Expression of Microbial Cysteine Biosynthesis Genes in Sheep by Transgenesis.

Christopher Simon Bawden
B.Sc.(Hons.), Grad. Dip. Ed.

A thesis submitted for the degree of Doctor of Philosophy in the University of Adelaide
Department of Biochemistry
Thesis Summary

The main body of work undertaken in this thesis involves analysis of expression of the cysteine biosynthesis genes of the yeast, *Saccharomyces cerevisiae*, and the bacterium, *Salmonella typhimurium*, in various mammalian systems including cell cultures, transgenic mice and transgenic sheep.

The ultimate goal of the work is to establish a heritable trait of increased wool growth in the sheep by transgenesis with the genes for cysteine biosynthesis. This has arisen from a knowledge of the limitation imposed on wool growth by an insufficient supply of the sulphur-containing amino acid cysteine or its precursor, methionine, to the wool follicle. This limitation has been well established experimentally, and is a result of many factors, not the least of which is a requirement for large quantities of cysteine to support synthesis of the keratin proteins of the wool fibre.

Since the physiological mechanisms involved with cysteine metabolism in the rumen of the sheep limit wool growth, a logical solution would be to provide the sheep with a capacity to synthesize larger quantities of cysteine endogenously. This could be achieved by transgenesis of the sheep with a set of cysteine biosynthesis genes not found in the mammalian genome. In contrast to mammals, plants and microbial species can synthesize cysteine in a two-step reaction. The microbial enzymes of this pathway, serine acetyltransferase and O-acetyl L-serine sulphydrylase, have been well characterised. The genes encoding the respective enzymes in yeast and bacteria are CYS1 and MET25 (yeast) and cysE and cysM (bacteria). The latter three of these have been isolated and positively identified.

Testing the fidelity of expression of the cysteine synthesis genes from the species *S. cerevisiae* and *S. typhimurium* in a mammalian environment is a prerequisite to their use in such a transgenesis programme. This thesis first describes analysis of the expression of the yeast gene, MET25, in transient and permanent mammalian cell culture systems, and finds it unsuitable as a transgene. Subsequently, the analysis of expression of the cysE gene from *Salmonella* in the same cell culture systems and transgenic mice is described. Detection of tissue-specific expression of functional enzyme molecules *in vivo* (transgenic mice) demonstrated this to be a suitable candidate for transgenesis of the sheep.
Finally, analysis of expression of a combination of the cysE and cysM genes from Salmonella in a permanent cell culture system, in transgenic mice and in transgenic sheep, is described. Determination of expression of both genes in cloned cell lines, through detection of the desired catalytic conversion of serine to cysteine, revealed a capacity to synthesise cysteine. Following this, the expression of the same gene combination in transgenic mice and sheep was analysed.

The provision of a mammalian species with a new amino acid synthesis pathway would indeed be a unique situation, purely from the point of view of the presence of such an extra metabolic option within a cell. The consequences of the availability of such a pathway to a normal mammalian cell is unknown but will become apparent at the stage of testing the cysteine biosynthesis genes in a tissuespecific manner in the epithelial cells lining the rumen, where the vital substrate sulphide is found in abundance. In the final chapter, this thesis discusses the possible ramifications of such an achievement for the physiology of the sheep.
# Contents

**Thesis Summary**

**Declaration**

**Acknowledgements**

**Abbreviations**

**Chapter 1 : Introduction.**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 General Introduction.</td>
<td>1</td>
</tr>
<tr>
<td>1.2 The requirement for cysteine in wool growth.</td>
<td>2</td>
</tr>
<tr>
<td>1.3 The relationship between cysteine and sub-optimal wool growth.</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Factors which contribute to a sulphur deficit in the wool follicle.</td>
<td>6</td>
</tr>
<tr>
<td>1.5 The use of Genetic Engineering to overcome the sulphur deficit.</td>
<td>8</td>
</tr>
<tr>
<td>1.6 The cysteine biosynthesis pathways of microorganisms.</td>
<td>10</td>
</tr>
<tr>
<td>1.6.1 The cysteine biosynthesis genes of <em>Saccharomyces cerevisiae.</em></td>
<td>10</td>
</tr>
<tr>
<td>1.6.2 The cysteine biosynthesis genes of <em>Salmonella typhimurium.</em></td>
<td>12</td>
</tr>
<tr>
<td>1.7 Availability of substrates for cysteine biosynthesis : the requirement for tissue-specific expression of the transgenes.</td>
<td>13</td>
</tr>
<tr>
<td>1.8 Transfer of the cysteine biosynthesis genes to the sheep genome.</td>
<td>14</td>
</tr>
<tr>
<td>1.9 The research aims embodied in this thesis.</td>
<td>15</td>
</tr>
</tbody>
</table>

**Chapter 2 : Materials and Methods.**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Materials.</td>
<td>16</td>
</tr>
<tr>
<td>2.1.1 Chemicals.</td>
<td>16</td>
</tr>
<tr>
<td>2.1.2 Enzymes.</td>
<td>17</td>
</tr>
<tr>
<td>2.1.3 Bacterial Strains.</td>
<td>17</td>
</tr>
<tr>
<td>2.1.4 Plasmids and Bacteriophage strains.</td>
<td>18</td>
</tr>
<tr>
<td>2.1.5 Bacterial Growth Media.</td>
<td>18</td>
</tr>
<tr>
<td>2.1.6 Buffers and Solutions.</td>
<td>19</td>
</tr>
<tr>
<td>2.1.7 Radiochemicals.</td>
<td>20</td>
</tr>
<tr>
<td>2.1.8 Molecular Biology Kits.</td>
<td>20</td>
</tr>
<tr>
<td>2.1.9 Equipment.</td>
<td>20</td>
</tr>
<tr>
<td>2.1.10 Cell Culture Materials.</td>
<td>21</td>
</tr>
</tbody>
</table>
2.1.11 Animals used in the Transgenesis programmes.

2.2 Methods.

2.2.1 DNA Methods.

2.2.1.1 Recombinant DNA methods.

2.2.1.2 General DNA methods.

2.2.2 RNA Methods.

2.2.3 Protein Methods.

2.2.4 Cell culture methods.

2.2.5 Transgenesis Methods.

2.2.5.1 Production of transgenic mice.

2.2.5.2 Production of transgenic sheep.

2.2.6 Computer-assisted analysis methods.

Chapter 3 : Expression of the MET25 Gene.

3.1 Introduction.

Results.

3.2 Efficiency of translation of MET25 gene transcripts.

3.2.2 Manipulation of the MET25 gene to improve translation.

3.2.3 Effect of removal of the 5'-untranslated region of MET25.

3.2.4 Effect of addition of the Xenopus β-globin 5'-untranslated region (and associated 3'-untranslated sequences).

3.2.5 Comparison with transcripts known to be efficiently translated.


3.4 Production of assayable quantities of the MET25 gene product in vitro.

3.4.1 Production of assayable quantities of the MET25 gene product in the RRL.

3.4.2 Production of assyable quantities of the MET25 gene product in cell culture.

Discussion.

3.5 The suitability of MET25 as a transgene.

3.5.1 Determination of translation efficiency.

3.5.2 Improvement of translation efficiency.
3.5.3 Determination of the catalytic capacity of MET25 protein translated in the RRL.
3.5.4 Production of the MET25 gene product in cell culture.
3.5.5 Factors contributing to lack of enzyme activity.
3.5.6 An alternative to the MET25 gene.

Chapter 4 : Expression of the cysE Gene.
4.1 Introduction.
Results.
4.2 Expression of the cysE gene \textit{in vitro}.
4.2.1 Translation of cysE gene transcripts in the RRL.
4.2.2 Expression of the cysE gene in mammalian cells.
4.3 Expression of the cysE gene \textit{in vivo}.
4.3.1 Generation and identification of founder transgenic mice.
4.3.2 Analysis of transgene expression in F0 mice.
4.3.3 Heritability of the transgene.
4.3.4 Quantitation of SAT enzyme activity in the tissues of transgenic mice.
Discussion.
4.4 The suitability of cysE as a transgene.
4.4.1 Activity of the cysE gene product in a mammalian environment.
4.4.2 Expression of the cysE gene in transgenic mice.
4.4.3 The effects of the host genome on expression of the transgene.
4.4.4 Heritability of functional transgenes.

Chapter 5 : Coexpression of Cysteine Biosynthesis Genes.
5.1 Introduction.
Results.
5.2 Detection of expression of the cysteine biosynthesis genes.
5.3 Construction of a linked-gene eukaryotic expression plasmid.
5.4 Coexpression of the cysE and cysM genes \textit{in vitro}.
5.4.1 Coexpression in transient cell cultures.
5.4.2 Coexpression in permanent cell cultures.
5.4.2.1 Initial characterisation of permanent cell lines.
5.4.2.2 Analysis of coexpression in the permanent cell lines.  
5.4.2.3 Analysis of the cysteine synthesis capacity of cell line pLT-EM.4.  
5.5 Coexpression of the cysE and cysM genes in vivo.  
5.5.1 Coexpression of the cysE and cysM genes in transgenic mice.  
5.5.2 Coexpression of the cysE and cysM genes in transgenic sheep.  
5.5.2.1 Production and identification of transgenic sheep.  
5.5.2.2 Analysis of expression of the transgenes in sheep.  
Discussion.  
5.6 Coexpression of the cysteine biosynthesis genes of *Salmonella typhimurium*  
in mammalian systems in vitro and in vivo.  
5.6.1 Coexpression of the cysE and cysM genes in vitro.  
5.6.2 Coexpression of the cysE and cysM genes in vivo.  

Chapter 6: General Discussion.  
6.1 Introduction.  
6.2 Sulphur metabolism in mammals.  
6.3 Sulphur metabolism in sheep: rumen physiology and the sulphur balance.  
6.4 Improvements to the transgenesis regime.  
6.4.1 Optimisation of expression of cysteine biosynthesis transgenes.  
6.4.2 Site-specific transgene integration.  
6.5 Testing for cysteine synthesis and improved wool growth.  

Bibliography