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Comparative genomics identifies new alpha class genes within the avian glutathione *S*-transferase gene cluster

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ABSTRACT

Glutathione *S*-transferases (GSTs: EC2.5.1.18) are a superfamily of multifunctional dimeric enzymes that catalyze the conjugation of glutathione (GSH) to electrophilic chemicals. In most animals and in humans, GSTs are the principal enzymes responsible for detoxifying the mycotoxin aflatoxin B_1 (AFB_1) and GST dysfunction is a known risk factor for susceptibility towards AFB_1. Turkeys are one of the most susceptible animals known to AFB_1, which is a common contaminant of poultry feeds. The extreme susceptibility of turkeys is associated with hepatic GSTs unable to detoxify the highly reactive and electrophilic metabolite *exo*-AFB_1-8,9-epoxide (AFBO). In this study, comparative genomic approaches were used to amplify and identify the α-class tGST genes (*tGSTA1.1, tGSTA1.2, tGSTA1.3, tGSTA2, tGSTA3* and *tGSTA4*) from turkey liver. The conserved GST domains and four α-class signature motifs in turkey GSTs (with the exception of *tGSTA1.1* which lacked one motif) confirm the presence of hepatic α-class GSTs in the turkey. Four signature motifs and conserved residues found in α-class tGSTs are (1) xMExxxWLLAAAGVE, (2) YGKDxKERAxIDMYVxG, (3) PVxEKVLKxHGxxxL and (4) PxIKKFLXPGSxxKPxxx. A BAC clone containing the α-class GST gene cluster was isolated and sequenced. The turkey α-class GTS genes genetically map to chromosome MGA2 with synteny between turkey and human α-class GSTs and flanking genes. This study identifies the α-class tGST gene cluster and genetic markers (SNPs, single nucleotide polymorphisms) that can be used to further examine AFB_1 susceptibility and resistance in turkeys. Functional characterization of heterologously expressed proteins from these genes is currently underway.

1. Introduction

Glutathione *S*-transferases (GSTs; E.C.2.5.1.18), a superfamily of multifunctional dimeric proteins, are important phase II biotransformation enzymes involved in cellular detoxification and excretion of a variety of xenobiotic substances (Eaton and Bammler, 1999; Frova, 2006). Carcinogens, environmental toxins and products of oxidative stress are detoxified by GSTs which principally catalyze the conjugation of reactive, electrophilic atoms with reduced glutathione (GSH) (Konishi et al., 2005; Salinas and Wong, 1999). Because of their importance in disease resistance, cancer susceptibility, and responsiveness to drug therapy, mammalian GSTs have been intensively studied. GSTs are primarily cytosolic enzymes, but microsomal forms also exist (Kelner et al., 1996). Cytosolic GSTs exist as dimeric subunits of 23–30 k Da with an average length of 199–244 amino acids (Hayes and Pulford, 1995; Mannervik and Danielson, 1988). Each subunit is composed of two spatially distinct domains. The N-terminal domain I has an α/β structure consisting of four β-strands and three α-helices. Domain II contains a larger α domain with five to six α-helices. There are two ligand-binding sites per subunit: a specific GSH-binding site (G-site) and the hydrophobic substrate binding site (H-site) (Frova, 2006; Sun et al., 1998).

Cytosolic GSTs from human, rat, and mouse have been well studied and are assigned to one of seven classes [alpha (α), mu (μ), pi (π), theta (τ), sigma (σ), zeta (ζ), omega (ο)] based on amino acid similarities (Frova, 2006; Hayes et al., 2005). Human GSTs are diverse and most abundantly expressed in the liver. Members of each class tend to have high sequence identity (>60%)(Board, 1998) and individual genes for each human GST class are clustered together on the same chromosome (Board and Webb, 1987). Human α-class GSTs (*hGSTA*) are well documented with five functional genes (*hGSTA1-hGSTA5*) and seven pseudogenes on chromosome 6p12.1-6p12.2 (Coles and Kadlubar, 2005; Morel et al., 2002).

Avian GSTs comprise a complex isoenzyme system that has received much less attention (Yeung and Gidari, 1980). According to electrophoretic mobility on SDS/PAGE, five groups of GST subunits (designated CL1–CL5) have been identified in the cytosolic fraction of Leghorn chick livers (Chang et al., 1990). Searches of Expressed Sequence Tag (EST) databases have isolated α (Chang et al., 1990; Chang et al., 1992; Liu et al., 1993), μ (Liu and Tam, 1991; Sun et al.,

Abbreviations: GST, glutathione S-transferase; AFB1, Aflatoxin B_1; AFBO, *exo*-AFB_1-8,9-epoxide; tGST, turkey glutathione *S*-transferase.

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1998), τ (Hsiao et al., 1995) and σ (Thomson et al., 1998) classes from cDNA sequences of the domestic chicken. Full-length cDNA of α-class GSTs was isolated and heterologously expressed in baculovirus and *Escherichia coli* system using GST-specific substrates (Liu et al., 1997; Liu et al., 1993). Nine class-alpha isozymes with distinctive molecular masses were affinity purified from chicken livers and partially cloned and characterized (Hsieh et al., 1999); clustering of chicken ESTs accessioned in Genbank suggests expression of six separate α-class transcripts. The nomenclature of chicken α-class GSTs was recently re-named (GenBank accession nos.) as *cGSTA1* (NM_001001777), *cGSTA2* (NM_001001776), *cGSTA3* (NM_204818), and *cGSTA-CL3* (M38219) based on subunit nomenclature proposed by (Mannervik et al., 1992).

In nearly all animals studied, GSTs are the principal detoxification enzymes for aflatoxin B_1 (AFB_1), a ubiquitous food and feed-borne mycotoxin that is a potent animal and human hepatotoxin and carcinogen (Coulombe, 1993; Newberne and Butler, 1969). To exert its toxic and carcinogenic effects, AFB_1 requires metabolic activation to the highly reactive electrophilic and carcinogenic intermediate, the *exo*-AFB_1-8,9-epoxide (AFBO), catalyzed by hepatic cytochromes P450 (CYPs) (Hayes et al., 1991b; Swenson et al., 1975). When functional, GSTs catalyze the conjugation of GSH to AFBO, thereby rendering it non-toxic and easily excretable. We recently amplified and cloned from turkey *CYP1A5* and *CYP3A37*, high-affinity enzymes mostly responsible for AFB_1 bioactivation in turkey liver (Rawal et al., 2009; Yip and Coulombe, 2006).

In rodents, α-class GSTs are the most efficient isozymes in detoxifying the AFBO (Hayes et al., 1991a; Hayes et al., 1991b). Mouse liver cytosol almost exclusively conjugates the *exo*-AFBO through the activity of its α-class GST (Raney et al., 1992), designated *Gsta3*, which has a high affinity toward AFBO, has been shown to be critical in the relative resistance of mice toward AFB_1 (Ramsdell and Eaton, 1990). Rat constitutively express only small amount of α-class GST with high AFBO activity (*rGSTA5-5*) and thus are sensitive to AFB_1-induced hepatocarcinogenesis (Hayes and Pulford, 1995; Wang et al., 2002). In contrast to rodents, constitutively expressed human hepatic α-class GSTs has little or no AFBO detoxifying activity(Raney et al., 1992; Slone et al., 1995).

Turkeys are one of the most susceptible animals known to AFB_1 (Giambrone et al., 1985; Hamilton et al., 1972). Even small amounts in the diet cause severe hepatotoxicosis and reduction in growth rate, feed efficiency and hatchability, acute hepatic necrosis, and increased susceptibility to bacterial and viral diseases(Kubena et al., 1995; Pier et al., 1980).

There is currently little information available on the nature of GSTs in turkeys. Using prototype substrates we have demonstrated that turkey liver possesses active GST activity, but none with measurable GST-mediated detoxification of AFB_1 (Klein et al., 2000; Klein et al., 2002, 2003). The purpose of this study was to fully characterize the α-class GSTs of the turkey. We amplified six α-class GSTs (tGSTs) from turkey liver mRNA by RACE and genetically mapped them to turkey chromosome MGA2. The CHORI-260 turkey BAC library was screened to identify clones containing the α-class gene cluster and one clone was fully sequenced and assembled. The six α-class GST genes were annotated according to gene structure, sequence similarity and synteny with chicken and human α-class GSTs. This study provides the complete sequence of the α-class genes and genetic markers that will be important in future studies of AFB_1 susceptibility and resistance in turkeys.

2. Materials and methods

2.1. RNA extraction and Rapid Amplification of cDNA Ends (RACE)

Male day-old turkey poults (Nicholas commercial strain) were obtained from Moroni Feed Co. (Moroni, UT). Freshly isolated turkey livers were stored in *RNAlater* (Ambion). Samples were homogenized using a Polytron (Brinkman) and mRNA was extracted using Oligotex Direct mRNA kit (Qiagen). The first strand cDNA was synthesized using MMLV reverse transcriptase (Clontech) and each 5′-CDS primer and 3′-CDS primer provided in SMART™ RACE cDNA Amplification Kit (Clontech), respectively, to carry out RACE. Gene-specific primers (Table 1) were designed based on sequences of chicken GST α-class transcripts, *cGSTA1*, *cGSTA2*, *cGSTA3* and *cGSTA-CL3* to perform 5′- and 3′-RACE. Fragments were amplified in PCR reactions with both universal primer mix (UPM, long primer: 5′-CTAATACGACTCACTA-TAGGGCAAGCAGTGGTATCAACGCAGAGT-3′, and short primer: 5′-CTAATACGACTCACTATAGGGC-3′) and nested universal primers (NUP, 5′-AAGCAGTGGTATCAACGCAGAGT-3′) provided in SMART™ RACE cDNA Amplification kit (Clontech). The following PCR profile was performed with Advantage 2 PCR kit (Clontech): 2 min at 94 °C, 30 s at 94 °C, 30 s at optimal annealing temperature 58–68 °C (34 cycles for first reaction and 25 cycles for nested PCR), and 45 s^{-90} s at 72 °C, followed by a final extension at 72 °C for 8 min.

PCR products were subcloned in TA cloning vector pDrive (Qiagen) and transformed into chemically competent *E. coli*, DH5a (Invitrogen). Presence of RACE fragments within the clones was confirmed by colony PCR and sequence analysis. For confirmation of GST gene coding regions, PCR was performed using the proofreading enzyme *pfuUltra* high-fidelity DNA polymerase (Stratagene) with gene-specific primers (Table 2) and cloning with Zero Blunt PCRII vector (Invitrogen). The PCR profile for this reaction was 2 min at 94 °C, 30 s at 94 °C, 30 s at optimal annealing temperature, 56–60 °C (25 cycles), and 1 min and 30 s at 72 °C, followed by a final extension at 72 °C for 8 min. Clones were confirmed by colony PCR and sequence analysis. All alignments of genes were analyzed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html#).

2.2. BAC library screening and sequencing

Sequences of turkey GST genes were used to design probes to screen the CHORI-260 BAC library array by overgo hybridization (Ross et al., 1999). Overgo sequences were as follows: *GSTA2* O2-CAGAGTAGAATTACATTACGTTGCTG; and *GSTA2* O1-CTGTAT-TACTGTTCTGCAGTTACCCA; *GSTA3* O1-CAGGCAGATGTAAGAGGAG-CACTTC; and *GSTA3* O2-TTAGAAGACTTCATTGCGTGGAAGT. Positive BAC clones were identified and grown overnight in LB media containing 25 μg/mL chloramphenicol. BAC DNA was prepared using QIAprep columns (Qiagen) and presence of GSTs was confirmed by PCR (Table 3A). Two GST-positive clones were identified (37H15 and 08C04) and end sequenced with vector-specific primers (Genbank accession nos. FI907948, FI907949, FI907950, FI907951). Based on position in the chicken genome of end sequences, 37H15 was chosen for full sequencing. BAC DNA was prepared using a large construct kit (Qiagen) and submitted to the Advanced Genetic Analysis Center, University of Minnesota for sublibrary construction and sequencing with Roche 454 GS FLX technology.

2.3. BAC contig assembly

Approximately 16,000 454 sequence reads (~12X coverage) were generated. Sequences were initially assembled with GS Assembler (Roche) and Sequencher software (Gene Codes, Corp) with the tGST cDNAs used to aid alignments. Assembled contigs were confirmed by overlapping gene-specific PCR and resequencing using primers anchored within exons (Table 3B). PCR reactions were performed with BAC DNA as template using Hotstar *Taq* polymerase (Qiagen). Amplifications were performed for 35 cycles with 58 °C annealing temperature and 1 min/kb extension times. PCR products were purified with PCR cleanup columns (Qiagen) and directly sequenced.

Due to the duplicated nature of the three A1 genes, additional large-insert clones were constructed to aid in assembly of the