INVESTIGATION OF THE GENE RESPONSIBLE FOR RECESSIVE PIGMENTATION IN AUSTRALIAN MERINO SHEEP

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SUMMARY

Our interest in coat colour genetics stems from the financial losses associated with pigment contamination of the Australian wool clip. We are working on a project to develop a diagnostic test to identify rams heterozygous (carriers) for the gene allowing recessive expression of pigmented wool in Merino sheep. Several pigmentation genes, including Agouti, Kit and Steel, have been identified as possible candidates, the most likely being Agouti. Initial work has involved the development of an ovine Agouti gene probe for molecular genetic studies and microsatellite analysis of ovine chromosomal regions. Results indicate the gene for recessive pigmentation maps to ovine chromosome 13 making Agouti a positional candidate for this pigmentation locus.

Keywords: Agouti, self-colour, ovine, microsatellites.

INTRODUCTION

Pigmentation in commercial wool flocks can lead to financial losses for breeders and wool processors. Because self-colour pigmentation is a recessive condition, carriers cannot be identified phenotypically and the gene frequency has remained at a significantly high level, despite this being an undesirable trait, in commercial wool flocks. Progeny testing is the only method available to breeders to test for carriers and this is a costly and lengthy process. We are involved in a collaborative project to identify and characterise the gene responsible for recessive pigmentation in Australian Merino sheep. The ultimate aim is to develop a genetic test that will enable breeders to identify carriers of the recessive condition and remove these animals from their breeding programs.

The Agouti locus (A) is believed to be responsible for most of the coat colour variation in sheep breeds worldwide (Sponenberg et al. 1996). However, the gene has not been isolated from the ovine genome or characterised at the molecular level. The mouse agouti locus codes for a small protein that acts as a molecular signal in the hair follicle to switch on production of yellow/red pigment (phaeomelanin) production (Siracusa 1994). In mice over 25 agouti alleles have been identified. Resulting pigmentation in mice ranges from completely yellow to completely black. In the recessive conditions (nonagouti types) mostly dark pigment (eumelanin) is produced (Siracusa 1994). In modern sheep breeds the most dominant Agouti allele, the white/tan (Awt) allele, is believed to be the most common mechanism for the white wool phenotype (Sponenberg et al. 1996). As in the mouse there seems to be a dominance hierarchy among the Agouti alleles
with the most recessive allele, nonagouti \((A^a)\), resulting in uniformly eumelanic (black/brown) wool (Sponenberg et al. 1996).

Our research to date has involved the development of an ovine Agouti gene probe to determine the relationship between the ovine Agouti gene and recessive pigmentation at the molecular level. At the same time we have utilised comparative mapping data to investigate the homologous map position in the sheep genome. The mouse agouti gene has been mapped to chromosome 2 (Bultman et al. 1991) and the homologous human gene to chromosome 20 (Kwon et al. 1994). Genes that map closely on both sides of this gene in mouse and humans have been mapped to bovine chromosome 13 (Eggen and Fries 1995) which is homologous to ovine chromosome 13 (Echard et al. 1994).

**METHODS**

**Flock.** The sheep used in this research were bred specifically for this project on a commercial sheep station. A back cross design was used to generate half-sib pedigrees from a single pigmented (self-colour) ram (Figure 1). The grand progeny of this ram demonstrated a 1:1 segregation of pigmented: white woolled fleece, as expected for a simple Mendelian condition.

\[
\begin{align*}
\text{Self-colour grandsire } & (A^a A^a) \quad \times \quad 6 \text{ white dams } (A^W_t A^W_t) \\
& \downarrow \\
6 \text{ ram sires } & (A^W_t A^a) \quad \times \quad 92 \text{ self-colour dams } (A^a A^a) \\
& \downarrow \\
39 \text{ self-colour } & (A^a A^a) \quad + \quad 36 \text{ white } (A^W_t A^a) \text{ progeny}
\end{align*}
\]

**Figure 1. Pigmentation back cross flock**

**Molecular techniques** Much of the molecular work was based on the polymerase chain reaction (PCR). This technique involves exponential amplification of short stretches of DNA that can then be utilised for genetic analysis. PCR primers were designed from human and mouse DNA sequence data (Bultman et al. 1992, Kwon et al. 1994) to amplify coding regions corresponding to exons 2 and 3 of the mouse agouti gene. PCR products were used in hybridisation studies to detect genetic variation between individuals, and to isolate longer fragments of the ovine Agouti gene. PCR technology was also employed in microsatellite analysis to determine pedigree relationships within the pigmentation flock, and to investigate ovine chromosome 13 markers for linkage to the recessive pigmentation locus. A total of 12 microsatellite markers from ovine chromosomes 1, 3, 6, 13 and 26 (Crawford et al. 1995) were used to generate typing data.

**Linkage analysis** Linkage between the pigmentation locus and microsatellite markers was analysed using the Animap lodtable computer program (Georges et al. 1995), which compares genetic loci in pairs, calculating lod scores based on maximum likelihood estimations in half-sib pedigrees. Further analysis was carried out for those markers which gave positive lod scores using
the Liped computer program (Ott 1974) in order that the available three generation typing data could be utilised.

RESULTS
PCR products were generated for the human, bovine and ovine exon 2 region and the bovine and ovine exon 3 region. Sequence identity of ovine and bovine PCR generated products showed a relatively high degree of homology to published human and mouse sequences (Bultman et al. 1992, Kwon et al. 1994) at both the protein and DNA level (Table 1). Hybridisation studies using the ovine Agouti exon 2 PCR fragment indicates a single coding region present in the sheep genome.

Table 1. Homology between PCR generated products and human/mouse agouti sequence data

<table>
<thead>
<tr>
<th>Genomes</th>
<th>Exon 2 Protein sequence</th>
<th>Exon 2 DNA sequence</th>
<th>Exon 3 Protein sequence</th>
<th>Exon 3 DNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine/Bovine</td>
<td>96%</td>
<td>98%</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>Ovine/Human</td>
<td>80%</td>
<td>86%</td>
<td>86%</td>
<td>88%</td>
</tr>
<tr>
<td>Ovine/Murine</td>
<td>69%</td>
<td>80%</td>
<td>88%</td>
<td>88%</td>
</tr>
<tr>
<td>Human/Murine</td>
<td>77%</td>
<td>81%</td>
<td>86%</td>
<td>86%</td>
</tr>
</tbody>
</table>

Pairwise linkage analysis was carried out between the recessive pigmentation gene and all typing data using the pedigree relationships as determined from parentage analysis. The results gave significant evidence of linkage between the recessive pigmentation locus and microsatellite markers that have been previously mapped to ovine chromosome 13 (Crawford et al. 1995). The maximum lod score of 3.45 was found with the HUJ616 marker at a recombination fraction of 0.25 (Table 2). A lod score of +3 is generally accepted as significant evidence of linkage between two loci. The recombination fraction gives an indication of how closely linked two loci are: 0.0 indicates tight linkage, 0.5 indicates that two genes do not physically map close together. Positive results of pairwise linkage analysis with other markers that have been mapped to the same chromosomal region give additional support to the linkage evidence. Results for the microsatellite marker BM4621 that has been mapped close to the Kit gene locus on ovine chromosome 6 (Crawford et al. 1995) gave no evidence of linkage.
Table 2. Results of pairwise linkage analysis between recessive pigmentation and microsatellite markers

<table>
<thead>
<tr>
<th>Locus*</th>
<th>Chromosome</th>
<th>PIC**</th>
<th>Maximum lod score***</th>
<th>Recombination fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM6438</td>
<td>1</td>
<td>0.78</td>
<td>-0.18</td>
<td>0.4</td>
</tr>
<tr>
<td>CP34</td>
<td>3</td>
<td>0.80</td>
<td>0.25</td>
<td>0.4</td>
</tr>
<tr>
<td>BM4621</td>
<td>6</td>
<td>0.87</td>
<td>0.00</td>
<td>0.4</td>
</tr>
<tr>
<td>JMP8</td>
<td>6</td>
<td>0.73</td>
<td>-0.07</td>
<td>0.4</td>
</tr>
<tr>
<td>HUJ616</td>
<td>13</td>
<td>0.68</td>
<td>3.45</td>
<td>0.25</td>
</tr>
<tr>
<td>McM152</td>
<td>13</td>
<td>0.77</td>
<td>1.93</td>
<td>0.31</td>
</tr>
<tr>
<td>McM253</td>
<td>13</td>
<td>0.77</td>
<td>1.40</td>
<td>0.29</td>
</tr>
<tr>
<td>IL2RA</td>
<td>13</td>
<td>0.68</td>
<td>-0.18</td>
<td>0.4</td>
</tr>
<tr>
<td>TGLA23</td>
<td>13</td>
<td>0.50</td>
<td>0.53</td>
<td>0.1</td>
</tr>
<tr>
<td>MAF18</td>
<td>13</td>
<td>0.49</td>
<td>0.00</td>
<td>0.4</td>
</tr>
<tr>
<td>BM6526</td>
<td>26</td>
<td>0.67</td>
<td>-0.13</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Previously published ovine microsatellite markers (Crawford et al. 1995, Smith et al. 1995)
** Polymorphic Information Content values indicate the informativeness of the marker.
*** A lod score of +3 provides statistical evidence of linkage.

DISCUSSION

In order to determine the relationship between the Agouti gene and recessive pigmentation in Merino sheep we have investigated this gene using molecular genetic techniques in animals from a pigmentation back-cross flock. We have developed an ovine Agouti gene probe that shares significant homology with known human and mouse agouti sequences at both the protein and DNA level. This probe can now be used to identify genetic variation between individuals, providing a DNA marker for future mapping studies. In addition we are in the process of isolating the complete coding sequence of the ovine Agouti gene to investigate any sequence variation between individual animals.

The locus responsible for recessive pigmentation in Merino sheep has been shown to be linked to DNA markers on ovine chromosome 13. Mapping data for closely linked genes on the homologous bovine chromosome, together with comparative data from human and mouse research, indicate that ovine chromosome 13 is homologous to the mouse chromosome 2 and human chromosome 20 region that contains the agouti gene locus (Eggen and Fries 1995, Kwon et al. 1994, Siracusa and Abbot 1993). The linkage mapping of recessive pigmentation to ovine chromosome 13 is in accord with historical breeding data that suggest that recessive black in Merino sheep is caused by action of the Agouti gene.

Characterisation of this gene at the molecular level will enable us to search directly for the mutation responsible for the expression of black wool and bring us closer to the development of a
diagnostic test for carrier rams. Until a genetic carrier test is developed, and widely used, it is unlikely that there will be any appreciable reduction in expression of black pigmentation in Australian wool flocks.

ACKNOWLEDGMENT
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REFERENCES