated by heme, the end product of the heme biosynthetic pathway [1–3]. This effect is largely exerted at the level of ALAS,** the first enzyme of the pathway, which exists as tissue-specific isozymes [4–7]. Namely, heme represses the level of ALAS-N in the liver [8–10], while it up-regulates ALAS-E in the erythroid bone marrow [11, 12]. The regulation of heme biosynthesis in other non-erythroid tissues remains unclear, but it is generally assumed to be similarly regulated as in liver, since ALAS-E is expressed exclusively in erythroid cells, whereas ALAS-N is thought to be expressed in an ubiquitous fashion.

The Harderian gland, a bi-lobed alveolar gland located within the orbit in rodents, is known to contain extremely high levels of protoporphyrin [13–16], and ALAS activity [17–20]. It is generally thought that the gland secretes lipids for lubricating the cornea, but the basis for the marked porphyrin synthetic activity in this organ has not been elucidated. To examine the reason(s) for the markedly elevated porphyrin biosynthesis in this organ, we determined the levels of mRNAs encoding enzymes in the heme biosynthetic pathway, HO-1, and the concentrations of protoporphyrin and heme in the rat Harderian gland. We report here that the Harderian gland expresses high levels of mRNAs encoding heme biosynthetic enzymes, except for FeC, and that ALAS-N mRNA in this tissue is constitutively expressed and not influenced by treatment with either AIA or hemin, an inducer and a repressor of ALAS in

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** *Abbreviations:* AIA, 2-allyl-2-isopropylacetamide; ALAD, δ-aminolevulinate dehydratase; ALAS, δ-aminolevulinate synthase; ALAS-E, erythroid-specific ALAS; ALAS-N, non-specific ALAS; CPO, coproporphyrinogen oxidase; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; EPP, erythropoietic protoporphyria; FeC, ferrochelatase; HO-1, heme oxygenase-1; PBGD, porphobilinogen deaminase; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; UROD, uroporphyrinogen decarboxylase.

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the liver, respectively. These findings thus account for an extremely high level of protoporphyrin and a low heme content in the Harderian gland, and indicate that the mode of regulation of heme biosynthesis in this organ is different from that in the liver and erythroid tissues.

MATERIALS AND METHODS

Treatment of Animals and Preparation of RNA

AIA, a gift of the Nippon Roche Co., Tokyo, was dissolved in physiological saline at a concentration of 20 mg/mL. Rats were fasted for 24 hr, and then were administered 300 mg/kg body weight AIA subcutaneously. Animals were decapitated 4.5 hr after injection when a maximal induction of ALAS in rat liver takes place [21], and tissues were quickly removed, rinsed in physiological saline, and processed for total RNA extraction.

Probes

cDNA probes for ALAS-N and ALAS-E were a rat ALAS-N cDNA, pKRA2cA [21], and a rat ALAS-E cDNA, pREAL1 [22], respectively. The pEL link was constructed by ligating a 370 bp cDNA fragment that corresponds to the 5'-terminal region of pKRA2cA to the *Eco*RI site in pES0.4 (a truncated subclone of a rat ALAS-E cDNA clone, pREAL1) (Fig. 1A) [22]. Other cDNA probes used were pALAD1 (for the rat ALAD) [23], p44SB1 (for the rat PBGD), pUD3 (for the human UROD, a gift of Dr. H. de Verneuil) [24], mouse CPO (a gift of Dr. Shigeru Taketani) [25], mouse FeC (a gift of Dr. Taketani) [26], and rRHO1 (for the rat HO-1, a gift of Dr. Shigeki Shibahara) [27]. HG126 clone of a human ribosomal DNA fragment was obtained from the Japanese Cancer Research Resources Bank, Tokyo. The probes were labeled by the random primer extension method [28]. Hybridization and washing of nitrocellulose filters were performed as described previously [29].

For ALAS-N, HO-1, and FeC, an RNA probe was prepared by inserting cDNA fragments into pBluescript IIKS+. Antisense RNAs of ALAS-N, HO-1, and FeC were prepared by *in vitro* transcription of the cDNAs in pBluescript IIKS+ vector, according to the method of Melton *et al.* [30].

Northern Blot Analysis

Total RNA was isolated according to the method of Chomczynski and Sacchi, [31]. RNA (30 μg) was applied to a 1.0% agarose/formaldehyde gel, electrophoresed, transferred to a sheet of Hybond C-extra (Amersham Japan, Tokyo), and hybridized to the probes. When an RNA probe was used (for ALAS-N, HO-1, and FeC), a sheet of nylon

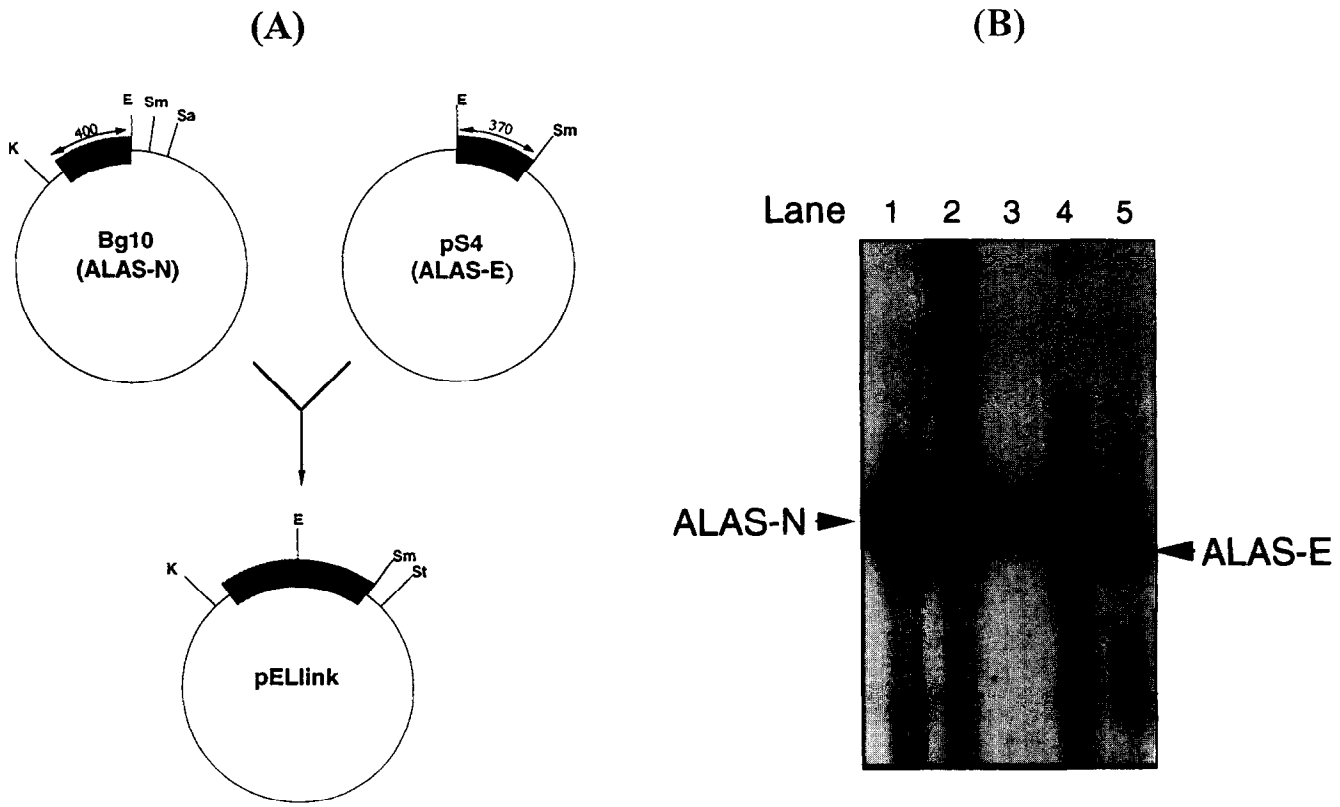

FIG. 1. Northern blot analysis of ALAS mRNAs. (A) Construction of pEL link probe. (B) Northern blot analysis of ALAS using pEL link probes. Details are described in Materials and Methods. Lane 1: Harderian gland from an untreated rat; lane 2: Harderian gland from an AIA-treated rat; lane 3: liver from an untreated rat; lane 4: liver from an AIA-treated rat; and lane 5: spleen from an untreated rat.

filter (Zeta probe blotting membrane, Bio-Rad, Hercules, CA) was used instead of Hybond C-extra. Hybridized filters were rinsed briefly in 2× SSC twice, incubated in 2× SSC/0.1% SDS at 50° for 30 min, 0.1% SSC/0.1% SDS at 50° for 30 min, 2× SSC at room temperature for 5 min, and 2× SSC/RNase (1 μg/mL) at 37° for 7 min, followed by rinsing in 0.1% SSC/0.1% SDS three times, and incubation in 0.1% SSC/0.1% SDS at 50° for 30 min. mRNA concentrations were determined by an LKB Ultroscan XL densitometer (LKB Pharmacia, Uppsala, Sweden). There was a linear relationship between the amount of mRNA and the absorbance at 633 nm. Experiments were repeated at least twice using separate preparations of RNA, and similar results were observed in each experiment. Since there were no major significant changes in ribosomal RNA levels, results were expressed without normalization based on ribosomal RNA content.

Western Blot Analysis

Mitochondrial fractions were isolated at ≈8° as follows. Tissues were homogenized in 4 vol. of a solution that contained 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.2 mM dithiothreitol, 0.1 mM pyridoxal 5′-phosphate, 0.5 mM EDTA, and 10 μg/mL each of the following protease inhibitors, i.e. leupeptin, chymostatin, antipain, and pepstatin (Peptide Institute, Osaka). The homogenate was centrifuged at 600 *g* for 10 min, and the supernatant fraction was centrifuged again at 10,000 *g* for 10 min. The precipitate was rinsed again by resuspending in the same solution and by re-centrifugation. The final precipitate was dissolved in 1 vol. of a solution containing 20 mM Tris–HCl (pH 7.4), 0.2 mM dithiothreitol, 0.1 mM pyridoxal 5′-phosphate, and 0.5 mM EDTA. For immunoblot analysis of ALAS, 100 μL of this solution was mixed with 100 μL of a buffer containing 100 mM Tris–HCl (pH 6.8), 3.2% (w/v) SDS, 10% (w/v) glycerol, and 8% (v/v) 2-mercaptoethanol and heated at 100° for 3 min. Protein concentration was determined using the Bio-Rad Protein Assay kit (Nippon Bio-Rad, Tokyo). Samples equivalent to 20 μg of protein for the liver and 5 μg of protein for the Harderian gland were applied to an 18% (w/v) Laemmli gel after supplementing with 0.0025% (w/v) bromophenol blue. After electrophoretic separation, proteins were transferred to polyvinylpyrrolidone difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA). The membrane was treated with Tris-buffered saline (TBS) containing 3% (w/v) skim milk at 4° overnight, incubated with a rabbit anti-ALAS antibody diluted at 1:500 with TBS containing 1% (w/v) BSA, and then incubated with horseradish peroxidase (HRP)-labeled rat anti-rabbit IgG; the color reaction was performed using a Konika Immunostain HRP kit (IS-50B, Seikagaku Kogyo, Tokyo), and quantified by densitometry. The rabbit antibody against ALAS was prepared by immunizing rabbits with a BSA-conjugate of a peptide, EKAYFSGMSKMVSAQA, which corresponded to the carboxyl terminus of the rat ALAS-N. The antibody was affinity-purified from rabbit serum using the synthetic peptide.

In Situ *Hybridization*

Fresh frozen blocks of the Harderian glands from adult male rats were sectioned at 30 μm thickness using a cryostat. The sections, which were mounted on silane-coated glass slides, were fixed with paraformaldehyde and pretreated as described previously [32]. The slides were incubated with a fluid containing 50% (v/v) formamide, 4× SSC, 1× Denhardt's solution, 1% (w/v) sarcosyl, 0.1 M sodium phosphate buffer (pH 7.2), 100 mM dithiothreitol, and 250 μg/mL heat-denatured salmon sperm DNA for 2 hr at room temperature. Hybridization was performed in the same solution (50 μl/slide) containing 10% (w/v) dextran sulfate and 0.5 to 1.0 × 10^6 cpm of the probe labeled with [^{35}S]dATP by nick-translation at 42° for 16 hr in a humidified chamber. After hybridization, slides were rinsed sequentially in 2× SSC, 0.1% (w/v) sarcosyl at room temperature for 30 min, three times in 0.1% (w/v) sarcosyl at 42° for 40 min each, and dehydrated in 70% and 100% ethanol. The sections were exposed to Hyperfilm-β_{max} for 2–3 weeks.

Organ Culture

Four rats were used per experiment for these studies. Harderian glands were dissected quickly from animals after decapitation, and the organs were rinsed in Earle's buffer devoid of Ca^{2+} and Mg^{2+}. Each gland was cut into four approximately equivalent pieces, and each piece was added to a 60-mm petri dish containing 5 mL of Minimum Essential Medium supplemented with 10% heat-inactivated calf serum (HyClone, Logan, UT). Hemin was added to two dishes at a final concentration of 100 μM, while the other dishes were treated with the same volume of Earle's buffer containing Ca^{2+} and Mg^{2+} as control. Cultures were incubated for 1.5 hr, and 3 hr, at 37°, 5% CO_2, and then tissues were harvested for RNA isolation. Twenty micrograms of total RNA was used for northern blot analysis.

Other Assays

Porphyrin content was determined fluorometrically as described previously [33]. Heme content was determined by the pyridine hemochromogen assay according to the method described by Porra and Jones [34]. Cytochrome P450 determination was made according to Omura and Sato [35]. Protein concentration of microsomes was determined by the method of Lowry *et al.* [36]. Results of all experiments were confirmed by repeating experiments at least twice.

RESULTS

Markedly Elevated Expression of ALAS-N, but not ALAS-E, in the Harderian Gland

The species and the levels of mRNA encoding ALAS were examined in the Harderian gland. As shown in Fig. 1B, ALAS-N, but not ALAS-E, was expressed in the Harderian gland as in the liver. In contrast, ALAS-E mRNA was

expressed in the spleen, an erythropoietic organ in the rodent (lane 5, Fig. 1B), but not in the liver or in the Harderian gland. The level of ALAS-N mRNA in the Harderian gland was extremely high (15-fold higher than the level of the untreated liver), which is even higher than the maximally induced level of ALAS-N mRNA in the liver following treatment of animals with AIA. While ALAS-N mRNA underwent a marked induction in the liver by treatment with AIA (>10-fold) (lanes 3 and 4, Fig. 1B), the level of ALAS-N mRNA in the Harderian gland was unchanged after AIA treatment (lanes 1 and 2, Fig. 1B).

Localization of ALAS-N mRNA expression in the tissue was examined by *in situ* hybridization analysis. ALAS-N mRNA was found uniformly expressed in the gland, but not in the surrounding connective tissue (Fig. 2). Consistent with the results of the northern blot analysis, ALAS-E mRNA was undetectable in these tissues (Fig. 2).

To examine whether the increased level of ALAS-N mRNA was also reflected by increased protein content, ALAS-N protein levels were examined by immunoblot analysis. Results demonstrated that ALAS-N was expressed at a high level in the Harderian gland from both untreated and AIA-treated animals, and its level was at least 8-fold higher than the level in the AIA-treated liver (Fig. 3). Both the band at 75 kDa, a precursor of ALAS-N [9], and the band at 65 kDa, the mature form of the enzyme [9], in the liver increased 20- and 14-fold, respectively, after AIA treatment of animals compared with the untreated animals. In contrast, the band at 65 kDa, the major species in the Harderian gland, was not influenced by AIA treatment.

Markedly Elevated Expression of mRNAs for ALAD, PBGD, UROD, and CPO, but not FeC, in the Harderian Gland

Since ALAS-N mRNA in the Harderian gland was constitutively expressed, mRNAs for other enzymes of the heme biosynthetic pathway were examined by northern blot

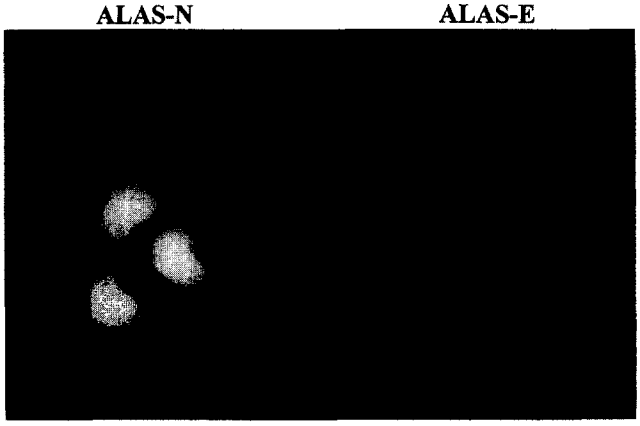

FIG. 2. *In situ* hybridization of ALAS mRNAs. *In situ* hybridization of ALAS mRNAs was carried out using a rat ALAS-N and a rat ALAS-E cDNA probe, as described in Materials and Methods.

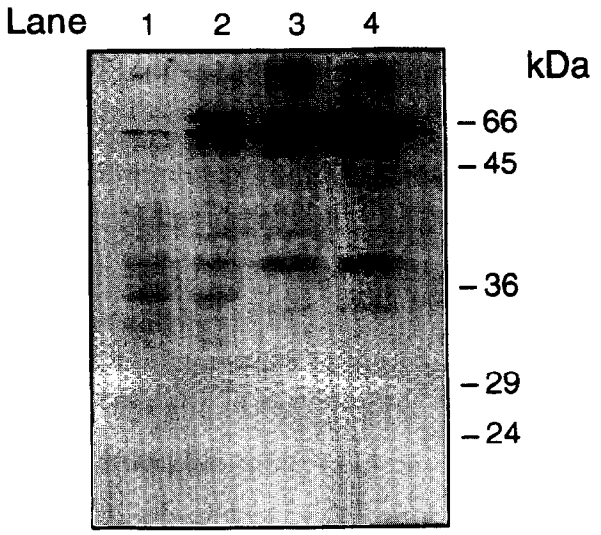

FIG. 3. Western blot analysis of ALAS protein. Five micrograms of protein of the Harderian gland, and 20 μg of protein of the liver were loaded onto a 18% (w/v) Laemmli gel, and separated by electrophoresis, as described in Materials and Methods. Lane 1: liver from an untreated rat; lane 2: liver from an AIA-treated rat; lane 3: Harderian gland from an untreated rat; and lane 4: Harderian gland from an AIA-treated rat.

analysis using cDNA probes, as described in Materials and Methods. Levels of mRNAs in the gland were 5-, 10-, 20-, and 8-fold higher than those expressed in the liver, for ALAD, PBGD, UROD, and CPO, respectively (Fig. 4). Levels of these mRNAs in the gland were not altered by treatment of animals with AIA [33, 37, 38]. ALAD mRNA expressed in the Harderian gland was exclusively that of the ALAD-N [39], but not of the ALAD-E, as determined by reverse transcriptase-polymerase chain reaction (data not shown).

In contrast to the markedly elevated expression of these heme pathway enzymes, FeC mRNA level was much lower than that in the liver (Fig. 4). Low FeC mRNA levels (<40% of the level in the liver) were further confirmed using an RNA probe that permitted detection with higher sensitivity and specificity (data not shown).

Levels of HO-1 mRNA, Protoporphyrin, and Heme Content in the Harderian Gland

HO-1 mRNA levels were examined in the Harderian gland and the liver, using northern blot analysis. The level of HO-1 mRNA in the Harderian gland was >10-fold higher than that in the liver. Consistent with this finding as well as with low FeC gene expression, heme content in the gland was undetectable by the pyridine hemochromogen assay. Assuming an absorbance unit of 0.005 as the lowest level of detection by this method, the level of heme in this organ was below 0.012 nmol/mg protein. This is in marked contrast to heme content in the rat liver microsome, which is ⩾1 nmol/mg microsomal protein [40].

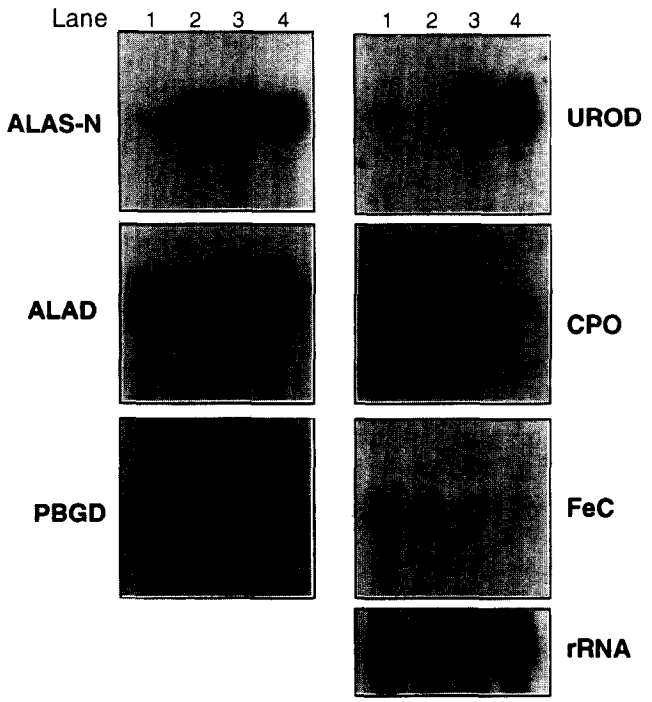

FIG. 4. Northern blot analysis of mRNAs encoding heme pathway enzymes. mRNAs encoding ALAD, PBGD, UROD, CPO, and FeC were examined by northern blot analysis, as described in Materials and Methods. ALAS-N was additionally examined for comparison. rRNA was also examined as a control. Lane 1: liver from an untreated rat; lane 2: liver from an AIA-treated rat; lane 3: Harderian gland from an untreated rat; and lane 4: Harderian gland from an AIA-treated rat. All mRNA bands were detected at the appropriate size for each mRNA, and identical results were confirmed by repeated experiments.

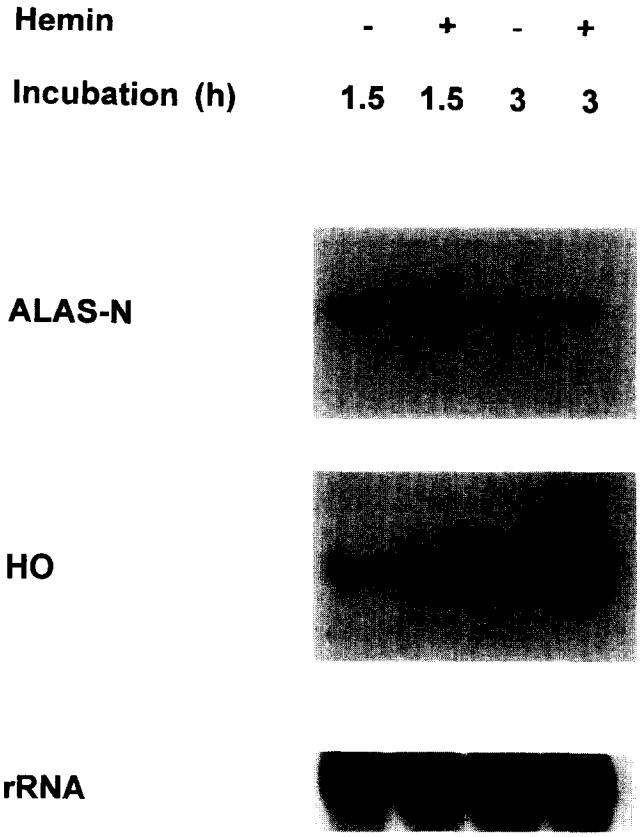

FIG. 5. Effects of hemin treatment on the levels of ALAS-N and HO-1 mRNAs. Harderian glands were incubated *in vitro* in the presence of Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum, with or without 100 μM hemin, for 1.5 and 3 hr, as described in Materials and Methods.

In contrast to the low heme content, the level of porphyrin, which was almost exclusively protoporphyrin (data not shown) in the Harderian gland, was elevated markedly (4.58 nmol/mg microsomal protein), a level >1000 times higher than that in the liver (0.0044 nmol/mg microsomal protein). Thus, the molar ratio between protoporphyrin and heme, which is usually less than 0.01 in other tissues, is >380 in the Harderian gland.

Effect of Hemin on ALAS-N and HO-1 in the Harderian Gland

ALAS-N mRNA in the liver is known to be repressed by heme [21, 33], while HO-1 mRNA is known to be induced by heme [41]. The effect of hemin on ALAS-N mRNA and HO-1 levels was examined in organ cultures of the Harderian gland, using northern blot analysis. ALAS-N mRNA levels in the untreated organ showed a rapid decline with time ($T_{½}$ < 1.5 hr), while HO-1 mRNA showed an induction (Fig. 5). The reason(s) for the rapid decline of ALAS-N mRNA is not clear; however, its half-life was similar to that for ALAS-N mRNA in the liver after cessation of RNA synthesis (~20 min) [21], and it is possible that the markedly elevated level of ALAS-N mRNA in the Harderian gland is maintained by continued synthesis *in vivo* in animals, and its level may decline rapidly when the organ is removed from the animal and placed in culture. Incubation of the gland with hemin for 1.5 and 3 hr significantly increased HO-1 mRNA levels (Fig. 5), as observed in the liver [41]. In contrast, ALAS-N mRNA levels were not affected by hemin treatment at either 1.5 or 3 hr (Fig. 5). These findings indicate that, while HO-1 mRNA is inducible by hemin treatment, ALAS-N mRNA expression in the Harderian gland is not repressible by the same treatment. Thus, it can be concluded that the markedly elevated level of ALAS-N mRNA in the gland is not the result of its derepression by heme deficiency.

DISCUSSION

It is known that ALAS activity in the normal liver is very low, but its induction takes place when a demand for increase in heme synthesis occurs [42]. ALAS induction also takes place in erythroid cells while cells are undergoing erythroid differentiation from stem cells [43]. The induction process of ALAS is different between these two tissues, however, reflecting the fact that ALAS-N and ALAS-E are two different gene products [42]. It is known that the Har-

derian gland in rodents contains high levels of protoporphyrin [13–16] and ALAS activity [17–20], but the reason(s) for such elevated porphyrin synthesis has not been elucidated. In this study, we examined the expression and the regulation of ALAS in this gland and demonstrated for the first time that ALAS in this gland is exclusively of the ALAS-N isoform, and that it is constitutively expressed and not influenced by AIA and hemin, the inducer and the repressor of the enzyme in the liver, respectively. The isozymic phenotype of ALAD in the Harderian gland was also shown to be that of the ALAD-N (our unpublished observation).

In addition to ALAS-N, mRNAs encoding ALAD, PBGD, UROD, and CPO were expressed at much higher levels than those in the liver (Fig. 4). This point is illustrated in a hypothetical scheme of the regulation of heme biosynthesis in the Harderian gland (Fig. 6). The exception was FeC mRNA in the Harderian gland, which was much lower than that in the liver (less than 40%). Our findings also demonstrated that mRNAs for heme pathway enzymes in the Harderian gland were entirely refractory to AIA treatment (Fig. 4). Constitutive expression of the ALAS-N gene is different from that in the liver, suggesting the existence of a different transcriptional mechanism for ALAS-N regulation in the Harderian gland.

It should also be noted that HO-1 mRNA in the Harderian gland was much higher than that in the liver (>10-fold than in the untreated liver; data not shown). The markedly elevated level of HO-1 mRNA in the Harderian gland is clearly not due to a heme-mediated induction, since the gland contains little heme. It is more likely due to another mechanism(s) such as stress-mediated induction, since HO-1 is known to be inducible in response to various stress stimuli, including porphyrin treatment [44]. The markedly elevated level of HO-1 expression, however, may further contribute to the destruction of heme in this tissue. These findings account for the marked accumulation of protoporphyrin as well as the lack of heme in the Harderian gland (Fig. 6).

ALAS-N mRNA in the liver is known to be inducible by treatment of animals with porphyrogenic chemicals such as AIA or DDC, and is suppressible by treatment with hemin [8, 33, 42, 45–48]. Since there is no detectable amount of heme in the Harderian gland, it is possible that the elevated level of ALAS-N mRNA may be the result of derepression of the enzyme by heme deficiency. However, treatment of the Harderian gland *in vitro* with hemin had no effect on the level of ALAS-N mRNA. This finding is specific to ALAS-N, because HO-1 mRNA was inducible in the gland by the same treatment (Fig. 5). It is also consistent with the fact that treatment of animals with AIA, a strong inducer of ALAS in the liver [45], did not influence the level of ALAS-N mRNA in the Harderian gland (Figs. 1 and 4). The refractoriness of ALAS-N mRNA to hemin or to AIA treatment is not due to the stability of the mRNA, since it turns over rapidly in the gland (Fig. 5; $T_{1/2} < 1.5$ hr), which presumably reflects its rapid turnover together with the cessation of mRNA synthesis in the isolated gland. Thus, the markedly elevated level of ALAS-N mRNA in the Harderian gland appears to be maintained on the basis of its continuous and increased synthesis (Fig. 6).

While ALAS-N expression in the liver is regulated in a negative feedback manner by heme [8–10, 33, 37, 49, 50], ALAS-E expression in erythroid cells is not [11, 12]. Our findings in this study demonstrated that ALAS-N mRNA levels in the Harderian gland are refractory to treatment with either hemin or AIA (Fig. 4). Our findings are consistent with those reported by Margolis [16] that treatment of mice with AIA or DDC did not influence porphyrin concentrations in the gland. The exact mechanism for the constitutive induction of ALAS-N in the Harderian gland is unclear at present, but these findings strongly suggest that, in this tissue, there may be as yet another different mechanism of transcriptional regulation of ALAS-N. How-

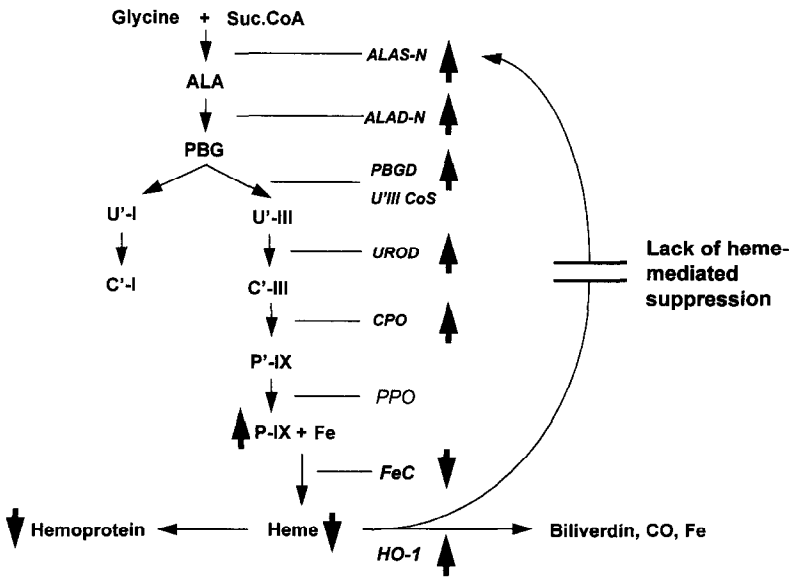

FIG. 6. A hypothetical scheme on the regulation of heme biosynthesis in the Harderian gland. Key: U': uroporphyrinogen; C': coproporphyrinogen; P': protoporphyrinogen; and P: protoporphyrin. Thick bold arrows, (↑) and (↓), indicate up- and down-regulation, respectively. Abbreviations not defined previously: ALA, δ-aminolevulinate; PBG, porphobilinogen; and PPO, protoporphyrinogen oxidase.

ever, the fact that ALAS activity in the Harderian gland can be influenced by gonadal hormones [13, 14, 51], adrogenic steroids [13], and pantothenic acid deficiency [15] also suggests that the regulatory mechanism of ALAS-N in the Harderian gland may be different from that in the liver.

It should also be noted that, not only ALAS-N, but also ALAD, PBGD, UROD, and CPO mRNAs were increased markedly in the Harderian gland (Fig. 6). Such a global up-regulation of heme pathway enzyme genes has not been described in other tissues, suggesting that the regulation of heme biosynthesis itself may also be different in this gland.

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