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

F Vilchis, L Ramos, C Timossi and B Chávez

Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga no. 15, Del. Tlalpan C.P. 14000, México D.F., Mexico

(Requests for offprints should be addressed to B Chávez; Email: bachavez@prodigy.net.mx)

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 17 β -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.

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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 (Ponka 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 P450_{scc} (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 ovariectomized 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 GTCCTCAG-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-His-TOPO (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 ($[\alpha\text{-}^{32}\text{P}]\text{ddNTP}$) 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'-CGTCCATCCTTTGACAGAAGAAGC-3'; 5'-GATTTACACAGAGCGGGCAGC-3'), was labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ 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 and hybridized under similar conditions to those described for Northern blot. For the study of ferrochelatase expression levels in males and females, 1.0 μl (1/20) of each RT reaction (from liver and HG) was used to amplify a 730 bp fragment from cDNA with 30 cycles of the following PCR conditions: 94 °C for 60 s, 58 °C for 45 s, 72 °C for 45 s and a final extension step at 72 °C for 7 min. A control PCR was performed with all cDNAs using primers for the ubiquitous protein cyclophilin [5'-CCCCACCGTGTTCTTCGA-3' (sense) and 5'-AGGTCCTTACCGTTCTGGTCG-3' (antisense)], which yielded a 453 bp fragment. Aliquots of 5.0 μl from each RT-PCR reaction were analysed on 1% agarose gel containing ethidium bromide and evaluated by densitometry with the Eagle-Eye II still video system (StrataGene, La Jolla, CA, USA).

Statistical analysis

Densitometric data were expressed as the mean value $\pm$ S.D. of three independent RT-PCR determinations. For each experimental group, the relative density value

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 $P < 0.05$.

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 NH_2 presequence with a cleavage site at residues 26–27. In Fig. 1, the first AUG codon is the initiator codon, although the flanking region around the second methionine conforms to the Kozak consensus (Kozak 1987).

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 ^{32}P -labelled probe of ferrochelatase showed that expression of a 2.1-kb mRNA was

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-70 gccctgggttcgtaggcgctcgtggtccttgctgtagcgccgcgcagaggatccctggcgtcccgaa

ATG CTT TCG GCC GGC GCC AAC ATG GCC GCA GCC CTG CGG GCT GCC GGC GCG CTG CTC CGC 60
Met Leu Ser Ala Gly Ala Asn Met Ala Ala Ala Leu Arg Ala Ala Gly Ala Leu Leu Arg 20

CAG CCG CTG GCG CAT GGC AGC TCG AGG GCC TGT CAG CCA TGG AGG TGC CAG TCG GGT GCG 120
Gln Pro Leu Ala His Gly Ser Ser Arg Ala Cys Gln Pro Trp Arg Cys Gln Ser Gly Ala 40

GCG GCA GCG GCC ACC ACA GAG AAA GTG CAT CAT GCC AAA ACT GCA AAG CCT CAA GCT CAA 180
Ala Ala Ala Ala Thr Thr Glu Lys Val His His Ala Lys Thr Ala Lys Pro Gln Ala Gln 60

CCA GAA AGG AGG AAG CCA AAA ACC GGC ATA TTA ATG TTA AAC ATG GGG GGC CCC GAA ACC 240
Pro Glu Arg Arg Lys Pro Lys Thr Gly Ile Leu Met Leu Asn Met Gly Gly Pro Glu Thr 80

CTT GGA GAA GTT CAA GAC TTC CTT CAA AGA CTC TTC TTG GAC CGA GAC CTC ATG ACA CTT 300
Leu Gly Glu Val Gln Asp Phe Leu Gln Arg Leu Phe Leu Asp Arg Asp Leu Met Thr Leu 100

CCC ATT CAA AAC AAG CTG GCA CCA TTC ATC GCC AAA CGC CGA ACC CCC AAA ATT CAA GAG 360
Pro Ile Gln Asn Lys Leu Ala Pro Phe Ile Ala Lys Arg Arg Thr Pro Lys Ile Gln Glu 120

CAG TAT CGC AGG ATT GGA GGT GGC TCC CCC ATC AAG ATG TGG ACT TCC AAG CAA GGA GAA 420
Gln Tyr Arg Arg Ile Gly Gly Gly Ser Pro Ile Lys Met Trp Thr Ser Lys Gln Gly Glu 140

GGC ATG GTG AAG TTG CTG GAT GAG CTG TCC CCT GAC ACA GCA CCT CAC AAA TAC TAT ATT 480
Gly Met Val Lys Leu Leu Asp Glu Leu Ser Pro Asp Thr Ala Pro His Lys Tyr Tyr Ile 160

GGA TTC CGG TAC GTC CAT CCT TTG ACA GAA GAA GCA ATT GAA GAG ATG GAG AGA GAT GGC 540
Gly Phe Arg Tyr Val His Pro Leu Thr Glu Glu Ala Ile Glu Glu Met Glu Arg Asp Gly 180

CTA GAA AGG GCC ATT GCT TTC ACA CAG TAT CCA CAG TAT AGC TGC TTC ACC ACC GGC AGC 600
Leu Glu Arg Ala Ile Ala Phe Thr Gln Tyr Pro Gln Tyr Ser Cys Phe Thr Thr Gly Ser 200

AGT TTA AAT GCC ATT TAC AGA TAC TAT AAC GAG ATG GGA CGG AAG CCC ACC ATG AAG TGG 660
Ser Leu Asn Ala Ile Tyr Arg Tyr Tyr Asn Glu Met Gly Arg Lys Pro Thr Met Lys Trp 220

AGC ACA ATT GAC AGG TGG CCC ACG CAT CCC CTC CTC ATC CAG TGC TTT GCA GAC CAC ATT 720
Ser Thr Ile Asp Arg Trp Pro Thr His Pro Leu Leu Ile Gln Cys Phe Ala Asp His Ile 240

CTG AAA GAA CTG GAC CAT TTT CCG CGG GAG AAG AGA AGC GAG GTG GTG ATT CTG TTT TCT 780
Leu Lys Glu Leu Asp His Phe Pro Arg Glu Lys Arg Ser Glu Val Val Ile Leu Phe Ser 260

GCC CAC TCC CTG CCA ATG TCC GTT GTC AAC AGA GGA GAT CCC TAT CCC CAG GAA GTA GGT 840
Ala His Ser Leu Pro Met Ser Val Val Asn Arg Gly Asp Pro Tyr Pro Gln Glu Val Gly 280

GCC ACT GTC CAC AAA GTC ATG GAA GAA CTA GGT TAT CCC AAC CCC TAC CGA CTG GTT TGG 900
Ala Thr Val His Lys Val Met Glu Glu Leu Gly Tyr Pro Asn Pro Tyr Arg Leu Val Trp 300

CAG TCC AAG GTT GGC CCA GTA CCC TGG CTG GGT CCT CAA ACA GAT GAG GCC ATC AAA GGG 960
Gln Ser Lys Val Gly Pro Val Pro Trp Leu Gly Pro Gln Thr Asp Glu Ala Ile Lys Gly 320

CTT TGT GAG AGA GGG AGG AAG AAT ATT CTC TTG GTT CCA ATA GCA TTT ACC AGT GAT CAC 1020
Leu Cys Glu Arg Gly Arg Lys Asn Ile Leu Leu val Pro Ile Ala Phe Thr Ser Asp His 340

ATC GAG ACC CTG TAT GAA CTG GAT ATT GAA TAC TCC CAA GTG TTA GCC AAG AAG TGT GAA 1080
Ile Glu Thr Leu Tyr Glu Leu Asp Ile Glu Tyr Ser Gln Val Leu Ala Lys Lys Cys Glu 360

GCT GAA AAC ATC AGA AGA GCG GAG TCT CTT AAT GGA AAT CCA TTG TTC TCT AAG GCC TTG 1140
Ala Glu Asn Ile Arg Arg Ala Glu Ser Leu Asn Gly Asn Pro Leu Phe Ser Lys Ala Leu 380

GCT GAT CTG GTA TAC TCA CAC ATC CAG TCA AAC AAG CTG TGC TCT ACG CAG TTG ACT CTG 1200
Ala Asp Leu Val Tyr Ser His Ile Gln Ser Asn Lys Leu Cys Ser Thr Gln Leu Thr Leu 400

AGC TGC CCG CTC TGT GTA AAT CCT GTC TGC AGG GAG ACT AAA TCC TTC TTC ACC AGC CAG 1260
Ser Cys Pro Leu Cys Val Asn Pro Val Cys Arg Glu Thr Lys Ser Phe Phe Thr Ser Gln 420

CAG CTG TGA ccctgcctgaggaccctgtgggattcacagatgcgccagcctccagacacctccaatgggaatggaag 1336
Gln Leu ***
ccacatagccttgagacagcctgtggacaaagatagtattttcttctgctttatttatgttactttatttttgt 1415
ttcctgtgtagtcacagctggcctccaaatgggcctcctgttcgaatttcctgaatgctaattagcatcacagacacac 1494
aaaattgtgtttctcggggaatagactctgaaattcttactgcggtttctattttcatacacacatacagtgaatactc 1573
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atgagacacacacagattctgcacttactgccccaaacagcctcctaaaatagtatttcgagtcgccactatgtgtctac 1731
tagtggtgaatgagatgtctacaacctcatggatgcaggttaagtgaatatagttatattatgaatatataattgttaa 1810
agaattggcctgagggcatagcttagtgattgtgtgctggcctagcattcatgaggctcctagtttgatccctgaaat 1889
acatgtcgatattatgttacctattctttccctaagtcocatgggtaccaggatactggtagttggcctctattgcag 1968
tgaagtcacagtcatcggtcattgtgtgtcctgtgaaagagtcctcactctcctccctgaaactgaaatgagtgctca 2047
tttcaaaagtgtctgagattttccaggccacacagcccataaaatatatggaaattc..... 2105

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Hamster	MLSAGANMAAALRAAGALLRQPLAHGSSRACQPW---RCQS-GAAAAATTEKVHHAKTAKPQAQPERRKPKTGILML
Mouse	MLGSANMAAALRAAGALLREPLVHJSSRACQPW---RCQS-GAAVAATTEKVHHAKTKPQAQPERRKPKTGILML
Human	MRLSGANMAAALRAAGVLLRDPLASSSWRVCQPW---RWKSGAAAAAVTTEAQHAQGAQPVQVQPKRKPKTGILML
Bovine	-----MAAALRSAGVLLRDRLLYGGSRACQPR---RCQSGAATAAAATETAQRARSFKPQAQPGNRKPKRTGILML
Chicken	-----MAAAGRAARPLVAGGRQLRVPLRWRG-----QVAAAAPSTKKQAEPETRRKPKTGILML
Xenopus	-----MAAFRAAHRLIGHILRNESAGLVLTQ---RWSS-----SAVASVPKSSDPKPHAQPDKRRKPKTGILML
Danio	-----MAVLGGACRLVQLVRCGSPVGLCLSS-----SLRRQSTATAAFTNTATPETKESRKPKTGILML
Hamster	NMGGPETLGEVQDFLQRLFLDRDMLTLPIONKLAPPIAKRRTPKIQEQYRRIGGGSPIKMWTSSKQEGGMVKLLDELSP
Mouse	NMGGPETLGEVQDFLQRLFLDRDMLTLPIONKLAPPIAKRRTPKIQEQYRRIGGGSPIKMWTSSKQEGGMVKLLDELSP
Human	NMGGPETLGDVHDFLLRLFLDQDMLTLPIONKLAPPIAKRRTPKIQEQYRRIGGGSPIKMWTSSKQEGGMVKLLDELSP
Bovine	NMGGPETVEEVQDFLQRLFLDQDMLTLPVQDKLGPPIAKRRTPKIQEQYRRIGGGSPIKMWTSSKQEGGMVKLLDELSP
Chicken	NMGGPERLDDVHDFLLRLFLDRDMLTLPQNKLAAPPIAKRRTPRIQEQYSRIGGGSPIKKWTAVQEGGMVKLLDSMSP
Xenopus	NMGGPETLDDVHDFLLRLFLDQDMLTLPQSKLAAPPIAKRRTPKIQEQYSKIGGGSPIKKWTETQQEGGMVKLLDELSP
Danio	NMGGPEKLEDVHDFLLRLFLMDTDFMLPVQNKLGPPPIAKRRTPKIQEQYSKIGGGSPIKAWTMTQEGGMVKLLDEMCP
Hamster	DTAPHKYYIGFRVYHPLTEEAIEEMERDGLERAIAFTQYPQYSCSTTGSSSLNAIYRYYNEMGRKPTMKWSTIDRWPTH
Mouse	ATAPHKYYIGFRVYHPLTEEAIEEMERDGLERAIAFTQYPQYSCSTTGSSSLNAIYRYYNEVGQKPTMKWSTIDRWPTH
Human	NTAPHKYYIGFRVYHPLTEEAIEEMERDGLERAIAFTQYPQYSCSTTGSSSLNAIYRYYNQVGRKPTMKWSTIDRWPTH
Bovine	HTAPHKYYIGFRVYHPLTEEAIEEMERDGLERAIAFTQYPQYSCSTTGSSSLNAIYRYYNEVGRKPTMKWSTIDRWPTH
Chicken	QTAPHKYYIGFRVYHPLTEEAIEEMEDDGLERAIAFTQYPQYSCSTTGSSSLNAIYRYYNKKGKKPKMKWSIDRWPTH
Xenopus	ATAPHKYYIGFRVYHPLTEEAIEEMERDGLERAIAFTQYPQYSCSTTGSSSLNAIYRYYNAKGTQPKMKWSIDRWPTH
Danio	DTAPHKYYIGFRVYHPLTEEAIEEMERDGLERAIAFTQYPQYSCSTTGSSSLNAIYRYYNRDRPKMRWSIDRWPTH
Hamster	PLLIQCFADHILKELDHFPPEKRSEVVILFSAHSLPMSVNVNRGDPYPQEVGATVHKVMEELGYPNPYRLVWQSKVGPV
Mouse	PLLIQCFADHILKELNHFPEEKRS EVVILFSAHSLPMSVNVNRGDPYPQEVGATVHKVMEELGYPNPYRLVWQSKVGPV
Human	HLLIQCFADHILKELDHFELEKRS EVVILFSAHSLPMSVNVNRGDPYPQEVGATVHKVMEELGYPNPYRLVWQSKVGPV
Bovine	PLLIQCFADHILKELDHFPPEKRREVILFSAHSLPMSVNVNRGDPYPQEVGATVQVMDKLGYSNPYRLVWQSKVGPV
Chicken	PLLIQCFADHILKELDLFPDPKRDVVILFSAHSLPMSVNVNRGDPYPQEVGATVQVMDKLGYSNPYRLVWQSKVGPV
Xenopus	PLLIQCFADHILKELDMFPADKREVVILFSAHSLPMSVNVNRGDPYPQEVGATVQVMDKLGYSNPYRLVWQSKVGPV
Danio	PLLIQCFADHILKELDMFPADKREVVILFSAHSLPMSVNVNRGDPYPQEVGATVQVMDKLGYSNPYRLVWQSKVGPV
Hamster	PWLGPQTDIAIKGLCERGRKNILLVPIAFTSDHIETLYELDIYSQVLAKKCEAENIRRAESLNGNPLFSKALADLVY
Mouse	PWLGPQTDIAIKGLCERGRKNILLVPIAFTSDHIETLYELDIYSQVLAKKCEAENIRRAESLNGNPLFSKALADLVH
Human	PWLGPQTDIAIKGLCERGRKNILLVPIAFTSDHIETLYELDIYSQVLAKKCEAENIRRAESLNGNPLFSKALADLVH
Bovine	PWLGPQTDIAIKGLCERGRKNILLVPIAFTSDHIETLYELDIYSQVLAKKCEAENIRRAESLNGNPLFSKALADLVH
Chicken	PWLGPQTDIAIKGLCERGRKNILLVPIAFTSDHIETLYELDIYSQVLAKKCEAENIRRAESLNGNPLFSKALADLVH
Xenopus	PWLGPQTDIAIKGLCERGRKNILLVPIAFTSDHIETLYELDIYSQVLAKKCEAENIRRAESLNGNPLFSKALADLVH
Danio	PWLGPQTDIAIKGLCERGRKNILLVPIAFTSDHIETLYELDIYSQVLAKKCEAENIRRAESLNGNPLFSKALADLVH
Hamster	SHIQSNKLCSTQLTSLCPLCVNPVCRETKSFFTSQQQL-
Mouse	SHIQSNKLCSTQLTSLCPLCVNPVCRETKSFFTSQQQL-
Human	SHIQSNKLCSTQLTSLCPLCVNPVCRETKSFFTSQQQL-
Bovine	SHIQSNKLCSTQLTSLCPLCVNPVCRETKSFFTSQQQL-
Chicken	SHIQSNKLCSTQLTSLCPLCVNPVCRETKSFFTSQQQL-
Xenopus	SHIQSNKLCSTQLTSLCPLCVNPVCRETKSFFTSQQQL-
Danio	SHIQSNKLCSTQLTSLCPLCVNPVCRETKSFFTSQQQL-

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/).

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

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 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 ³²P-labelled ferrochelatase cDNA probe. Molecular size markers are 0.24–9.5 kb RNA ladder. Arrow indicates 2.1 kb mRNA.

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 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 $\pm$ s.d. ($n=7$). Arrow indicates 100 bp DNA marker. * $P<0.001$ vs dioestrus (D).

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 ³²P-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 6 Expression levels of ferrochelatase mRNA in hamster Harderian gland (HG). 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 $\pm$ 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 17 β -oestradiol to spayed females, or of testosterone to castrated males, does not modify significantly the amount of mRNA of liver. Figure 8 shows the ferrochelatase:cyclophilin ratio in the HGs from gonadectomized, treated animals. Here, the administration of progesterone or progesterone plus 17 β -oestradiol to spayed females was shown to increase

Figure 7 Effect of sex-steroid hormones on the ferrochelatase mRNA levels in liver of gonadectomized hamsters. Samples of liver cDNA from spayed females treated with oestradiol benzoate (E₂), progesterone (P₄), oestradiol plus progesterone (E₂+P₄) or vehicle alone (oil); intact males (I), castrated males (C), and males castrated for 7 (C+T) or 30 days (C1+T) and treated with testosterone, were used to amplify specific transcripts of ferrochelatase (FECH) and cyclophilin (CY). Normalization of the relative optical densities of RT-PCR products for 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 $\pm$ s.d. ($n=6$). Arrow indicates 100 bp DNA marker.

significantly ($P<0.05$) 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);

Figure 8 Effect of sex-steroid hormones on the ferrochelatase mRNA levels in Harderian gland (HG) of gonadectomized hamsters. Samples of HG cDNA from spayed females treated with oestradiol benzoate (E₂), progesterone (P₄), oestradiol plus progesterone (E₂+P₄) or vehicle alone (oil); intact males (I), castrated males (C), and males castrated for 7 (C+T) or 30 days (C1+T) and treated with testosterone (FECH) and cyclophilin (CY). Normalization of the relative optical densities of RT-PCR products for 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=6). Arrow indicates 100 bp DNA marker. *P<0.05 vs control group (oil).

a glutamic acid residue (Glu₂₈₈), considered to be involved in catalysis (Ferreira *et al.* 1999); and the four cysteine residues (Cys₁₉₅, Cys₄₀₂, Cys₄₀₅ and Cys₄₁₀), considered to be the [2Fe-2S] cluster ligands (Sellers *et al.* 1998, Dailey *et al.* 2000). While the key function of His262 and Glu188 are recognized in a number of studies, the results from site-directed mutagenesis experiments have brought into question the putative role of His262 in substrate iron binding (Dailey *et al.* 2000).

At present, relatively little is known about the regulation of porphyrin synthesis and the role of ferrochelatase in the physiology of the rodent HG. Unlike what occurs in other porphyrinogenic tissues, such as the liver and the adrenal gland, it is not known whether HG ferrochelatase uses iron from intracellular sources or whether it depends on 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

has been identified from invertebrates up to mammals (Ventura *et al.* 2005). Nevertheless, the role of frataxin or its possible expression in HG has not been examined and remains to be investigated. There is evidence that iron is present at large concentrations in the male HG (Hoffman & Jones 1981); and it is known that ferrochelatase activity is under the control of intracellular iron, which possibly is correlated with formation of the [2Fe-2S] cluster at the C-terminal region (Taketani *et al.* 2000).

In nonerythroid cells, the rate-limiting step of haem production is mediated by ALA-S1, an enzyme whose synthesis is under feedback inhibition by haem. There is also evidence that the activity of this enzyme is regulated by testosterone and 5 α -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 (Sassa & 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 5 α -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 content of haem is nearly undetectable and because HG ferrochelatase activity is minimal (Thompson *et al.* 1984, Nagai *et al.* 1997). Although the incorporation of ferrous iron into protoporphyrin IX to form haem has not been clearly demonstrated in Harderian tissue, the specific synthesis of considerable amounts of androgen-induced haemoproteins, whose production shows gender-associated differences (Vilchis *et al.* 1996), along with the relative content of glandular cytochrome P450 scc (Vilchis *et al.* 2002), is indirect evidence of local ferrochelatase activity.

As an initial step to define more clearly the role of this enzyme in the HG, we first assessed its transcriptional activity by analysing mRNA levels and determined whether ferrochelatase is expressed differentially in males and females. Northern blot analyses showed positive hybridization in samples of liver RNA with little or almost undetectable hybridization signal in HG preparations, suggesting a low transcriptional activity in this tissue. Similarly, low expression levels have also been detected by Northern blot analysis of murine ferrochelatase (Brenner & Frasier 1991). As shown in Fig. 5, the level of ferrochelatase mRNA was slightly higher in the liver of males than the livers of females. Apparently, the amount of hepatic mRNA is not influenced by the sex-steroid

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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