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Attempt to express hGSTA3Delta3 *in vitro*

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SUMMARY

Glutathione transferases (GSTs) are most known for their detoxification properties. In these reactions the GST conjugates a potentially harmful electrophilic substrate with a tripeptide called glutathione (GSH). The reaction neutralizes the electrophile, which may otherwise harm macromolecules in the cell. GSTs are involved in many other reactions. This report concerns a human GST in the alpha class (*hGSTA3*) which catalyzes a double-bond isomerase reaction in the biosynthesis of progesterone and testosterone. In this reaction, GSH functions as a cofactor which helps proton transfer and an associated change of double-bond position in the substrate molecule. In this study I have tried to express an alternatively spliced variant of the enzyme *in vitro*. The spliced variant lacks exon 3 and is termed hGSTA3Δ3. It is not known if the spliced form of the enzyme is present as a protein in the cell but the corresponding RNA has been detected in steroidogenic tissues such as testis, ovary, adrenal gland and placenta. This suggests that hGSTA3Δ3 has a function either as a protein or as untranslated RNA. If the spliced variant can be expressed *in vitro* it would be interesting to see if the protein can be expressed *in vivo* and to functionally characterize the enzyme. hGSTA3Δ3 has been subcloned to an expression vector. A coupled transcription/translation reaction was used for expression but no expression products were detected under the conditions tested.

ABBREVIATIONS

GSH	glutathione (reduced state)
GSSG	glutathione disulfide (oxidized state of glutathione)
GS^-	thiolate anion of glutathione
GST	glutathione transferase
GST A3-3	protein homodimer expressed from GSTA3
G-site	active site for binding of GSH
H-site	active site for binding of hydrophobic substrate
hGSTA3	human GST A3 gene
hGSTA3Δ3	human GST A3 RNA without exon 3
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HRP	horseradish peroxidase
UTR	untranslated region (of RNA)
3βHSD	3β-hydroxysteroid dehydrogenase/isomerase
Δ^4-AD	Δ^4-androstene-3,17-dione
Δ^5-AD	Δ^5-androstene-3,17-dione
Δ^4-PD	Δ^4-pregnene-3,20-dione
Δ^5-PD	Δ^5-pregnene-3,20-dione

INTRODUCTION

Glutathione transferases

The understanding of the structure of glutathione transferases (GSTs) has increased rapidly since the first GST structure was determined in 1991 and today GSTs are the best-characterized enzymes in detoxification (as reviewed by Oakley, 2011). Still relatively few of the GST genes have been characterized and more research is needed to further understand how the genes are regulated since the GSTs is a large family (Higgins & Hayes, 2011). The GST family is characterized by broad substrate specificity and low catalytic efficiency which is thought to be evolutionary beneficial to achieve a broader protection against electrophilic substrates that may be harmful for the cell (Tew & Ronai, 1999). GST is normally saturated with gluthatione (GSH) at the physiological conditions in the cell and GSH is therefore in complex with the enzyme. GSH consists of glutamic acid, cysteine and glycine; this water-soluble tripeptide is the most abundant intracellular small molecule thiol (Townsend *et al*., 2003). GSTs play a central role in detoxification reactions where GSH (in the base form GS^-) reacts with an electrophilic center in the substrate molecule, which may be a range of structurally diverse endogenous or exogenous compounds. This is a conjugation where GSH neutralizes the electrophile and protects macromolecules in the cell from the reactive electrophile.

Another function of GSTs is to catalyze the reduction of organic hydroperoxides. In this reaction GSH will be oxidized to GSSG (as reviewed by Higgins & Hayes, 2011). The GSH:GSSG ratio is strictly regulated in the cell since it is critical to cell survival and an imbalance of GSH is observed in for example cancer, neurodegenerative disorders, cystic fibrosis, HIV and aging (Townsend *et al*., 2003). The cell will be at risk for oxidative damage in the absence of GSH and GSH depletion increases the rate of GSH synthesis (Townsend *et al*., 2003). Research has found that GSTs are involved in several other processes in the cell such as biosynthesis and metabolism of prostaglandins, steroids and leukotrienes (as reviewed by Laborde, 2010). Further, GSTs also handle toxic products generated by oxidative stress and are involved in cell signaling pathways that control cell proliferation and apoptosis (as reviewed by Laborde, 2010).

Classification and general characteristics

Three separate families of GSTs with distinct evolutionary origins have been recognized: cytosolic, mitochondrial and microsomal transferases (as reviewed by Higgins & Hayes, 2011). Furthermore, the cytosolic GSTs form the largest superfamily and the microsomal GSTs are integral membrane proteins now also termed membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEGs). Cytosolic and mitochondrial transferases both form dimers and the families are strongly related evolutionarily (Oakley, 2011). Human cytosolic transferases consist of 7 classes: alpha, zeta, theta, mu, pi, sigma and omega which are localized on 7 chromosomes (Oakley, 2011, Tars *el al*., 2010). The functional cytosolic GSTs are dimers, the alpha class and mu class form several different isoenzymes due to their special ability to not only form homodimers but also heterodimers (Laborde, 2010). Each subunit contains a GSH-binding site (G-site) and a site that can bind a hydrophobic substrate (H-site) (Laborde, 2010).

The GST alpha locus spans approximately 300 kilobases on chromosome 6 and contains five genes (*hGSTA1-hGSTA5*) and seven pseudogenes (as reviewed by Morel *et al*., 1994, Morel *et al*., 2002). The pseudogenes may have different characteristics such as an absence of exon(s), single nucleotide deletion and stop codons in the open reading frames (Morel et al.,

2002). This report concerns expression of an alpha class human *GSTA3* gene (*hGSTA3*) product that is alternatively spliced. This splice variant´s transcript lacks exon 3 and is termed hGSTA3Δ3.

GSTs in steroid metabolism

Cholesterol serves as the precursor of all steroid hormones and many enzymatic reactions are required to produce active steroid hormones, Figure 1 (Raffalli-Mathieu & Mannervik, 2005). Human *GSTA3* has by RT-PCR been shown to be expressed in certain tissues that are characterized by production of steroid hormones (i.e. testis, ovary, adrenal gland and placenta) and to play an essential role in the biosynthesis of progesterone and testosterone (Johansson & Mannervik, 2001). The protein GST A3-3 is expressed from the *GSTA3* gene and it is a homodimer composed of two GST A3 subunits. GST A3-3 is thought to be a major contributor to catalyze an obligatory Δ^5- Δ^4 double-bond isomerization since it was discovered in 2001 by Johansson and Mannervik that the catalytic efficiency (k_{cat}/K_M) for GST A3-3 exceeds by 230 times the corresponding value for the 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD), which was originally thought to be the principal enzyme catalyzing this step (Johansson and Mannervik, 2001). In this reaction GSH serves as a catalytic cofactor but the GST A3-3 has significant steroid isomerase activity even without GSH (Johansson & Mannervik, 2002, Tars *el al*., 2010).

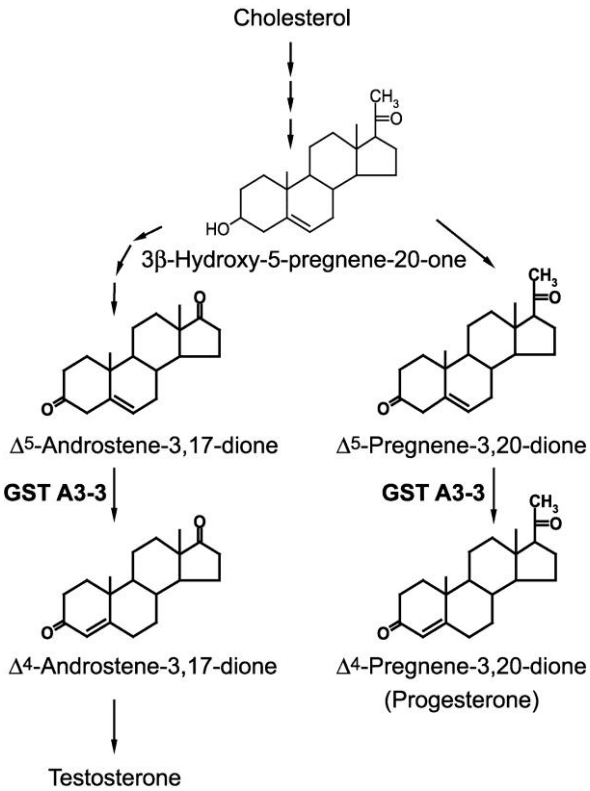

Figure 1. Isomerization reactions catalyzed by GST A3-3. Cholesterol is the precursor of all steroid hormones. GST A3-3 catalyzes two isomerization reactions to produce Δ^4-AD and Δ^4-PD. Copied from Johansson & Mannervik, 2001.

Similarity to other alpha class GSTs regarding sequence similarity and isomerase activity

Concerning the other enzymes of the alpha class it has been found that GST A3-3 share high sequence similarity with GST A2-2 (89%) but only a small number of residues are identical in the active site (50%), which explains why GST A2-2 has 5000 times less catalytic efficiency

with Δ^5-AD (Tars *el al*., 2010). In comparison, the residues of the active site of GST A1-1 are more identical to the residues constituting the GST A3-3 active site even though the sequence similarity is approximately the same (91%); the strong similarity in the active site explains that GST A1-1 has higher catalytic efficiency than GST A2-2 (Johansson & Mannervik, 2001, Tars *el al*., 2010). The catalytic efficiency of GST A3-3 is 20 times higher with both Δ^5-AD and Δ^5-PD than the catalytic efficiency of GST A1-1 and since GST A3-3 is selectively expressed Johansson and Mannervik (2001) suggest that GST A3-3 has evolved to participate in the biosynthesis of steroid hormones, Figure 2. GST A4-4 has much lower sequence identity with GST A3-3 (54%) compared to the sequence identity between GST A3-3 and GST A1-1 or GST A2-2 (Johansson & Mannervik, 2001). The GSH-binding site was proved to be strictly conserved when comparing GST A1-1, GST A2-2 and GST A3-3 (Johansson & Mannervik, 2001).

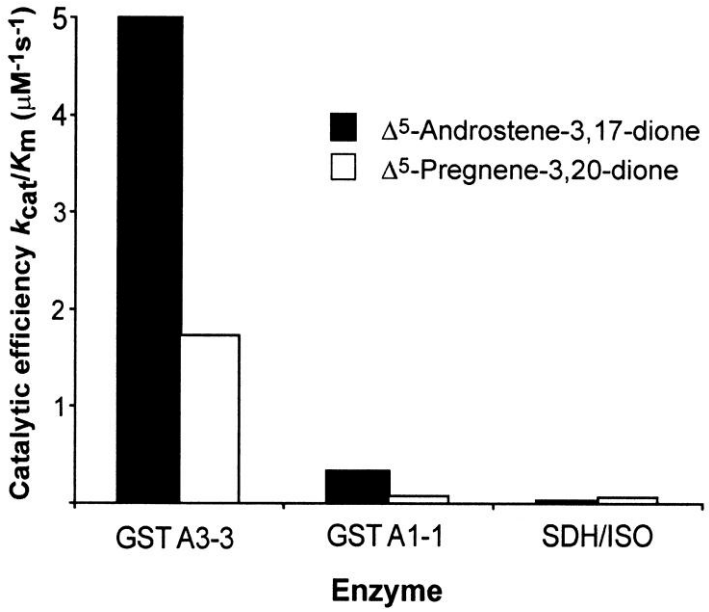

Figure 2. Comparison of catalytic efficiencies. GST A3-3 has higher catalytic efficiency than GST A1-1 and 3β-hydroxysteroid dehydrogenase/isomerase. GST A3-3 has higher catalytic efficiency with Δ^5-AD as a substrate than with Δ^5-PD. Copied from Johansson & Mannervik, 2001.

The mechanism of the double-bond isomerization reaction

The mechanism of the isomerization reaction is expected to occur in the same way in the two reactions using different substrates as seen in Figure 1. The first reaction converts Δ^5-pregnene-3,20-dione (Δ^5-PD) to progesterone (Δ^4-PD) and the second reaction converts Δ^5-androstene-3,17-dione (Δ^5-AD) to androstenedione (Δ^4-AD). Androstenedione is the immediate precursor of testosterone. Several attempts have been made to explain the mechanism of the isomerase reaction by solving crystal structures of hGST A3-3·GSH (Gu *et al*., 2004) and hGST A3-3·GSH·Δ^4-AD (Δ^5-AD is not stable in the presence of GST) (Tars *el al*., 2010). The main outcome of the reaction is a proton transfer from C4 to C6 in the substrate (i.e. Δ^4- AD or Δ^5- AD) which means that the double bond changes its position in the molecule (Gu *et al*., 2004, Tars *el al*., 2010). GSH is expected to have a very central role in the isomerase reaction acting as an acid/base catalyst (Tars *el al*., 2010). Notably GSH will not be conjugated with the substrate, which is otherwise common in detoxification reactions (Tars *el al*., 2010). GSH is in its base form (GS^-) when bound to the enzyme and the negatively charged sulfur of the GS^- attracts a hydrogen atom from C4, the hydrogen protonates GS^- and the acid form of glutathione (GSH) is obtained (Tars *el al*., 2010). Further, in the last step of the reaction the hydrogen is donated to C6 and GSH is now in its base form

(GS^-) again. As noted earlier GST A3-3 has significant isomerase activity in the absence of GSH. The explanation for this is that water may replace GSH as an acid/base catalyst and since water is not as strong acid/base catalyst this will lower the rate of the reaction (Tars *el al*., 2010). To further understand how this reaction occurs it is of interest to look into what could possibly be a stabilizing factor for the negatively charged oxygen that will be formed in position C3. Gu *et al.* (2004) suggests that a water molecule plays this role. However, Tars *et al.* (2010) did not find a water molecule in the relevant position on their electron density maps from X-ray crystallography so the answer to this question is not clear yet.

Expression of GSTs may be tissue specific and depend on age

For some GSTs the expression levels may vary depending on developmental stage; for example a decrease with increased age was found for the pi class GSTs in liver while the alpha class GSTs do have similar expression level in adult and fetal tissues (as reviewed by Laborde, 2010).

Morel *et al.* (2002) investigated the expression pattern for genes *hGSTA1-hGSTA5* in various tissues by RT-PCR analysis. Interestingly they showed that *hGSTA1* and *hGSTA2* are often expressed in the same tissues, kidney, liver, lung, small intestine, prostate, testis, fetal liver, adrenal gland, pancreas and trachea. hGSTA1 transcripts were also detected in stomach, placenta and mammary gland in this analysis. Furthermore hGSTA3 transcripts were found in lung, stomach, testis, placenta, adrenal gland, trachea and mammary gland, hGSTA4 transcripts were detected in all tissues analyzed and no transcripts at all could be found for hGSTA5. Very remarkably, Morel *et al.* (2002) found three hGSTA3 transcripts in lung, stomach, testis, placenta, adrenal gland, trachea and mammary gland. Sequencing of the PCR products showed that one of them lacks exon 3 and another lacks both exon 2 and 3. Other studies found hGSTA3 transcripts in adrenal gland, ovary, placenta and testis; in this analysis a 52 bp shorter PCR product was obtained for all tissues mentioned in addition to GST A3-3 mRNA (Johansson & Mannervik, 2001). Sequencing showed that this shorter PCR product missed exon 3.

Importance of specific primers to determine expression levels

In a recent study Larsson *et al.* (2011a) demonstrate that available antibodies and previously reported qPCR primers that have been used in previous studies to determine expression levels of *GSTA3* in tissues are not accurate because the primers or antibodies used do not have the required specificity. For example, the primers may also promote amplification of *GSTA1*, *GSTA2*, *GSTA4* or *GSTA5* (Larsson *et al*., 2011a). As they emphasize the importance of knowing the gene expression levels to understand gene function they further designed quantitative PCR (qPCR) primers which aimed to anneal to regions of the transcripts that have the lowest similarity in sequence compared to the other alpha class enzymes; untranslated regions (UTRs) tended to be less similar than coding regions in this analysis. These new primers were used to test the levels of GSTA3 transcripts in human embryonic liver and steroidogenic cell lines and it turned out that GSTA3 is not present in fetal liver but, as expected, GSTA3 was detected in both placenta and adrenal gland.

GSTA3 and alternative splicing

Three splice variants of GSTA3 transcripts have been found, Figure 3. Similar discoveries have not been reported for the other *GSTA* genes (Johansson & Mannervik, 2001, Morel *et al*., 2002, Raffalli-Mathieu & Mannervik, 2005). The splice variants lack certain exons; the first splice variant lacks exon 3, the second has lost both exons 1 and 2 and the third splice variant

does not have exons 2 and 3 (Raffalli-Mathieu & Mannervik, 2005). This phenomenon is called alternative splicing and will be discussed in the next paragraph.

Through the mechanism of alternative splicing, different forms of a protein (i.e. isoforms) can be obtained from a single gene (Lodish *et al*., 2007). Most genes in higher multicellular eukaryotes contain multiple introns in contrast to bacterial and archaeal genes (Lodish *et al*., 2007). Interestingly it is estimated that approximately 60 percent of human genes give rise to alternatively spliced mRNAs and current knowledge states that alternative mRNA splicing is of importance to produce a specific protein isoform in a specific cell type in many cases (Lodish *et al*., 2007). Alternative mRNA splicing may give rise to different proteins from the same gene occurring in different cell types as expressed but it may also give rise to different proteins in the same cell type depending on different developmental or environmental signals (Lodish *et al*., 2007).

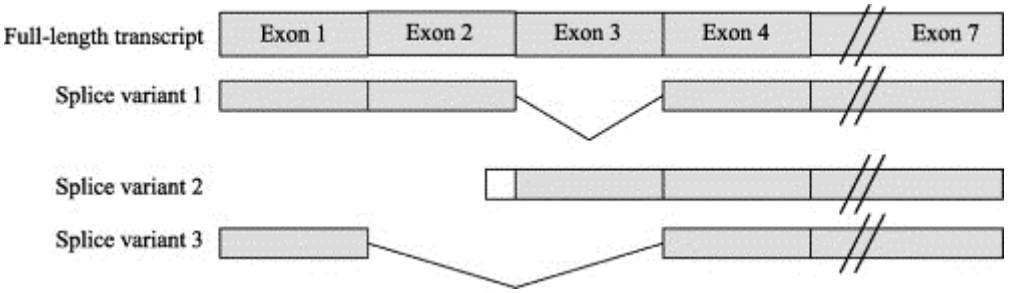

Figure 3. Full-length transcript and splice variants of hGSTA3. Today three different splice variants of hGSTA3 transcripts have been detected. Copied from Raffalli-Mathieu & Mannervik, 2005.

Different expression levels of hGSTA3 and hGSTA3Δ3 in skin

Skin is an important steroidogenic organ which produces androgens (Zouboulis, 2009 as cited by Larsson *et al*., 2011b). In a current study Larsson *et al.* (2011b) investigated if GSTA3 transcripts could be detected in skin cells, using the cell types keratinocytes and sebocytes. In this study, primers were designed to specifically detect both the full length transcript of GSTA3 and the splice variant lacking exon 3 GSTA3Δ3 (Larsson *et al*., 2011b). Very interestingly and quite unexpectedly they discovered that in the two skin cell lines examined, transcripts were only found in the spliced form and could not be detected in the full length form (Larsson *et al*., 2011b). This result is unique for all tissues analyzed today; in no other tissue were the spliced variant present and the full-length form absent (Larsson *et al*., 2011b).

AIM

In previous studies, hGSTA3Δ3 and GST A3-3 mRNA have been detected in steroidogenic tissues. In skin tissue hGSTA3Δ3 was detected but GST A3-3 mRNA has not been found. This suggests that hGSTA3Δ3 either is expressed and has a function as a protein or that hGSTA3Δ3 has a function as untranslated RNA. The aim of this study was to see if it is possible to express hGSTA3Δ3 using a cell free system. The first step is to create a construct with hGSTA3Δ3 as an insert through subcloning. Further a coupled transcription/translation reaction was used for expression. The detection method was based on a light reaction that marks the expression products on a film.

RESULTS

Restriction digestion of the plasmids

The first goal of this project was to produce a construct with the hGSTA3Δ3 sequence inserted into the expression vector pCMVTNT. The plasmid pJ204:57413-hGSTA3Δ3 was ordered with the hGSTA3Δ3 sequence as an insert. To obtain large amounts of both insert and pCMVTNT vector required for upcoming reactions, both plasmids were amplified using the following procedure. At first they were transformed into bacteria (i.e. XL1-Blue *E.coli*) using electroporation. They were cultured overnight on Luria Broth (LB)/ampicillin (amp) plates and colonies were picked and placed in LB/amp media for overnight culture. Subsequently, the plasmids were purified from the cultures using kits for plasmid purification. The insert (i.e. hGSTA3Δ3) was excised from the pJ204:57413-hGSTA3Δ3 vector using restriction enzymes (i.e. EcoRI and NotI) that cleaved on each side of the insert. The pCMVTNT vector containing these restriction sites in the multiple cloning sites was cut with the same enzymes. Both digestion products were analyzed using agarose gel electrophoresis and their sizes verified (Figure 4 and Figure 5).

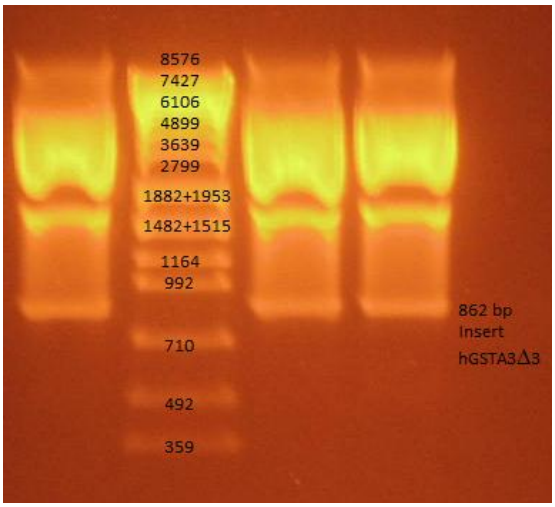

Figure 4. Analysis of excised insert. The pJ204:57413-hGSTA3Δ3 vector was cleaved with restriction enzymes (i.e. EcoRI and NotI) and separated on 1% agarose gel. Three wells were loaded with identical samples of reaction mixtures (2 µg DNA per well). Marker 0,37-8,0 kbp (Roche Diagnostics) was used.

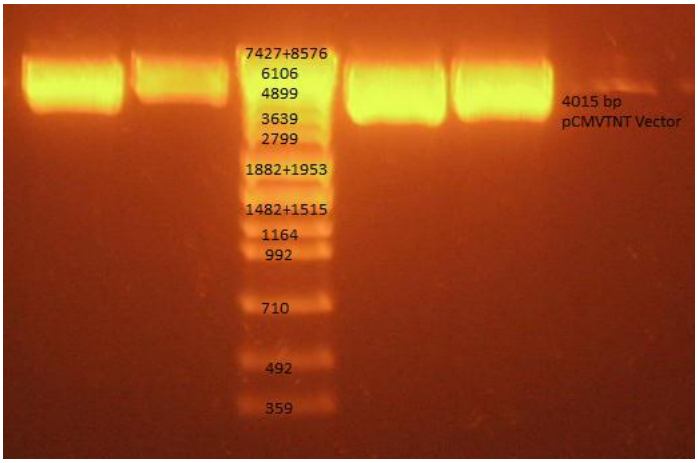

Figure 5. Analysis of cleaved expression vector. The pCMVTNT vector was cleaved with restriction enzymes (i.e. EcoRI and NotI) and separated on 1% agarose gel. Four wells were loaded with identical samples of reaction mixtures (1 µg DNA per well). Marker 0,37-8,0 kbp (Roche Diagnostics) was used.

Ligation

In the next step the insert (see Figure 4) and the pCMVTNT vector (see Figure 5) were purified from the agarose gel. Sufficient amounts of DNA could be gained, and the insert was ligated into the pCMVTNT vector. To verify that the correct construct had been produced in this process the resulting construct was cleaved again with the same restriction enzymes and the products were analyzed with agarose gel electrophoresis. The size of the insert and cleaved vector corresponded to the expected sizes of these DNA sequences, Figure 6.

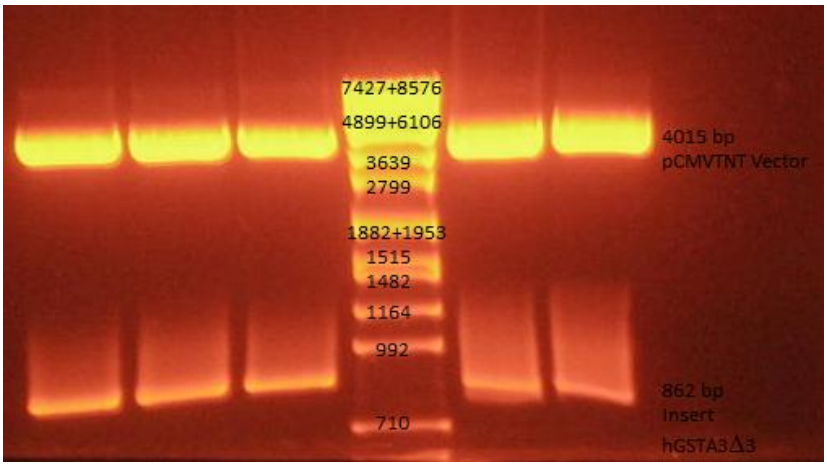

Figure 6. Analysis of cleaved construct. The construct (i.e. pCMVTNT vector with hGSTA3Δ3 insert) was cleaved with restriction enzymes (i.e. EcoRI and NotI) and separated on 1% agarose gel. Five wells were loaded with reaction mixtures (1 µg vector DNA per well) where the vectors had been obtained from five different colonies. Marker 0,37-8,0 kbp (Roche Diagnostics) was used.

In vitro transcription/translation

When the construct had been successfully produced, this vector was amplified and purified from the bacteria media using similar methods as described earlier (see the Materials and Methods section). In the first experiments the plasmids were used without any further purification. In the later experiments the plasmids were purified from an agarose gel to ensure that the samples were not contaminated with ethanol from the last step in the plasmid purification. The hGSTA3Δ3 sequence inserted into the pCMVTNT vector was used as a template in a coupled standard reaction for transcription and translation. A positive control with DNA of known size was used in this experiment. Two additional reaction conditions were used to try to optimize the reaction following the manufacturer´s recommendations. First additional transcend tRNA was added to some samples during incubation to try to get stronger signals. Second RNase A was added to some samples after completion of the incubation to digest RNA that may form interfering bands on the gel. Different amounts of samples from the reaction mixture were loaded on the gel (i.e. 2 µL, 4 µL and 8 µL) to ensure a clear signal. 4 µL appeared to be optimal for detection in this experiment.

Detection

To detect if proteins were successfully expressed, all proteins on the gel were transferred to a nitrocellulose membrane after SDS-PAGE. All lysine molecules in the reaction mixture that were used in the translation step are coupled to biotin. Hence all proteins that will be produced that contain lysine will also get a biotin coupled to these residues. The membrane was first blocked and in the next step streptavidin coupled to horseradish peroxidase (HRP) was added. Streptavidin has high affinity for biotin. Therefore streptavidin-HRP binds specifically to the proteins produced in the transcription/translation reaction. The function of

HRP is to catalyze a reaction that emits light which can be detected on a chemiluminescence film in a dark room.

The analysis did not show any expression of hGSTA3Δ3, Figure 7. When comparing the samples with different reaction conditions the RNase A appears to have the effect to eliminate one of the interfering bands on the gel. Adding more transcend tRNA did not appear to have any obvious effect in this experiment.

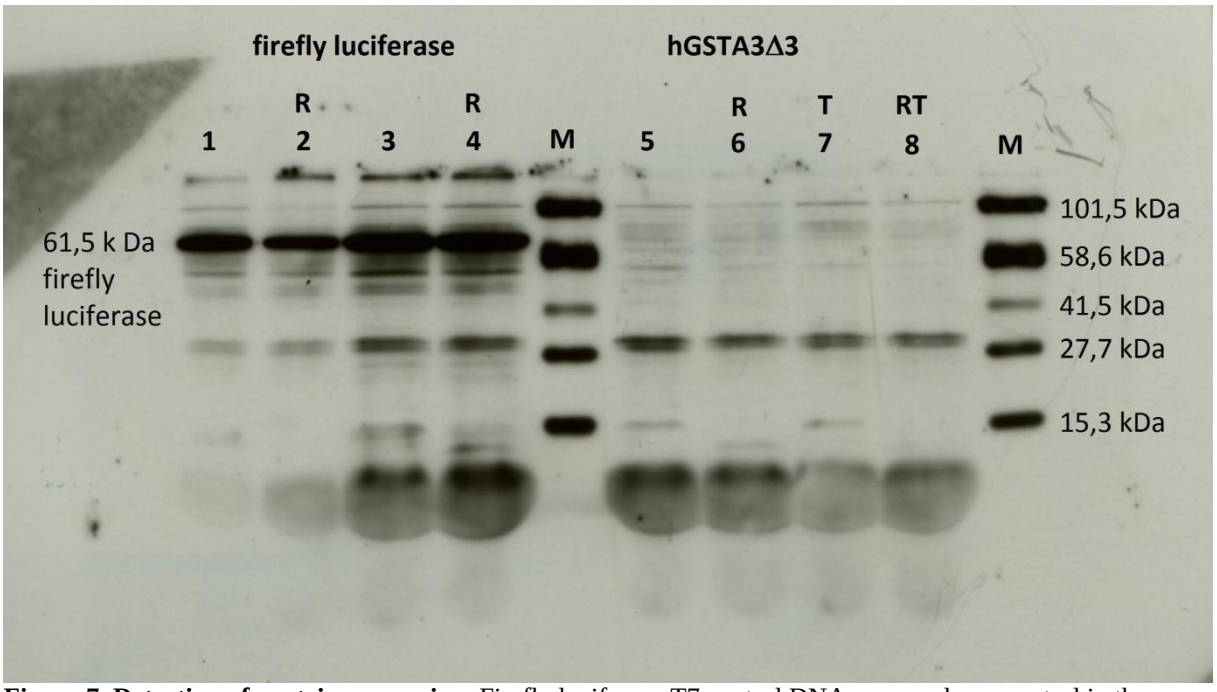

Figure 7. Detection of protein expression. Firefly luciferace T7 control DNA was used as a control in the experiment. Luciferase was detected in samples 1-4. No expression of hGSTA3Δ3 could be detected in samples 5-8. The GST A3-3 functional enzyme is a dimer of 2 × 25,3 kDa and the expected molecular weight of expression products from hGSTA3Δ3 was expected to be less than this. Several interfering bands of varying sizes were detected for all samples. Samples 1-2 contain 2 µL from the reaction mix and samples 3-8 contain 4 µL from the reaction mix. RNaseA (R) was added to samples 2, 4, 6 and 8 after the end of reaction. Additional transcend tRNA (T) was added to the reaction mixture (1,5 µL was added instead of 1 µL). Marker SDS-PAGE Standards, Low Range (Bio-Rad) was used. GSTA3Δ3 templates were purified from agarose gel prior to detection to avoid contamination by ethanol.

DISCUSSION

In this project I addressed the question whether hGSTA3Δ3 can be expressed and in the experiments hGSTA3Δ3 could not be successfully expressed in vitro. However, to be able make the statement that it is not possible to express hGSTA3Δ3, additional replicates and further investigations would be required. As mentioned in the introduction, hGSTA3Δ3 has been detected in several steroidogenic tissues. This suggests that hGSTA3Δ3 has a function in the cell either as a protein or as untranslated RNA.

However, if proteins can be expressed in vitro I can see two possible reasons for not detecting expression in vitro. Either the signal could not be detected or there was something wrong with the construct or the experiment procedure that prohibited expression in my experiment. If a smaller amount of proteins were produced in the hGSTA3Δ3 samples than in the control samples it may be possible that the amounts produced were too small to be detected. Since the luciferase T7 control DNA gave signals in all experiments I know that the reaction mixture is active for transcription and translation. Another possibility would be that signals were achieved but that these signals were hidden by interfering bands from the reaction mix on the gel. The streptavidin turned out to bind to other components in the reaction mixture which gave rise to these bands. Therefore the experiment has to be improved in order to get rid of them for example by using a different detection method. Furthermore, the hGSTA3 samples were treated in the same way as the control so the experimental procedure should be the same and cannot explain this result. The DNA construct was cleaved again and analyzed with agarose gel electrophoresis before it was used in the transcription/translation reaction to guarantee that the sequences were of expected sizes. DNA sequencing was not used to verify the exact DNA sequence because mutations were not likely since I used bacterial culture for amplification.

Before continuing with additional replicates in vitro it would be interesting to check if it was the transcription or translation step that did not succeed in the experiment. To determine this it would be possible to purify and detect RNA from the reaction mix. We already know that RNA is present in the cells so this kind of experiment is only interesting as a control for the experimental procedures. Other control experiments could be to run the transcription/translation reaction without any DNA to get a reference for the components in the reaction mixture that was detected as bands on the gel and to express *hGSTA3* to detect the full length protein.

The hGSTA3Δ3 RNA might have an active function as untranslated RNA and the protein might not be expressed. To be able to speculate if hGSTA3Δ3 has a function as RNA and further which this function would be in the cell more research is required. A possibility is that proteins are not expressed and RNA is present in the steroidogenic tissues without any particular function.

MATERIALS AND METHODS

Vectors
The pJ204:57413-hGSTA3Δ3 vector was provided by DNA 2.0. This plasmid contained a splicing variant of the human *hGSTA3* gene lacking the third exon. The plasmid also contains a gene for ampicillin resistance. The vector was dissolved from the filter paper according to the manufacturer´s instructions.

For expression, the pCMVTNT vector provided by Promega was used. This vector also contains a gene for ampicillin resistance.

Transformation of bacterial cells
Electroporation was used to transform plasmids into bacteria cells. The electrocompetent XL1-Blue *E.coli* were thawed on ice. 50 µL bacteria were transferred to the cuvette and 1 µL plasmid (containing 1 ng DNA) was added. After ligation 1 µL ligation product was used (concentration of formed constructs in the mix unknown). The voltage of 1.25 kV was used and afterwards 750 µL 2 TY (16.0 g Tryptone, 10.0 g yeast extract and 5.0 g NaCl diluted with deionized water to a volume of 1.0 L and autoclaved) was added two times to rinse the cuvette. The culture was saved in a culture tube and the bacteria recovered 1h in the rotation incubator at 37 ºC. The bacteria were plated on LB (10.0 g Bacto Tryptone, 5.0 g Bacto-yeast extract and 5.0 g NaCl diluted to a volume of 1.0 L) / agar (15.0 g/L) / ampicillin plates (1 mg/L). Further, single colonies were picked and cultured in 3 mL LB media containing 100 µg ampicillin/mL.

Culture of bacterial cells
Electrocompetent XL1-Blue *E.coli* (produced using standard protocols) was used as host in the experiments. Bacteria cells were first spread on LB/ampicillin plates overnight. In the second step single colonies were picked and grown in 3 mL of LB/ampicillin (1 µg/mL) media. Plasmids that were purified using Qiagen Plasmid Mini kit (provided by Qiagen) were grown for 12-16 h according to instructions and then purified with the kit, see manufacturer´s protocol. Plasmids that were purified using Qiagen Plasmid Maxi kit were grown for 8 h, then 150 µL of this starter culture were transferred to 100 ml LB/ampicillin media with the same ampicillin concentration and grown for 12-16 h according to the manufacturer´s instructions prior to purification.

Plasmid purification and precipitation of DNA
Qiagen Plasmid Mini kit (provided by Qiagen) was used to purify the plasmids from the bacteria. 1.5 mL culture was used in the plasmid preparation that was performed according to the manufacturer´s protocol. 10 samples were prepared for each of the two vectors. The concentrations were measured with NanoDrop (ND-1000 Spectrophotometer) and the samples pooled. 1/10 volume of 3 M Na-Acetate pH 5.2 and 2.3 volumes 99.9% ethanol were added to the pooled samples. The two samples were incubated 1 h at -20° to precipitate the plasmid DNA. The samples were centrifuged 20 min at 21000×g in a microcentrifuge at 4 °C. The pellet was washed twice with room temperature 70% ethanol and centrifuged 5 min at 21000×g at 4 °C. The pellet was left to dry in the air until suitable consistency, approximately 15 min, and dissolved in buffer QLE from Qiagen Plasmid Mini kit.

Restriction enzymes
The pJ204:57413-hGSTA3Δ3 vector was cut with restriction enzymes EcoRI and NotI to excise the hGSTA3Δ3insert. The pCMVTNT vector was cut with the same restriction

enzymes in multiple cloning region. 2 µg plasmid DNA, 2 µL enzymes (1 µL EcoRI and 1 µL NotI), 2 µL 10× FastDigest Green Buffer and nuclease-free water to a total volume of 20 µL was used in the protocol for digestion of DNA. The optimal reaction times for the enzymes differ and therefore all reagents except the enzyme EcoRI were added, mixed and incubated 25 min at 37 °C. Then the enzyme EcoRI was added and the reaction continued at the same temperature for additional 5 min. After a total reaction time of 30 min the enzymes were inactivated for 5 min at 80 °C. All reagents were provided by Fermentas.

Analysis and purification of expression vector and insert
The cleaved vectors were run on 1% agarose gel with 0.5×TBE buffer. DNA was purified from the gel using QIAquick Gel Extraction Kit (provided by Qiagen) according to the manufacturer´s instructions.

Ligation
Ligation was performed with different ratios between linear vector DNA and insert DNA (1:1, 3:1 and 5:1, insert:vector). In this experiment the ratio 3:1 appeared most optimal. 75 ng linear vector DNA (recommended amount was 20-100 ng), insert DNA to a molar ratio of 3:1 over vector, 2 µL 10 T4 DNA Ligase Buffer, 1 u T4 DNA Ligase and nuclease free water to a total reaction volume of 20 µL was added. The reaction mixture was incubated at 22 °C for 10 min. The T4 DNA Ligase was inactivated by incubation at 65 °C for 10 min. All reagents were provided by Fermentas.

Amplification and purification of construct
To amplify the construct (i.e. pCMVTNT vector with hGSTA3Δ3 insert) the Qiagen Plasmid Maxi kit (provided by Qiagen) was used according to the manufacturer's instructions. The plasmid was considered a high copy plasmid since 3-5 µg DNA could be obtained from 1 mL of LB culture. Bacterial culture with the vector was saved with half the same volume of 80% glycerol added and stored at -80°C.

Purification of vectors from agarose gel before expression of proteins
To ensure that the vector DNA would not be contaminated with ethanol from the Plasmid Maxi preparation the plasmid was further purified after agarose gel electrophoresis, (1 % agarose) using 0,5×TBE buffer. The vector was purified using QIAquick Gel Extraction Kit (provided by Qiagen) according to the manufacturer´s protocol.

Expression of proteins
A TNT Lysate Coupled standard reaction was used for expression. The reaction was a standard reaction using transcend tRNA. All components were from Promega except RNasin Ribonuclease Inhibitor (Sigma). Luciferase T7 control DNA (also provided by Promega) was used as a positive control in the experiment. All components were mixed and the reaction mix incubated for 90 min at 30 °C. The reaction was performed according to the manufacturer´s protocol using a total reaction volume of 50 µL. Two additional reaction conditions were also used. Additional transcend tRNA was added in some reactions (i.e. 1.5 µL instead of 1.0 µL). Further, RNase A was added to a final concentration of 0.2 mg/ml after completion of the incubation. These samples were incubated additionally 5 min at 30 °C.

SDS-PAGE
Separation and stacking gel were prepared using standard protocols. Separation gel was prepared containing 0.35 M Tris pH 8.8, 15% acrylamide, 0.1% SDS, 0.05% APS, 0.05% TEMED and diluted with deionized water to a volume of 15.0 mL. The cassette was filled up

with separation gel until 1 cm below the bottom of the comb. After the separation gel had polymerized the stacking gel was added to the top and the comb was inserted. Stacking gel was prepared containing 0.13 M Tris pH 6.8, 3.9% acrylamide, 0.1% SDS, 0.05% APS, 0.11% TEMED and diluted with deionized water to a volume of 5.0 mL. The chambers were filled with running buffer (25 mM trizmabase, 0.19 M glycine, 3.5 mM SDS).

The samples were prepared according to the following procedure. At first SDS-sample buffer was prepared containing 63 mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol. The solution was diluted with deionized water to a volume of 100 µL. The volume of SDS-sample buffer added to the each sample was twice the sample volume from the reaction mixture. Sample volumes of the reaction mix of 2 µL, 4 µL and 8 µL were used in the experiments. SDS-PAGE was performed at 80V for 2h.

Transfer of proteins to nitrocellulose membrane
Proteins were transferred from the SDS-PAGE gel to a hybond-ECL nitrocellulose membrane provided by Amersham Biosciences. To prepare for the transfer, the gel was incubated in blotting solution (25 mM trizmabase, 0.19 M glycine, 20% MeOH) for 30 min in the cold room with gentle shaking to let the gel absorb the blotting solution. The transfer was performed at 100 V for 1h using blotting solution.

Blocking, Streptavidin-HRP binding and washing
The membrane was blocked using TBS-T (0,05 M Tris pH 7,4, 0,15 M NaCl, 0,05% Tween 20) overnight in the cold room with gentle shaking. According to the manufacturer this step should be performed for 1 h in room temperature instead. Though, no known disadvantage of performing this step overnight in cold room appeared.

Then the membrane was incubated in 15 mL of a 1:10000 dilution of Streptavidin-HRP (provided by Promega) in TBST 45-60 min with gentle shaking at room temperature. The membrane was later washed three times with 15 mL TBST for 5 min with gentle shaking at room temperature. Subsequently the membrane was washed three times with 15 mL deionized water for 5 min with gentle shaking at room temperature.

Detection
To detect the proteins, ECL High performance chemiluminescence film (provided by GE Healthcare) was used. Transcend chemiluminescent substrate A and transcend chemiluminescent substrate B (provided by Promega) were mixed in dim light using 2.5 mL of each substrate. The mix was poured on the membrane and incubated at room temperature for 1 min. The membrane was then placed in a cassette and the room was darkened. A film was placed on the membrane and the cassette was closed. The cassette was left for about 10-15 min to allow exposure of the film. The room was darkened when the cassette was opened and the exposed film transferred to box filled with developer solution. The film was left in the developer solution for approximately 2 min until the picture had developed properly and it was then transferred to a box filled with fix solution where it was left approximately 10 min before the film was washed with deionized water and dried. Several developments were done from the same membrane and longer exposure times were used when the light emitting reaction was starting to decrease to give stronger signals on the film.

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