Conservation of glutathione S-transferase mRNA and protein sequences similar to human and horse Alpha class GST A3-3 across dog, goat, and opossum species

Shawna M. Hubert^{a,b}, Paul B. Samollow^{c,d}, Helena Lindström^e, Bengt Mannervik^e, Nancy H. Ing^{a,d,f*}

^a Department of Animal Science, Texas A&M AgriLife Research, Texas A&M University, College Station, TX 77843-2471, USA

^b Department of Thoracic Head & Neck Medical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

^cDepartment of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biosciences, Texas A&M University, College Station, TX 77843-2471, USA

^d Faculty of Genetics, Texas A&M University, College Station, TX 77843-2128, USA

^e Department of Biochemistry and Biophysics, Stockholm University, Arrhenius Laboratories, SE-10691, Stockholm, Sweden

^f Faculty of Biotechnology, Texas A&M University, College Station, TX 77843-2128, USA

*Corresponding authors at:

Nancy H. Ing, Department of Animal Science, Texas A&M University, 2471 TAMU, College Station, TX 77843-2471, United States. Tel.: +1 979 862 2790; Fax: +1 979 862 3399

Bengt Mannervik, Department of Biochemistry and Biophysics, Stockholm University, Arrhenius Laboratories, SE-10691, Stockholm, Sweden.

E-mail addresses:

n-ing@exchange.tamu.edu (N.H. Ing)

bengt.mannervik@dbb.su.se (B. Mannervik)

- 27 SMHubert@mdanderson.org (S.M. Hubert)
- 28 psamollow@cvm.tamu.edu (P. Samollow)
- 29 helenasemail@yahoo.com (H. Lindström)

Abstract

Recently, the glutathione S-transferase A3-3 (GST A3-3) homodimeric enzyme was identified as the most efficient enzyme that catalyzes isomerization of the precursors of testosterone, estradiol, and progesterone in the gonads of humans and horses. However, the presence of GST A3-3 orthologs with equally high ketosteroid isomerase activity has not been verified in other mammalian species, even though pig and cattle homologs have been cloned and studied. Identifying *GSTA3* genes is a challenge because of multiple *GSTA* gene duplications (12 in the human genome), so few genomes have a corresponding *GSTA3* gene annotated. To improve our understanding of *GSTA3* gene products and their functions across diverse mammalian species, we cloned homologs of the horse and human *GSTA3* mRNAs from the testes of a dog, goat, and gray short-tailed opossum, with those current genomes lacking *GSTA3* gene annotations. The resultant novel *GSTA3* mRNA and inferred protein sequences had a high level of conservation with human *GSTA3* mRNA and protein sequences ($\geq 70\%$ and $\geq 64\%$ identities, respectively). Sequence conservation was also apparent for the 13 residues of the “H-site” in the 222 amino acid GSTA3 protein that is known to interact with the steroid substrates. Modeling predicted that the dog GSTA3-3 is a more active ketosteroid isomerase than the goat or opossum enzymes. Our results help us understand the active sites of mammalian GST A3-3 enzymes, and their inhibitors may be useful for reducing steroidogenesis for medical purposes, such as fertility control or treatment of steroid-dependent diseases.

1. Introduction

It is widely appreciated that reproduction of animal species important to man centers around sex steroid production by the gonads. While driven by peptide hormones from the hypothalamic/pituitary axis, production of testosterone by the Leydig cells of the testis and estradiol and progesterone by the granulosa cells and corpora lutea of the ovary are required for successful production of offspring [1-3]. Testosterone, estradiol and progesterone act in reproductive tissues by binding specific receptors which regulate the expression of sets of genes [4,5].

Testosterone, estradiol and progesterone are produced from cholesterol by a series of enzymatic modifications [6,7]. This reaction sequence differs among species: rodents utilize the Δ^4 pathway whereas humans and other larger mammals utilize the Δ^5 pathway [7]. The biosynthetic pathway for testosterone in human and horse testes involves the alpha class glutathione S-transferase A3-3 (GST A3-3) as a ketosteroid isomerase that acts as a homodimer to convert Δ^5 -androstene-3,17-dione to Δ^4 -androstene-3,17-dione, the immediate precursor of testosterone [8-15]. The recombinant human GST A3-3 enzyme is 230 times more efficient at this isomerization than hydroxy- Δ^5 -steroid dehydrogenase, 3 β -hydroxysteroid delta-isomerase 2 (HSD3B2), the enzyme to which this activity previously had been attributed [2]. Estradiol synthesis is likewise dependent upon GSTA3-3 in humans and horses because testosterone is further converted to estradiol by aromatase [7]. GST A3-3 also participates in the synthesis of progesterone in female mammals utilizing the Δ^5 pathway by isomerizing Δ^5 -pregnene-3,20-dione to Δ^4 -pregnene-3,20-dione (i.e., progesterone).

From a general perspective, the GST family of enzymes is recognized for having active roles in detoxification. The GSTA class of genes is best characterized in humans. It is represented by 12 genes and pseudogenes with almost indistinguishable sequences. These arose from gene duplications and in humans are clustered in a 282 kbp region on

chromosome 6 [16]. Because of the redundancy of GSTA genes, the *GSTA3* genes are
inexactly identified, if identified at all, in the current annotations of the genomes of most
mammals. This gap in knowledge limits studies of *GSTA3* gene expression because standard
GSTA3 mRNA and protein detection reagents crossreact with other four gene products in the
GSTA family, which are ubiquitously expressed [11].

Understanding the regulation of expression of *GSTA3* gene and the activities of
GSTA3-3 enzyme is critical to fully comprehending the synthesis of sex steroid hormones
that direct reproductive biology in animals. In extracts of a steroidogenic cell line, two
specific inhibitors of *GSTA3-3* (ethacrynic acid and tributyltin acetate) decreased
isomerization of delta-5 androstenedione with very low half maximal inhibitory
concentrations (IC₅₀) values of 2.5 uM and 0.09 uM, respectively [17]. In addition,
transfection of such a cell line with two different small interfering RNAs both decreased
progesterone synthesis by 26%. In stallions treated with dexamethasone, a glucocorticoid
drug used to treat inflammation, testicular levels of *GSTA3* mRNAs decreased by 50% at 12
hours post-injection, concurrent with a 94% reduction in serum testosterone [18,19]. In
stallion testes and cultured Leydig cells, dexamethasone also decreased concentrations of
steroidogenic factor 1 (SF1) mRNA [20]. In a human genome-wide study of the genes
regulated by SF1 protein, chromatin immunoprecipitation determined that the SF1 protein
bound to the *GSTA3* promoter to up-regulate the gene [13]. Notably, the SF1 transcription
factor up-regulates the expression of several gene products involved in steroidogenesis [2].
These data indicate that there are reagents and pathways by which *GSTA3* gene expression
and steroidogenesis can be altered *in vivo*. This could be useful for reducing steroidogenesis
for fertility control or other medical purposes.

The equine GST A3-3 enzyme is structurally similar to the human enzyme and
matches or exceeds the very high steroid isomerase activity of the latter [15]. Remarkably,
the cloning of *GSTA3* homologs from the gonads of domestic pigs (*Sus scrofa*) and cattle

(*Bos taurus*) yielded enzymes that were much less active ketosteroid isomerases for reasons yet to be determined. In the current work, *GSTA3* mRNA was cloned from the testes of the dog and goat (eutherian mammals), and gray short-tailed opossum (metatherian mammal). The purposes were (1) to compare mRNA and protein sequences with the human and horse counterparts to search for more highly active ketosteroid isomerases, (2) to generate *GSTA3*-specific reagents for future studies of the regulation of *GSTA3* genes, and (3) to understand the scope of the *GSTA3*-3 function in steroidogenesis across a range of mammalian species in which reproduction is important to humans.

2. Experimental

2.1. Testis tissue samples and RNA preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee. Adult testes used in this study were obtained from Texas A&M University owned research animals. Specifically, testes were from a large breed hound (*Canis lupus familiaris*), a goat (*Capra hircus*), and, a marsupial mammal, a gray short-tailed opossum (*Monodelphis domestica*).

Total cellular RNA was extracted from each testis with the Tripure reagent (Sigma-Aldrich; St. Louis, MO) protocol. The concentration and purity of the RNA was assessed using a Nanodrop spectrophotometer.

2.2. Reverse transcription

Reverse transcription reactions (25 µl) for the production of complimentary DNA (cDNA) were run with each RNA sample (500 ng) using random octamer primers (312 ng), dT₂₀ primers (625 ng), dNTPs, DTT, first strand buffer, Supersin and Superscript II Reverse Transcriptase (RT), following the manufacturer's instructions (ThermoFisher Scientific; Waltham, MA). The RT reactions incubated in an air incubator at 42 °C for three hours. The RT enzyme was inactivated by incubating at 70 °C for 15 minutes.

2.3. Cloning *GSTA3* cDNAs into the vector pCR2.1

Nested polymerase chain reaction (PCR) was utilized to selectively amplify the *GSTA3* mRNA targets because it increases the sensitivity and fidelity of the amplification (Fig. 1). Primers were designed from BLAST analyses with human and horse *GSTA3* mRNA sequences (GenBank accession numbers NM_000847.1 and KC512384.1, respectively; Table 1) to identify related dog, goat and opossum sequences for cloning cDNAs into the general use vector pCR2.1 (TA Cloning Kit; ThermoFisher Scientific). The primers were synthesized by Integrated DNA Technologies (Skokie, IL). PCR reactions (50 µl) were performed with Ex Taq enzyme (Takara Bio Inc.; Mountain View, CA) in a GeneAmp PCR System 9600 (PerkinElmer; Norwalk, CT). Primary PCR reactions (50 µl) were set up with outside primers and 1 µl of the appropriate RT reaction. Cycling parameters were 30 cycles of 94 °C for 15 seconds, 45 °C annealing for 30 seconds, and 72 °C for one minute, followed by a one-time hold at 72 °C for five minutes. Secondary PCR reactions (50 µl) were set up with inside primers and 5 µl of the primary PCR reactions as a cDNA template source instead of using the RT reaction. Cycling parameters were maintained for the secondary PCR. The PCR products were run on both a 1% agarose gel and a 0.8% low melting temperature agarose gel. Ethidium bromide staining of DNA bands was visualized under UV light. This procedure confirmed the presence of the intended PCR products of 670 to 700 base pairs in all of the secondary PCR reactions.

Table 1. PCR primer sequences (5'-3') for TA cloning *GSTA3* cDNAs into pCR2.1.

Species	Primer Set	Orientation	Sequence
Dog	Outside	Sense	GGAGACCTGCATCATGGCAGTGAAGCCCATG
		Antisense	AGGAGATTGGCCCTGCATGTGCT
	Inside	Sense	ATGGCGGGGAAGCCCAAGCTTCACTACTTCAATGG
		Antisense	CTGGGCATCCATTCTGTTCACTTAATCCT
Goat	Outside	Sense	GGAGACTGCATCATGGCAGTGAAGCCCATG
		Antisense	TCAAATTTGTCCCAAACAGCCCC
	Inside	Sense	ATGGCAGGGAAGCCCATTTCTCACTATTTCAATGG
		Antisense	CCCCGCCAGCCGCCAGCTTTATTAAACTT
Opossum	Outside	Sense	GGAGACTGCCATCATGGCAGTGAAGCCCATG

		Antisense	TGTGTTTAAGAAACACAGAGTCA
	Inside	Sense	GGTAAAGAAGATATTCAAGGTTGATGA
		Antisense	TCATCAACCTTGAATATCTTCTTTACC

GSTA3 cDNA bands were cut from the 0.8% low melting temperature agarose and melted at 70 °C for 10 minutes. Ligation of the cDNA inserts to pCR2.1 was performed according to the kit's instructions (TA Cloning kit; ThermoFisher). These ligation reactions were then utilized in transformation of *E. coli* competent cells. After incubation in SOC broth for 1 h at 37 °C, transformations were plated on LB-ampicillin agar containing isopropyl β-D-1-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for blue-white colony selection. Plates were incubated overnight at 37 °C. Selected white colonies were cultured overnight in LB-ampicillin broth and plasmid DNA was purified using the Qiagen QIAprep Spin Kit (Germantown, MD). *GSTA3* cDNAs were released from the pCR2.1 plasmids by digestion with EcoRI before being analyzed by gel electrophoresis. Plasmid samples that demonstrated the expected 670 to 700 base pair bands were sequenced.

2.4. Sequencing and analysis

The *GSTA3* cDNAs in the pCR2.1 clones were amplified with PCR through the use of Big-Dye mix (Applied Biosystems, ThermoFisher Scientific) using the M13 forward and M13 reverse primers (5'-TGTAACACGACGGCCAGT-3' and 5'-CAGGAAACAGCTATGAC-3', respectively). After PCR amplification, G-50 spin columns were used to remove unincorporated nucleotides. Samples were sequenced at the Texas A&M University Gene Technologies Laboratory on a PRISM 3100 Genetic Analyzer mix (Applied Biosystems, ThermoFisher Scientific). Each sequence was then compared to the NCBI GenBank database records for the human and horse *GSTA3* mRNA sequences (GenBank accession numbers NM_000847.1 and KC512384.1, respectively). The basic local alignment search tool (BLAST) on the NCBI website was also utilized to check for

alignments to reference mRNA sequences of other species. For each species, the sequence with the highest identity to the NCBI *GSTA3* mRNA reference sequences was translated to its amino acid sequence through the use of the ExPASy translate tool (<https://web.expasy.org/translate/>). Sequence validation of *GSTA3* mRNAs and proteins was obtained by locating the five amino acid residues that have been identified as key to the activity of the human GST A3-3 enzyme [9,21]. Following this analysis, *GSTA3* mRNA sequences were submitted to NCBI GenBank. Accession numbers for these sequences are given in the Results and Discussion section.

2.5. Cloning *GSTA3* cDNA in the bacterial expression vector pET-21a(+)

Another set of optimized primers was created for cloning into the bacterial expression vector pET-21a(+) (EMD Millipore; Billerica, MA). For directional in-frame cloning into pET-21a(+), the *Eco* RI (GAATTC) and *Xho*I (CTCGAG) restriction sites were added to the 5' ends of the inside sense and antisense primers, respectively (Table 2). Nested PCR was performed as described in section 2.3. Secondary PCR samples along with the pET-21a(+) vector were restricted with *Eco* RI and *Xho*I endonucleases. Purified bands from electrophoresis on an 0.8% LMT agarose gel were ligated. After transformation, colony growth in broth, and plasmid mini-preparation, the plasmid cDNAs were sequenced with the T7 promoter and the T7 terminator primers (5'-TAATACGACTCACTATAG-3' and 5'-GCTAGTTATTGCTCAGCGG-3', respectively). Sequence analysis was as described in section 2.4 and included confirming in-frame insertion of the sense strand sequence into the pET-21a(+) vector for protein expression in bacteria.

Table 2. PCR primer sequences (5'–3') for cloning *GSTA3* cDNAs into pET-21a+.

Species	Primer Set	Orientation	Sequence
Dog	Outside	Sense	CCAGAGACTACCATGGCGGGGAAGCCCAAG
		Antisense	TCTCAGGAGATTGGCCCTGCATG
	Inside	Sense	gcgaattcATGGCGGGGAAGCCCAAGCTTCACTACTTCAATGG
		Antisense	cgctcgagCTGGGCATCCATTCTGTTCAGTTAATCCT
Goat	Outside	Sense	AGAACTGCTATTATGGCAGGGAAGCCCAT
		Antisense	TCAAATTTGTCCCAGACAGCCC
	Inside	Sense	gcgaattcATGGCAGGGAAGCCCATTTCTTCACTATTTCAATGG

		Antisense	cgctcgagCCCCGCCAGCCGCCAGCTTTATTAAAACTT
Opossum	Outside	Sense	GAATGGAAGATCATGTCTGGGAAGCCCAT
		Antisense	TTGCATTACTTAGAACTCTTCCTGAATATTCAGCT
	Inside	Sense	gcgaattcGGTAAAGAAGATATTCAAGGTTGATGA
		Antisense	cgctcgagTCATCAACCTTGAATATCTTCTTACC

Lowercase letters designate the restriction enzyme site.

3. Results

3.1. Alignment of the cloned *GSTA3* mRNA sequences to those of human and horse *GSTA3* mRNAs

Fig. 2 shows the alignments of the newly cloned dog, goat, and gray short-tailed opossum *GSTA3* mRNA sequences to the known *GSTA3* mRNA sequences of human (GenBank accession number **NM_000847.4**) and horse (**KC512384.1**). The 5' ends of the sequences begin with the start codons (indicated by green font), and the 3' ends terminate with stop codons (in red font). As expected, there is a high degree of sequence conservation across the entire mRNA sequences (gray highlighted sequences in Fig. 2 are identical to those of human *GSTA3* mRNA). The GenBank accession numbers of the newly cloned *GSTA3* cDNAs are **KJ766127** (dog), **KM578828** (goat), and **KP686394** (gray short-tailed opossum). The overall percentages of identical residues at each position were compared pairwise between each *GSTA3* mRNA, and are presented in Table 3. Compared to the human *GSTA3* mRNA, the dog *GSTA3* mRNA has the highest sequence identity to that of the human, with 88% identical bases. Goat *GSTA3* mRNA was similar to that of the horse in having 84% and 85% identical residues, respectively, compared to the human *GSTA3* mRNA. The most divergent was the gray short-tailed opossum *GSTA3* mRNA, which had identities to the four eutherian *GSTA3* mRNAs ranging from 68% to 71% identical bases.

Table 3. The percentages of identical nucleotides at each position between *GSTA3* mRNAs of different mammalian species.

	Human	Dog	Horse	Goat	Opossum
Human	100	88	85	84	70
Dog	88	100	87	87	71
Horse	85	87	100	85	70
Goat	84	87	85	100	68
Opossum	70	71	70	68	100

3.2. Alignment of the predicted *GSTA3* protein sequences from five mammals

The amino acid sequences of the GSTA3 proteins were inferred from the cloned GSTA3 mRNA sequences. They are aligned with the human and horse GSTA3 protein sequences (GenBank accession numbers NP_000838.3 and AGK36275.1, respectively) in Fig. 3. Underneath the alignment, the “*” symbols identify sites with identical residues across all five species; these comprise 115 residues of the 222 total amino acids (52%) of the GSTA3 proteins. The “:” symbols identify sites with highly similar residues (53 residues of 222 total amino acids, or 24%) and the “.” symbols indicate positions with less similar residues (11 residues of the whole GSTA3 protein, or 5%). The conservation of amino acid residues between GSTA3 proteins of different species appears to be fairly consistent across the length of the proteins. The percentage of identical amino acids in the various positions was calculated between pairs of aligned GSTA3 protein sequences of all species (Table 4). The degree of conservation to human GSTA3 protein was maintained within eutherian species, with the dog GSTA3 protein having 85% identical residues, and horse and goat GSTA3 proteins both had 81% identical residues compared to the human protein. The metatherian gray short-tailed opossum GSTA3 protein had the lowest conservation with 64% identical residues with the human protein.

Table 4. The percentages of identical amino acids at each position between GSTA3 proteins of different mammalian species.

	Human	Dog	Horse	Goat	Opossum
Human	100	85	81	81	64
Dog	85	100	81	83	62
Horse	81	81	100	82	61
Goat	81	83	82	100	62
Opossum	64	62	61	62	100

3.3. Conservation of amino acids critical for steroid isomerase activity and binding of the cofactor glutathione

The G-site residues for binding the cofactor glutathione are Tyr9, Arg15, Arg45, Gln54, Val55, Pro56, Gln67, Thr68, Asp101, Arg131 and Phe220 in human GSTA3 [15].

The G-site residues were identical or conservatively changed among the GSTA3 proteins of the five species with the exception of Arg45 or Lys45 being Ile45 in the opossum enzyme (Fig. 3). Most of the H-site residues are nearly identical across all of the species. The five critical amino acids defining high ketosteroid isomerase activity in human GSTA3-3 as compared to the low activity of human GST A2-2 [9] are marked with underlining in Fig. 3 and “*” in Table 5, and are identical or similar to these human GST A3-3 residues in the three new sequences, with the exception of more bulky residues in position 208. Table 5 shows the H-site residues in the GSTA enzymes expressed in pig (“GSTA2-2”) and bovine (“GSTA1-1”) steroidogenic tissues and compared for ketosteroid isomerase activities to equine and human GSTA3-3 enzymes [15]. Both pig GSTA2-2 and bovine GSTA1-1 enzymes are similar to the dog, goat and opossum enzymes in having have more bulky residues at position 208 than do the human and equine GSTA3-3 enzymes. This may be part of the reason that the pig and bovine enzymes are only 0.01- to 0.003-fold as active with the substrate Δ^5 -androstene-3,17-dione compared to the equine GSTA3-3 enzyme. The divergent residues in positions 107 and 108 of the dog, goat, opossum GSTA3 and the bovine GSTA1 proteins are probably also relevant to isomerase activities.

Table 5. Conservation of H-site residues involved in steroid isomerization across GSTA3 proteins of different mammalian species.

Residue	Human	Horse	Dog	Goat	Opossum	Pig	Cattle
10*	F	F	F	F	F	F	F
12*	G	G	G	G	G	G	G
14	G	G	G	G	G	G	G
104	E	E	E	E	E	E	E
107	L	L	M	M	M	L	M
108	L	L	V	H	Y	L	H
110	P	P	P	P	P	P	P
111*	L	L	L	L	L	L	L
208*	A	G	L	T	M	T	T
213	L	V	L	I	L	L	I
216*	A	S	A	A	V	A	A
220	F	F	F	F	F	F	F
222	F	F	I	F	V	F	F

The residues marked by “*” were experimentally determined to be critical for the steroid isomerase activity of the human GSTA3 protein [9]. Identical amino acids at human GSTA3 residue positions

are highlighted. Pig and cattle data are from Lindström et al. (2018) and GenBank accession numbers **NP_999015.2** and **NP_001071617.1**, respectively.

3.4. Modeling the structures of the new GST A3-3

The crystal structure of human GST A3-3 in complex with Δ^4 -androstene-3,17-dione has been determined [22] and was used to model the structures of the related novel GST proteins. All GST A3-3 proteins appear to fold in a similar manner, and possibly could accommodate the steroid substrate in a productive binding mode without major clashes with the H-site residues. The dog GST model shows favorable interactions with the steroid except the close approach of Leu 208 to the D-ring of the substrate (Fig. 4). The high-activity human and equine GST A3-3 enzymes have smaller residues in position 208, Ala208 and Gly208, respectively. Similarly, the modeled opossum GST A3-3 enzyme indicates that its Met208 overlaps with the steroid, unless the overlap can be avoided by a rotation of the Met sidechain. The goat GST A3-3 has Thr208 in this highly variable position, and this residue is smaller than Leu208 in the dog enzyme. On the basis of the modeling, the dog, opossum, and goat GST A3-3 enzymes might have steroid isomerase activity, based on comparison with the structure of the high-activity human GST A3-3 in complex with androstenedione [22], but their more bulky 208 residues is a caveat.

4. Discussion

Complementary DNAs were cloned from *GSTA3* mRNAs in testes from the dog, goat, and gray short-tailed opossum. Given that the sequences of the *GSTA1*, *GSTA2* and *GSTA3* mRNAs are highly conserved [10], cloning of *GSTA3* mRNA was difficult even from testes, which has a distinctively high level of *GSTA3* gene expression [15]. The PCR primers used here were designed to have their 3' ends bind to codons or untranslated sequences that are unique to the *GSTA3* mRNA. Interestingly, there are no reagents for nucleic acid hybridization or antibody immunodetection that can distinguish *GSTA3* gene products from

those of *GSTA1* and *GSTA2* genes, both of which are ubiquitously expressed. The *GSTA3* mRNA can only be specifically amplified using PCR primers, such as those designed here and previously [11,18]. For example, in order to localize *GSTA3* gene expression to Leydig cells within horse testes, we developed an *in situ* reverse transcription-PCR protocol that was specific for *GSTA3* mRNA [18].

The human GSTA3-3 enzyme has well characterized catalytic efficiency with substrates Δ^5 -androstene-3,17-dione and Δ^5 -pregnene-3,20-dione, which is second only to the equine GSTA3 enzyme of the six GSTA proteins characterized so far [15]. The H-site residues of the GSTA3 proteins are critical for positioning the steroid substrate to be isomerized. The amino acids required for that activity were initially determined experimentally for human GSTA3-3 [9,21]. The human and horse GSTA3 proteins have very high conservation of H-site residues (Table 5). These differ from related GSTA enzymes, such as human GSTA2-2, which have very little activity [15]. When activity data become available for the dog, goat, and opossum enzymes, we may be able to relate enzymatic activity differences to specific amino acids or combinations of them. It will also be instructive to compare the steroid isomerase activities of the newly cloned GSTA3-3 enzymes with those of the bovine [23] and pig [10] cognates of the human GSTA3-3, both of which have significantly lower steroid isomerase activities than the human and horse GSTA3-3 enzymes [15].

As a recently discovered enzyme in steroid hormone biosynthesis, the GSTA3-3 enzyme and its corresponding mRNA sequence require extensive functional analyses across species. The mechanism of GSTA3-3 action and regulation of the *GSTA3* gene must be identified in order to fully understand the role played in steroid hormone biosynthesis and the possible implications of the enzyme function being impaired. Our work can contribute through identification of the *GSTA3* mRNA coding sequence in multiple species, as well as providing clones in the expression vector pET-21a(+) which can produce GST A3-3 proteins

to be evaluated for their activities as steroid isomerases. Evaluation of these recombinant enzymes will enable quantitative comparisons of the steroid isomerase activities among the species investigated, and allow further investigation of the effects of endogenous compounds like follicle stimulating hormone, luteinizing hormone, testosterone, estradiol, and glucocorticoids, as well as pharmaceuticals like phenobarbital or dexamethasone on the expression of the enzyme. For instance, expression of the *GSTA3* gene was down-regulated along with testosterone synthesis in testes of stallions treated with dexamethasone [18,19]. In cultured porcine Sertoli cells, follicle stimulating hormone, testosterone and estradiol increased *GSTA* gene expression [24]. In addition, inhibitors of the GSTA3-3 enzyme have been identified that may be medically useful for reducing fertility or progression of steroid-dependent diseases [15].

5. Conclusion

The three new *GSTA3* mRNA and protein sequences we report extend our comparative knowledge of the GST A3-3 enzymes of diverse mammalian species; and the information and reagents we have generated will help to facilitate future studies to characterize the GST A3-3 enzymes more generally. In addition, they may augment studies of the regulation of the expression of *GSTA3* genes in steroidogenic and other tissues. Mechanistic data of this kind can enable development of new approaches for manipulating GSTA3-3 enzyme activities *in vivo* for medical purposes. These could include inducing interruptions in fertility in animal species important to man or developing novel pharmacological treatments for steroid-dependent diseases in man and animals. It is essential that the new treatments that are prescribed are both effective and specific. It is, therefore, crucial that researchers continue to investigate steroidogenesis and its regulation in order to generate more complete mechanistic knowledge.

348 **Acknowledgments**

349 The authors acknowledge the assistance of Dr. John Edwards with tissue collection.

350 Additionally, B.M. was supported by the Swedish Research Council (grant 2015-04222).

351

352

References

1. Diemer T, Hales DB, Weidner W. Immune-endocrine interactions and Leydig cell function: the role of cytokines. *Andrologia*. 2003; 35:55-63. <https://doi.org/10.1046/j.1439-0272.2003.00537.x>.
2. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr. Rev.* 2004; 25:947-970. <https://doi.org/10.1210/er.2003-0030>.
3. Herrera-Luna CV, Budik S, Aurich C. Gene expression of ACTH, glucocorticoid receptors, 11BHSD enzymes, LH-, FSH-, GH receptors and aromatase in equine epididymal and testicular tissue. *Reprod. Dom. Anim.* 2012; 47:928-935. <https://doi.org/10.1111/j.1439-0531.2012.01993>.
4. Ing NH. Steroid hormones regulate gene expression posttranscriptionally by altering the stabilities of messenger RNAs. *Biol. Reprod.* 2005; 72:1290-1296. <https://doi.org/10.1095/biolreprod.105.040014>.
5. Lonard DM, O'Malley BW. Nuclear receptor coregulators: modulators of pathology and therapeutic targets. *Nat. Rev. Endocrinol.* 2012; 8:598-604. <https://doi.org/10.1038/nrendo.2012.100>.
6. Agular BM, Vinggaard AM, Vind C. Regulation by dexamethasone of the 3 β -hydroxysteroid dehydrogenase activity in adult rat Leydig cells. *J. Steroid Biochem. Mol. Biol.* 1992; 43:565-571. [https://doi.org/10.1016/0960-0760\(92\)90245-E](https://doi.org/10.1016/0960-0760(92)90245-E).
7. Conley AJ, Bird IM. The role of cytochrome P450 17 α -hydroxylase and 3 β -hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the Δ 5 and Δ 4 pathways of steroidogenesis in mammals. *Biol. Reprod.* 1997; 56:789-799. <https://doi.org/10.1095/biolreprod56.4.789>.
8. Johansson AS, Mannervik B. Human glutathione transferase A3-3, a highly efficient catalyst of double-bond isomerization in the biosynthetic pathway of steroid hormones. *J. Biol.*

- Chem. 2001; 276:33061-33065. <https://doi.org/10.1074/jbc.M104539200>.
9. Johansson A-S, Mannervik B. Active-site residues governing high steroid isomerase activity in human glutathione transferase A3-3. J. Biol. Chem. 2002; 277:16648-16654. <https://doi.org/10.1074/jbc.M201062200>.
10. Fedulova N, Raffalli-Mathieu F, Mannervik B. Porcine glutathione transferase alpha 2 is a human GST A3-3 analogue catalyzing steroid double-bond isomerization. J. Biochem. 2010; 431:159-167. <https://doi.org/10.1042/BJ20100839>.
11. Larsson E, Mannervik B, Raffalli-Mathieu F. Quantitative and selective polymerase chain reaction analysis of highly similar human alpha-class glutathione transferases. Anal. Biochem. 2011; 412:96-101. <https://doi.org/10.1016/j.ab.2011.01.024>.
12. Calvaresi M, Stenta M, Garavelli M, Altoe P, Bottoni A. Computational evidence for the catalytic mechanism of human glutathione transferase A3-3: A QM/MM investigation. ACS Catal. 2012; 2:280-286. <https://doi.org/10.1021/cs200369b>.
13. Matsumura T, Imamichi Y, Mizutani T, Ju Y, Yazawa T, Kawabe S., et al. Human glutathione transferase A (GSTA) family genes are regulated by steroidogenic factor 1 (SF-1) and are involved in steroidogenesis. FASEB J. 2013; 27:3198-3208. <https://doi.org/10.1096/fj.12-222745>.
14. Dourado DFAR, Fernandes PA, Mannervik B, Ramos MJ. Isomerization of Δ^5 -androstene-3,17-dione into Δ^4 -androstene-3, 17-dione catalyzed by human glutathione transferase A3-3: a computational study identifies a dual role for glutathione. J. Phys. Chem. A. 2014; 31:5790-5800. <https://doi.org/10.1021/jp410810q>.
15. Lindström H, Peer SM, Ing NH, Mannervik B. Characterization of equine GST A3-3 as a steroid isomerase. J. Steroid Biochem. Mol. Biol. 2018; 178:117-128. <https://doi.org/10.1016/j.jsbmb.2017.11.011>.
16. Islam MQ, Platz A, Szpirer J, Szpirer C, Levan G, Mannervik B. Chromosomal localization of human glutathione transferase genes of classes Alpha, Mu and Pi. Hum. Genet. 1989;

- 82:338-342. <https://doi.org/10.1007/BF00273994>.
17. Raffalli-Mathieu F, Orre C, Stridsberg M, Edalat M, Mannervik B. Targeting human glutathione transferase A3-3 attenuates progesterone production in human steroidogenic cells. *Biochem. J.* 2008; 414:103-109. <https://doi.org/10.1042/BJ20080397>.
18. Ing NH, Forrest DW, Riggs PK, Loux S, Love CC, Brinsko SP, et al. Dexamethasone acutely down-regulates genes involved in steroidogenesis in stallion testes. *J. Steroid Biochem. Mol. Biol.* 2014; 143:451-459. <https://doi.org/10.1016/j.jsbmb.2014.07.003>.
19. Ing NH, Brinsko SP, Curley KO, Forrest DD, Love CC, Hinrichs K, et al. Dexamethasone acutely regulates endocrine parameters in stallions and subsequently affects gene expression in testicular germ cells. *Anim. Reprod. Sci.* 2015; 152:47-54. <https://doi.org/10.1016/j.anireprosci.2014.11.007>.
20. Valdez R, Cavinder CA, Varner DD, Welsh TW, Vogelsang MM, Ing NH. Dexamethasone downregulates expression of several genes encoding orphan nuclear receptors that are important to steroidogenesis in stallion testes. *J. Biochem. Mol. Toxicol.* 2019; 33:1-8. <https://doi.org/10.1002/jbt.22309>.
21. Pettersson PL, Johansson A-S, Mannervik B. Transmutation of human glutathione transferase A2-2 with peroxidase activity into an efficient steroid isomerase. *J. Biol. Chem.* 2002; 277:30019-30022. <https://doi.org/10.1074/jbc.M204485200>.
22. Tars K, Olin B, Mannervik B. Structural basis for featuring of steroid isomerase activity in Alpha class glutathione transferases. *J. Mol. Biol.* 2010; 397:332-340. <https://doi.org/10.1016/j.jmb.2010.01.023>.
23. Raffalli-Mathieu F, Persson D, Mannervik B. Differences between bovine and human steroid double-bond isomerase activities of Alpha-class glutathione transferases selectively expressed in steroidogenic tissues. *Biochim. Biophys. Acta* 2007; 1770:130-136. <https://doi.org/10.1016/j.bbagen.2006.06.015>.
24. Benbrahim-Tallaa L, Tabone E, Tosser-Klopp G, Hatey F, Benamed M. Glutathione

431 transferase alpha expressed in porcine Sertoli cells is under the control of follicle-stimulating
432 hormone and testosterone. Biol. Reprod. 2002; 66:1734-1742.
433 <https://doi.org/10.1095/biolreprod66.6.1734>.
434
435

Figure Legends

Figure 1. Nested PCR for amplification of GSTA3 mRNA targets.

Figure 2. Aligned nucleotide sequences of the coding sequences of GSTA3 mRNAs from selected species including those used as reference. Human (*Homo sapiens*; NM_000847.5), horse (*Equus caballus*; KC512384.1), dog (*Canis lupus familiaris*; KJ766127), goat (*Capra hircus*; KM578828), and opossum (*Monodelphis domestica*; KP686394) those displayed here. Dog, goat, and opossum cDNA sequences were cloned in the current study. Shared identities with the human reference sequence are highlighted in gray. Start and stop codons are identified in bold, green and red type, respectively. The five critical codons defining high ketosteroid isomerase activity in human GSTA3-3 as compared to the low activity of human GST A2-2 [9] are identified in bold, multicolored type. Each full row has 60 nucleotides.

Figure 3. Aligned amino acid sequences for GSTA3 of all species investigated including those used as reference. Symbols: * = identical residues, : = similar residues, . = conservation of groups with weakly similar properties. G-site residues are highlighted in yellow and H-site residues are highlighted in blue. Phenylalanine in position 220 may contribute to both G- and H-sites. The underlined amino acids govern high or low 3-ketosteroid isomerase activity between the human GST A3-3 and GST A2-2.

Figure 4. Dog GST A3-3 active site modeled with bound Δ^4 -androst-3,17-dione. The crystal structure of the human GST A3-3 with the ligands glutathione and the steroid (PDB code 2vcv) was used as a template for modeling the dog enzyme. The ligands in the dog GST A3-3 were positioned by superpositioning the human GST A3-3 onto the dog model. Green arrows indicate the location of the residue Leu208 very close to the D-ring of Δ^4 -androst-3,17-dione, as well as the sulfur of glutathione and the oxygen of Tyr9 implicated in the catalysis of steroid isomerization. The image was created with the UCSF Chimera package (<http://www.rbvi.ucsf.edu/chimera>) developed by the

463 Resource for Biocomputing, Visualization, and Informatics at the University of California, San
464 Francisco (supported by NIGMS P41-GM103311).
465
466

Outside sense primer

Outside antisense primer

1° PCR

30 cycles:

Denaturation

Annealing

Extension

Inside sense primer

Inside antisense primer

2° PCR

30 cycles:

Denaturation

Annealing

Extension

Nested PCR product

Human	ATGTCAGGGGAAGCCCAAGCTTCTACTAC TTCA AT GGA CGGGGCAGAATGGAGCCCATCCGG
Dog	ATG GCGGGGAAGCCCAAGCTTCTACTAC TTCA AT GGA CGAGGCAGAATGGAGTCCATCCGG
Goat	ATG GCAGGGGAAGCCCATTTCTTCACTAT TTCA AT GGA CGCGGCAGAATGGAGTGCATTCCG
Opossum	ATG TCTGGGAAGCCCATACTTCAATTAC TTCA AT GGA AGAGGCAGAATGGAATCAGTGC
	***** * ***** ***** ** ***** * * * * * * * * *
Human	TGGCTCTTGGCTGCAGCTGGAGTGGAGTTTGAAGAGAAATTTATAGGATCTGCAGAAGAT
Dog	TGGCTCCTGGCTTCAGCTGGAGTAGAGTTTGAAGAGAAATTTATAAATACTCCAGAAGAC
Goat	TGGCTCCTGGCTGCGGCTGGAGTGGAGTTTGAAGAAAAATTTATAGAAAAACCAGAAGGC
Opossum	TGGCTCTTGGCAGCTGCTGGAGTCGAGTTTGAAGAAAAAC TAT TAAATCAGCTGAAGAT
	***** *
Human	TTGGGAAAGTTAAGAAATGATGGGAGTTTGATGTTCCAGCAAGTACCAATGGTTGAGATT
Dog	TTGGATAAATTAATAAATGATGGAAGTCGATGTTCCAGCAAGTGCCAATGGTGGAATTT
Goat	TTGGATAAGTTAATAAATGATGGGAGTTTGATGTTCCAGCAAGTGCCAATGGCTGAAATT
Opossum	TTTGATAACTTAATTAAGAGTGGAAACCTGATGTATCAACAAGTGCCAATGGTTGAAATT
	* *
Human	GATGGGATGAAGTTGGTACAGACCAGAGCCATTCTCAACTACATTGCCAGCAAATACAAC
Dog	GATGGAATGAAGCTGGTACAGGCCAGAGCCATTCTCAACTACATTGCCACCAAATACAAC
Goat	GATGGGATGAAGCTGGTGCAGACCAGAGCCATTCTCAACTACATTGCCGCCAAACACAAC
Opossum	GATGGACTGAACCTGGTACAAACCAGAGCCATCCTGAAGTATATAGCTGCCAAATACAAC
	***** *
Human	CTCTACGGGAAAGACATAAAGGAGAGAGCCCTAATTGATATGTATACAGAAGGTATGGCA
Dog	CTCTATGGGAAAGACATAAAGGAGAGAGCTCTGATAGATATGTACACAGAAGGTATAGTA
Goat	CTCTACGGGAAAGACATGAAGGAGAGAGCCCTGATTGATATGTACTCAGAGGGTGTGGCA
Opossum	TTGTATGGGAAGGACCTGAAGGAGAAAGCTCTGATTGACATGTATGTGGAAGGATGAGA
	* *
Human	GATTTGAATGAAATGATCCTTCTTCTGCCCT TTA TGTCGACCTGAGGAAAAAGATGCCAAG
Dog	GATTTGAATGAAATGATCATGGTTTTCCT CTA TGCCACCTGATCAAAAAGATGCCAAG
Goat	GATTTGGGTGAAATGATCATG CT ATTGCCA CTG TGCCACCTGCTGAAAAAGACGCCAAG
Opossum	GATCTGAATGAATGATCATGTACTACCCA CTG TGCTATCCTGGAGAAGGAAAAAGAAC
	*** *
Human	ATTGCCTTGATCAAAGAGAAAAACAAAAGTCGCTATTTCCTGCCTTCGAAAAAGTGTTA
Dog	ATTACTCTGATCAGAGAGAGAACACAGATCGTTATCTCCCGTGTTTGAAAAAGTGTTA
Goat	CTGACCTTAATCCGAGAAAAGACAACAAACCGTTATCTCCCTGCATTTGAAATGTGCTG
Opossum	CTCAACTTCATCCTGGAGAGAGCCACTGAAAGATTCTTCCAGTGATGAGAAGGCTTTA
	* *
Human	CAGAGCCATGGACAAGACTACCTTGTTGGCAACAAGCTGAGCCGGGCTGACATTAGCCTG
Dog	AAGAGCCATGGACAAGACTACCTTGTTGGCAACAAGCTGAGCAGGGCTGACATTCACCTG
Goat	AAGAGCCACGGACAAGACTACCTGGTGGCAACAAGCTGAGCAGGGCTGACATCCACCTG
Opossum	AAAAGTCATGGGAAAAATATCTTGTTGGCAACCAGATGAGCTGGGCAGATATACAGCTG
	* *
Human	GTGGAACTTCTCTACTATGTGGAAGAGCTTGACTCCAGCCTTATCTCCAACCTCCCTCTG
Dog	GTTGAACTTCTCTACTATGTGGAAGAGCTTGACTCCAGCCTTCTGGCCAACCTCCCTCTG
Goat	GTTGAACTTCTCTACTATGTGGAAGAGCTGGACCTAGCCTTTTGGCCAGCTTCCCTCTG
Opossum	TTTGAACCATCTTAATGGTTGAAGAATTAAAGTCGGATATTCTCTCTGCATTCCCTAAA
	* *
Human	CTGAAGGCCTGAAAACCAGAATCAGCAACCTGCCACGGTGAGAAGTTTCTACAGCCT
Dog	CTGAAGGCCTGAAAACCAGAGTCAGCAATCTCCCACCGTGAGAAGTTTCTGCAGCCT
Goat	CTGAAGGCCTTAAAGCCAGAGTCAGCAATCTCCCGCGCTGAAGAAGTTTCTGCAGCCC
Opossum	CTGCAGGAGTTCAAAGCTAGAATGAGCAAACTCCCAGCATTAGAAATTCCTTAAACCT
	*** *
Human	GGCAGCCCAAGGAAGCCTCCC GCA GATGCAAAAGCTTTAGAAGAA GCC AGAAAAGATTTTC
Dog	GGCAGCCCAAGGAAGCCTCCC TTG GATGAGAAAAGTTTAGAGCA AGC GAAGAAGATTTTC
Goat	GGTAGCCAGAGGAAGCCTCCC ACG GACGAGAAAAAATAGAAGAA GCC AGAGGGCTTTC
Opossum	GGCAGTCAAAGAAAAGAA--- ATG GATCCAATGGGTCTTGAAGAG GAG GTAAGAAGATA
	* *
Human	AGGTTT TAA ---
Dog	AGGATTAA CTGA
Goat	AAGTTT TAA ---
Opossum	TTCAAGGTT TGA

Human	MAGKPKLHYF	NGRGRMEPIR	WLLAAAGVEF	EEKFIGSAED	LGKLRNDGSL	MFQQQVPMVEI	60
Horse	MAVKPMLHYF	NGRGRMEPIR	WLLAAAGVEF	EETFIDTPED	FEKLKNDGSL	MFQQQVPMVEI	60
Dog	MAGKPKLHYF	NGRGRMESIR	WLLASAGVEF	EEKFINTPED	LDKLKNDGSL	MFQQQVPMVEI	60
Goat	MAGKPILHYF	NGRGRMECIR	WLLAAAGVEF	EEKFIEKPEG	LDKLKNDGSL	MFQQQVPMVEI	60
Opossum	MSGKPMMLHYF	NGRGRMESVR	WLLAAAGVEF	EEKLLKSAED	FDNLIKSGNL	MYQQQVPMVEI	60
	* * * * *	* * * * * *	* * * * *	* * : : . *	: : * : * *	* : * * * * *	

Human	DGMKLVQTRA	ILNYIASKYN	LYGKDIKERA	LIDMYTEGMA	DLNEMILLLP	LCRPEEKDAK	120
Horse	DGMKLVQSRA	ILNYVAAKHN	LYGKDIKERA	LIDMYIEGVA	DLNEMILLLP	ITPPAEKDAK	120
Dog	DGMKLVQARA	ILNYIATKYN	LYGKDIKERA	LIDMYTEGIV	DLNEMIMVLP	LCPPDQKDAK	120
Goat	DGMKLVQTRA	ILNYIAAKHN	LYGKDMKERA	LIDMYSEGVA	DLGEMIMHLP	LCPPAEKDAK	120
Opossum	DGLNLVQTRA	ILKYIAAKYN	LYGKDLKEKA	LIDMYVEGMR	DLNEMIMYYP	LCYPGEEEN	120
	** : : * * : *	** . * : * * : *	* * * * * : * * : *	* * * * * * * :	* * * * * : *	: * : : : :	

Human	IALIKEKTKS	RYFPAFEKVL	QSHGQDYLVG	NKLSRADISL	VELLYYVEEL	DSSLISNFPL	180
Horse	IMLIKDRTTN	RYLPFAFEKVL	KSHGEDYLVG	NRLSRADIHL	VELLYLVEEL	DPSLLTNFPL	180
Dog	ITLIRERTTD	RYLPVFEKVL	KSHGQDYLVG	NKLSRADIHL	VELLYYVEEL	DSSLANFPL	180
Goat	LTlireKTTN	RYLPAFENVL	KSHGQDYLVG	NKLSRADIHL	VELLYYVEEL	DPSLLASFPL	180
Opossum	LNfilerATE	RFFPVYEKAL	KSHGKNYLVG	NQMSWADIQL	FETILMVEEL	KSDILSAFPK	180
	: * : : : .	* : : * . : * : . *	: * * * : : * * * *	* : : * * * * *	. * : * * * *	. : : : * *	

Human	LKALKTRISN	LPTVKKFLQP	GSPRKPPADA	KALEEARKIF	RF- 223
Horse	LKALKARISN	LPTVKKFLQP	GGARKPPGDE	KSVEKSRKIF	KF- 223
Dog	LKALKTRVSN	LPTVKKFLQP	GSPRKPPLEDE	KSLEQAKKIF	RIN 223
Goat	LKALKARVSN	LPAVKKFLQP	GSQRKPPTDE	KKIEEARRAF	KF- 223
Opossum	LQEFKARMSK	LPSIQKFLKP	GSQRK-EMDP	MGLEEVKKIF	KVD 223
	* : : * : * : *	: * : : : * * * *	* . * * *	* : * : : : * : :