The influence of sex steroid hormones on ferrochelatase gene expression in Harderian gland of hamster (*Mesocricetus auratus*)

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**Abstract**

Ferrochelatase (protohaem ferrolyase, EC 4·99·1·1), the terminal enzyme of the haem biosynthetic pathway, catalyses the insertion of ferrous iron into protoporphyrin IX to form protohaem. The Syrian hamster Harderian gland (HG) is known for its ability to produce and accumulate large amounts of protoporphyrins. In this species, the female gland contains up to 120 times more porphyrin than the male gland. Data from biochemical studies suggest that this gland possesses the enzymatic complex for haem biosynthesis but lacks ferrochelatase activity. The abundance of intraglandular haem proteins does not support this idea. To gain more insight into this process, we isolated cDNA for ferrochelatase from hamster liver, using the 5′- and 3′- rapid amplification of complementary DNA ends (RACE), and investigated its expression in HG from males and females. The full-length cDNA comprises an open reading frame of 1269 bp encoding a polypeptide of 422 amino-acid residues. Hamster DNA sequence exhibits 92% identity to mouse and 87% identity to human sequences. The predicted hamster enzyme was shown to have structural features of mammalian ferrochelatase, including a putative NH₂-terminal presequence, a central core of about 330 amino-acid residues and an extra 30–50-amino-acid stretch at the carboxyl-terminus. RNA blotting experiments indicated that this cDNA hybridized to a liver mRNA of about 2·1 kb, while a weak hybridization signal was observed with mRNA from HG preparations. RT–PCR assays confirmed the expression of specific transcripts in both tissues. Male glands contained approximately twofold more enzyme mRNA than female glands. Likewise, the intraglandular content of mRNA varied during the oestrous cycle, with the highest levels found in the oestrous phase. These cyclic variations were less evident in liver. Ovariectomy plus treatment with progesterone or 17β-oestradiol plus progesterone increased ferrochelatase mRNA of the gland. In HG of short- or long-term castrated males, the administration of testosterone did not affect the ferrochelatase mRNA concentration. Based on mRNA expression levels, we conclude that Harderian ferrochelatase may play an active role in maintaining the physiological pool of haem required for processing cytochromes and other glandular haem proteins. Likewise, the sex-steroid hormones appear to have only a modest influence upon Harderian ferrochelatase.


**Introduction**

The Harderian gland (HG) is a compound tubuloalveolar structure located within the orbital cavities of most terrestrial vertebrates. Its function remains unclear, although it has been linked to a retino-pineal-gonadal system (Wetterberg *et al.* 1970) and may serve as a potential source of pheromones (Payne 1994) and as a secondary steroidogenic organ (Vilchis *et al.* 2002). Characteristically in Syrian hamster, the HG produces and accumulates large amounts of porphyrins, which can be microscopically visualized as solid intraluminal accretions (Christensen & Dam 1953, Hoffman 1971, Payne *et al.* 1977). The HG appears to express the whole enzymatic complex required for porphyrin synthesis from glycine and succinyl-CoA (Tomio & Grinstein 1968).

Interestingly, some steps of the biosynthetic pathway, including protoporphyrin IX production, are under hormonal influence. In this regard, Thompson *et al.* (1984) reported marked sex differences in five of the seven haem biosynthetic enzymes, with higher activities always being found in females. Porphobilinogen deaminase, uroporphyrinogen decarboxylase, coproporphyrinogen oxidase and protoporphyrinogen oxidase are among the enzymes whose activity is influenced by sex steroids (Spike *et al.* 1992, Gibson *et al.* 1999, Coto-Montes *et al.* 2001). In fact, the female hamster has considerably more porphyrins than the male (Spike *et al.* 1985, 1986). These sexual differences may be easily reversed by changes in sex hormones; castration of adult males increases the intraglandular content of these pigments, and administration of androgens to adult females decreases their intraglandular content.
(Hoffman 1971, McMasters & Hoffman 1984). In addition, the porphyrinogenic activity of the gland fluctuates throughout the oestrous cycle, pregnancy and lactation, suggesting that there is a link between HG activity and reproductive function (Moore et al. 1977, Payne et al. 1979). Previous reports have indicated that, apart from the gonadal steroids, other hormones from the pituitary (Buzzell et al. 1992), the thyroid (Hoffman et al. 1990) and the pineal gland (McMasters & Hoffman 1984) may regulate porphyrin synthesis in HG.

The rate-limiting step of the haem production is catalysed by 5-aminolevulinic acid synthase 1 (ALA-S1), an enzyme whose synthesis is under feedback inhibition by haem, and which is also negatively regulated by iron levels and androgens (Veltman & Maines 1985, Drew & Ades 1986, Menéndez-Peláez et al. 1991, Domínguez et al. 1994, Taketani et al. 2000). Haem not only serves as a prosthetic group of numerous haem proteins that synthesize important regulatory and signalling molecules, including cyclic GMP, steroid hormones and nitric oxide, but it also plays a key role in controlling protein synthesis and cell differentiation (Poroka 1999). In most tissues, porphyrins are converted to haem by ferrochelatase. However, the expression of this enzyme in HG is still a matter of controversy, especially because of its low or almost undetectable activity (Thompson et al. 1984, Nagai et al. 1997). Nevertheless, the relative abundance of cholesterol side-chain cleavage cytochrome P450scc (Vilchis et al. 2002), together with the large amount of androgen-regulated haem proteins (Vilchis et al. 1996) and the high concentrations of intraglandular iron (Hoffman & Jones 1981), strongly suggests a functional role for this mitochondrial enzyme. By isolating the specific cDNA, we provide experimental evidence of the transcriptional activity of ferrochelatase in HG.

Materials and Methods

Animals and tissues

Male and female adult hamsters (Mesocricetus auratus) weighing 130–180 g were housed under controlled conditions (temperature 21 ± 2 °C; lighting, 14 h:10 h/ light:darkness) with free access to food and water. The hamsters were orchidectomized under light ether anaesthesia and were allowed 7 days to recover before study. Other groups of male hamsters were castrated for 7 or 30 days and then injected with a daily dose of testosterone (1 mg) or vehicle alone (50 µl corn oil) for 6 consecutive days. In females, the ovarian cycle was monitored with vaginal smear patterns, considering that ovulation occurs at a mean of every 4 days with proestrus (P), oestrus (E), metoestrus (M) and dioestrus (D) phases. Other groups of females were ovarioctomized for 7 days and then treated with a daily dose of 17β-oestradiol (10 µg) or progesterone (1 mg) or 17β-oestradiol plus progesterone (10 µg plus 1 mg) or vehicle alone (50 µl corn oil) for 6 consecutive days. The experimental procedures were approved by the institutional ethical committee for investigation in animals. Animals were decapitated, and tissues were immediately excised, rapidly frozen and stored at −70 °C until assayed.

Extraction of RNA

Total cellular RNA was isolated with TRIZol reagent (Invitrogen). The amount and quality of RNA were estimated by spectrophotometry at 260/280 nm. Once total RNA was isolated, polyadenylated [poly (A)+] RNA was obtained with the Fast Track mRNA isolation kit (Invitrogen) by the manufacturer’s recommended protocol. Total RNA or poly(A)+ RNA was reverse transcribed into complementary DNA (cDNA) with the 1st Strand cDNA Synthesis kit for RT–PCR (Roche).

Isolation of hamster ferrochelatase cDNA

Initially, oligo-dT-primed cDNA from male liver and two sets of degenerate primers designed from the sequences reported for mouse (Taketani et al. 1990, Brenner & Frasier 1991), human (Nakahashi et al. 1990) and bovine (Shibuya et al. 1995) ferrochelatase were used to amplify specific transcripts of hamster mRNA. The PCR products were resolved on agarose gels, eluted and purified through Centricon-30 columns (Amicon Millipore Co, Bedford, MA, USA), and submitted for sequencing analysis. These partial cDNA sequences were then aligned with that of mouse ferrochelatase and shown to have a highly conserved nucleotide sequence, confirming their authenticity. Gene-specific primers derived from these fragments were used in further procedures to amplify the 5’- and 3’- end regions, by the method of rapid amplification of the cDNA ends (RACE).

Starting from male liver poly (A)+RNA, 5’- and 3’-RACE reactions were performed with the Gene Racer (RLM-RACE) kit from Invitrogen according to the manufacturer’s instructions. The complete cDNA sequence was obtained by PCR using gene-specific primers, designed from the 5’- and 3’-RACE fragments. The forward and reverse primers used in the reactions were as follows: FPLUS (5’-GATCCCTGGCGTCCCGGAAA TGCTT-3’) and FMIN (5’-TCTGTGAATCCCACGG GTCCCTCAG-3’). After 35 cycles of amplification (94 °C for 60 s, 65 °C for 45 s, 72 °C for 45 s, with a 7-min final extension at 72 °C), the PCR products (1320 bp) were ligated into the TA cloning vector pcDNA3·1/V5-HisTOPO (Invitrogen). After the plasmid was amplified by transforming competent cells, it was purified and subjected to restriction analysis with EcoRV and BamH1 to verify orientation of the inserted DNA. At least three positive clones were sequenced in both directions in order to establish the complete cDNA sequence.
The nucleotide sequences of PCR products and clones were determined with the Thermo Sequenase (α-33P]dNTP) Radiolabeled Terminator Cycle Sequencing kit (USB Co, Cleveland, OH, USA).

**Northern blot analysis**

Aliquots of total RNA (20 µg) isolated from each tissue sample were electrophoresed on a 1·2% agarose/6·0% formaldehyde gel, transferred to Hybond N+ nylon membrane (Amersham) by capillary diffusion and then fixed by UV cross-linking. Filters were prehybridized for 4 h at 65 °C in a Rapid-hyb buffer solution (Amersham) and further hybridized overnight at 65 °C in the same solution.

A 730 bp PCR product, amplified from ferrochelatase cDNA with the internal primers MA7; MA22 (5'-CGTCCATCTTGTGACAGAAGAAGC-3', 5'-GATTACACAGACCGGGCCACG-3'), was labelled with [α-32P]dCTP by random primer (RadPrime DNA Labeling System; Invitrogen) and used as probe in Northern and Southern blot assays. After hybridization and washing, the blots were exposed to Kodak BioMax XAR films at −70 °C for 2–8 h.

**RT–PCR/Southern blot analysis**

Oligo-dT-primed cDNA from each RNA was synthesized as described above and subsequently amplified with the primers FPLUS–FMINUS. The expected product size was 1320 bp. Parallel incubations containing no AMV-RT enzyme were used as controls in RT–PCR reactions. For Southern blots, 5·0 µl aliquots of RT–PCR products were separated by electrophoresis on 1% agarose gel containing ethidium bromide and visualized under UV light. The same gel was transferred to a nylon membrane (Amersham) by capillary diffusion and then fixed by UV cross-linking. Filters were prehybridized for 4 h at 65 °C in a Rapid-hyb buffer solution (Amersham) and further hybridized overnight at 65 °C in the same solution.

**Results**

**cDNA cloning**

To complete the hamster ferrochelatase cDNA sequence, 5'– and 3'– RACE was performed on adapter-ligated liver RNA. PCR products of 995 and 340 bp were obtained for the 5'– and 3'– RACE respectively; both products were sequenced with nested primers. Reverse transcription and PCR amplification of the poly (A)⁺RNA in the presence of gene-specific primers resulted in the amplification of a 1·3 kb fragment, which was inserted into the cloning vector. Overlay of these cDNA fragments provided the entire nucleotide sequence of hamster ferrochelatase mRNA.

The nucleotide sequence and deduced amino-acid sequence of hamster ferrochelatase cDNA are shown in Fig. 1. The overall sequence of the cDNA isolated in this study contained 2175 nucleotides, consisting of 70 bp of the 5'–untranslated region (UTR), 1269 bp of the open reading frame (ORF) and a long 3'–UTR of 836 bp. One canonical polyadenylation motif (AATAAA) is found at position 2085, 14 bp upstream of the poly (A)-tail. The ORF of the hamster ferrochelatase encodes for a protein of 422 amino acids and an estimated molecular weight of 47569 Da. Signal peptide prediction by the SignalP program identifies the first 53-amino-acid residues as a putative NH2 presequence with a cleavage site at residues 26–27.

**Protein sequence**

A comparison of the amino-acid sequence of hamster ferrochelatase with that of other species shows extensive homologies in certain regions (Fig. 2). Overall identities of amino-acid sequences between hamster and mouse, human, chicken, Xenopus and Danio ferrochelatase are 95%, 89%, 87%, 79% and 74% respectively. The predicted protein sequence contains the cysteine residues that contribute to the [2Fe-2S] cluster (Fig. 2), as well as a number of well-conserved amino-acid residues, believed to interact with the porphyrin substrate and with iron substrate binding.

**mRNA expression**

Northern blot hybridization of RNA samples from hamster tissues with the 32P-labelled probe of ferrochelatase showed that expression of a 2·1-kb mRNA was from six or seven samples (n) was evaluated. Differences between groups were assessed by Kruskal–Wallis one-way analysis of variance by ranks (Siegel & Castellan 1988). Significant differences were accepted at P<0·05.
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-70 gccctgttcgagctggggagacctgctttgtacagcagagctggccagctgcctgcctggcagacacactcgaatgtgggag

```cctgatctgttgagttgctgggagcgagctgcctgcctggcagacacactcgaatgtgggag```
limited to the liver and, to a lesser extent, to HG (Fig. 3). The RNA length coincided with that predicted by sequencing of the cDNA. Since the mRNA level in HG and other tissues (data not shown) was too low to be detected by Northern blot, the tissue distribution of ferrochelatase mRNA was further assessed by RT–PCR assays in combination with Southern blot hybridization. As shown in Fig. 4, the predicted 1320 bp cDNA product was detected in various tissues from males and females, including the liver, HG, adrenal glands and lung.

To explore the possibility that sex-steroid hormones were affecting ferrochelatase expression, semiquantitative RT–PCR assays were performed on tissue samples from intact and castrated male hamsters. Similar assays were performed on tissue samples from females.}

Figure 1 Nucleotide and predicted amino-acid sequences of hamster ferrochelatase cDNA. Position +1 is assigned to the first base of the putative initiator codon. Amino acids are numbered with position 1 assigned to the first methionine encoded. Numbering of the nucleotides (above) and amino acids (underneath) is shown on the right. The apparent polyadenylation signal (AATAAA) is underlined.

Figure 2 Amino-acid sequence comparison of hamster ferrochelatase to the mouse, human, bovine, chicken, *Xenopus* and *Danio* enzymes. The sequences are shown in single-letter code. Residues conserved among all species are in boldface. The conserved Cys residues at positions 195, 402, 405 and 410 in the hamster sequence are marked with an asterisk (*). Alignment was performed with the Multiple Sequence Alignment Program, Version 1.8 (clustalw.genome.jp).
performed on tissues derived from female hamsters in the different phases of the oestrous cycle. The relative abundance of ferrochelatase mRNA was obtained by normalizing the 753 bp band intensity with the band intensity of the constitutively expressed gene cyclophilin. As shown in Fig. 5, the results of densitometric analysis showed no significant variations in hepatic ferrochelatase mRNA during the oestrous cycle. The expression levels in liver of both intact and castrated males exhibited significant differences from those of females at dioestrus ($P<0.001$).

**Figure 3** Northern blot of ferrochelatase mRNA. Total RNA (approximately 20 μg) from female liver (lane 1), female HG (lane 2), male HG (lane 3) and male liver (lane 4) was electrophoresed on a formaldehyde-agarose gel and transferred to a nylon membrane. Hybridization was carried out with a $^{32}$P-labelled ferrochelatase cDNA probe. Molecular size markers are 0.24–9.5 kb RNA ladder. Arrow indicates 2.1 kb mRNA.

**Figure 4** Expression of ferrochelatase mRNA in hamster tissues as revealed by RT–PCR followed by Southern blot hybridization. (A) Ethidium bromide-stained agarose gel of RT-PCR carried out with primers FPLUS–FMINUS. (B) Autoradiography of Southern blot with a 730 bp $^{32}$P-labelled ferrochelatase probe. RNA from male liver (ML), female liver (FL), male HG (MHG), female HG (FHG), adrenal gland (AG) and lung (Lu) was used to prepare cDNA, in presence (+) or absence (−) of AMV reverse transcriptase. Lane mw, 100 bp DNA ladder as size standard.

**Figure 5** Expression levels of ferrochelatase mRNA in hamster liver. Complementary DNAs obtained from intact males (IM), castrated males (CM) and females at different phases of the oestrous cycle; proestrus (P), oestrus (E), metoestrus (M) and dioestrus (D), were used to amplify specific transcripts for ferrochelatase (FECH) and cyclophilin (CY). Normalization of the relative optical densities of RT–PCR products of FECH (753 bp) and CY (450 bp) is shown in the lower panel. Ethidium bromide-stained gels represent one experiment, and bars represent the mean ± s.d. ($n=7$). Arrow indicates 100 bp DNA marker. $^*P<0.001$ vs dioestrus (D).
On the other hand, the HG ferrochelatase mRNA showed marked variations during the oestrous cycle, with the highest levels at oestrus and lowest levels at dioestrus (Fig. 6). Consistently, the mRNA expression in male gland was higher than that found in female gland (Fig. 6). To assess the influence of sex steroid on the expression of ferrochelatase, groups of castrated hamsters were treated with different gonadal steroids. Figure 7 shows that the administration of progesterone or progesterone plus 17β-oestradiol to spayed females, or of testosterone to castrated males, does not modify significantly the amount of mRNA for ferrochelatase. In HGs from short- or long-term castrated males, testosterone had no significant effect.

**Discussion**

Here we have isolated and sequenced a cDNA-encoding ferrochelatase from hamster mRNA by RT–PCR and RACE methods. The results from this study show the transcriptional activity of ferrochelatase in Syrian hamster HGs. Differences in the HG mRNA content of males and females were found, as were variations in the female oestrous cycle. The isolated cDNA comprises 2175 nucleotides, including 70 bp of the 5′-UTR, 1266 bp of the open reading frame, and a long 3′-UTR of 839 bp followed by a poly(A) tail. Interestingly, it encodes a 422-amino-acid precursor molecule of ferrochelatase with a signal peptide of 48 amino acids followed by a mature protein of 374 amino acids. Some amino-acid residues are conserved among the vertebrate ferrochelatases and are present within the hamster sequence. These residues include a histidine at position 262 (His262), considered to be involved in substrate iron binding (Franco et al. 1995); significantly (P<0.05) the amount of mRNA for ferrochelatase. In HGs from short- or long-term castrated males, testosterone had no significant effect.
It is very likely that frataxin is also essential for iron-sulphur cluster biosynthesis. Frataxin can act as an extracellular source of iron (transferrin). Recent studies have demonstrated that frataxin, a nuclear-encoded protein, plays an important role in both haem and iron–sulphur cluster biosynthesis. Frataxin can act as a mitochondrial iron donor to transport Fe(II) not only to ferrochelatase but also to ISU proteins (He et al. 2004, Yoon & Cowan 2005). It is very likely that frataxin is also present in hamsters, since this mitochondrial protein content of haem is nearly undetectable and because HG porphyrin synthesis is under feedback inhibition by haem. There is also evidence that the activity of this enzyme is regulated by testosterone and 5α-DHT (Veltman & Maines 1985). Unlike ALA-S1, which is expressed ubiquitously, ALA-S2 is expressed only in differentiating erythroid precursor cells and appears not to be inhibited by haem (Sasa & Nagai 1996).

Data from previous reports have suggested an androgenic regulation of this pathway in HG. Thus, while the female HG expresses higher amounts of ALA-S1 mRNA than the male HG, the administration of testosterone or 5α-DHT to females led to a reduction in the levels of ALA-S1 mRNA. Moreover, the castration of males causes a marked elevation in ALA-S1 mRNA levels (Menéndez-Peláez et al. 1991, Rodríguez et al. 1993, Domínguez et al. 1994). Under these endocrine conditions, the concentrations of porphyrins in the HG show a strong correlation with the levels of ALA-S1 mRNA, indicating that factors other than haem may be modulating porphyrin synthesis. In this regard, the role of haem in HG physiology appears to be even less clear, particularly because the cellular production is mediated by ALA-S1, an enzyme whose regulation is under feedback inhibition by haem. There is also evidence that the activity of this enzyme is regulated by testosterone and 5α-DHT (Veltman & Maines 1985). Unlike ALA-S1, which is expressed ubiquitously, ALA-S2 is expressed only in differentiating erythroid precursor cells and appears not to be inhibited by haem (Sasa & Nagai 1996).

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hormones, since no significant differences were found during the distinct phases of the oestrous cycle, nor after the administration of sex-steroid hormones to spayed females (Fig. 7). In contrast to what occurs in liver, the content of ferrochelatase mRNA of the HG changed during the oestrous cycle. The intraglandular messenger levels were significantly higher at oestrus than at dioestrus. A similar cyclic pattern has been reported for the porphyrin concentrations in the female HG (Payne et al. 1979). The changes in the relative expression of ferrochelatase are probably modulated by circulating ovarian steroids, since this organ has been shown to be largely responsive to oestrogenic stimuli (Payne 1994), and the presence of specific receptors for oestrogens is well documented (Vilchis & Pérez-Palacios 1989, Vilchis et al. 1992). In HGs of cyclic hamsters, the maximal expression of ferrochelatase mRNA was observed at oestrus, a phase where the concentrations of oestradiol and progesterone have already declined to basal levels. This response may reflect a delayed effect induced by the high concentrations of oestradiol and/or progesterone that are normally present at proestrus (Smith et al. 1975, Vomachka & Greenwald 1979). On the other hand, in ovariectomized animals (Fig. 8), the levels of mRNA for the enzyme also increased significantly after treatment with progesterone or progesterone plus oestradiol, thus indicating involvement of ovarian steroids in the control of the Harderian ferrochelatase. However, these results could have been influenced by either the dose of steroids employed or the duration of treatment.

Both male gland histology and activity are known to be under hormonal control (Hoffman 1971, Payne 1979). Notably, male sex steroids sustain repressive effects on ALA-S1 activity and porphyrin levels (Domínguez et al. 1994) but do not change the abundance of type II cells (McMasters & Hoffman 1984) or the output of a haem-containing protein, which was considered to be involved in regulation of porphyrin synthesis (Vilchis et al. 1996). Here we found that the male HG contains less ferrochelatase mRNA than the liver, and the level of mRNA expression was not modified by castration or by the administration of testosterone to castrated males. These findings are consistent with the view that androgens have little influence on the rate of haem production. The low expression of ferrochelatase gene in the HG may explain its low haem content, although it is unlikely that haem levels inhibit HG porphyrinogenesis. However, the role of ferrochelatase in regulating porphyrin synthesis cannot be completely ruled out since haem concentrations as low as 10^{-7} M can suppress the formation of ALA-S (May et al. 1990, Spike et al. 1992). The data presented herein suggest that the Harderian ferrochelatase may function as a housekeeping enzyme to maintain the physiological pool of haem required for the cellular processing of cytochromes and other biologically active haemoproteins. In this organ, the control of porphyrin synthesis appears to be accomplished by mechanisms other than ferrochelatase-mediated haem production.

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