INVESTIGATION OF THE EFFECT OF GROWTH FACTOR AND PROTO-ONCOGENE VARIANTS ON WOOL QUALITY

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SUMMARY

Genetic variation has been explored in sheep at genes which are candidates for a major effect on wool quality. Work has focussed on epidermal growth factor (EGF), acidic and basic fibroblast growth factor (aFGF and bFGF), insulin-like growth factor-1 (IGF1), keratinocyte growth factor (KGF), transforming growth factor (TGF) alpha and beta-1, and the c-*myc* and N-*myc* proto-oncogenes.

Twenty restriction enzymes were used to digest DNA from a number of unrelated sheep and Southern blots were screened for variation using 16 mouse/human clones as labelled probes covering 8 candidate genes. Polymorphisms have been detected as RFLPs with EGF, EGF-receptor, aFGF, TGFα, TGFβ1, c-myc and N-myc clones. Variation was also investigated at a published microsatellite locus located 5 prime to IGF1. Large half-sibling families derived from super-fine wool Merino rams and strong wool Merino or Border Leicester ewes are currently being typed for polymorphisms detected using candidate gene markers and scored for average fibre diameter, variation in fibre diameter and fleece weight. Some difficulty has been experienced using heterologous cDNA clones as probes. Current work involves the cloning of sheep growth factor genes from a genomic cosmid library.

INTRODUCTION

The Merino's fleece has a lower fibre diameter than other breeds and this quality is preferred by manufacturers for the production of high quality clothing. Animals are currently selected at 1-2 years of age for breeding on the basis of fleece traits including average fibre diameter and fleece weight. Fibre diameter is vulnerable to seasonal conditions. Market premiums exist for wool which is low in fibre diameter and high in yield. The heritability of fibre diameter and yield traits is high (James *et al.* , 1990; Mortimer and Atkins, 1989), with positive genetic correlation between fleece weight and fibre diameter. Marked differences are known to exist between strains within the Merino breed (Atkins *et al.*, 1993). Although it is generally assumed that many genes are likely to be involved, single loci could be having a large effect on wool quality traits. Genotype based selection of animals therefore has potential for greatly accelerating improvement in wool quality traits.

Wool follicle development occurs in the crown of the foetus from around 50 days after conception and continues down the body of the foetus in a wave of initiation and subsequent differentiation (Hardy and Lyne, 1956). Nagorcka (1989) has proposed that there is a period of pre-pattern formation prior to initiation that is governed by a reaction diffusion mechanism. Interactions between the epidermis and mesenchymal layers are believed to be essential in the processes of initiation and growth (Moore *et al.*, 1991). It is likely that at least part of this interactive process effecting pre-pattern formation, initiation and differentiation is governed by known growth factors.

A number of known growth factors could be controlling the differentiation and organisation of wool follicles in such a way that wool quality is affected. Basic and acidic FGF, KGF, TGFα and EGF are believed to influence keratinocyte and fibroblast cell division (Moore,

1988). The FGF type proteins are known to affect cell migration/proliferation (McAvoy and Chamberlain, 1989) as are proto-oncogenes such as c-*myc* and N-*myc* (Blister and Jansen, 1986). Keratinisation is known to be affected by the TGFβ family of proteins (Lyons *et al.*, 1989; Choi and Fuchs, 1990). The presence and/or expression of all of these growth factors has been detected at various stages and in various layers of the developing wool/hair follicle (Pisansarakit *et al.*, 1989; Sutton, 1989; Sutton *et al.*, 1990; Sutton and Ward, 1990; Cam and Sutton, 1989). Recent experiments by other workers with transgenic mice have shown that the size, number and morphogenesis of hair follicles is affected by targeting and modulating the expression of genes coding for FGF receptor (Werner et al., 1993), IGF1 and type 1 IGF receptor (Baker *et al.*, 1993; Liu *et al.*, 1993), KGF (Guo *et al.*, 1993), TGFα (Mann *et al.*, 1993) and TGF-β1 (Sellheyer *et al.*, 1993).

The aim of our study has been to identify genetic variation in sheep at growth factor genes and proto-oncogenes which are candidates for a major effect on wool quality characteristics, determine if there are correlations between inheritance of particular alleles and inheritance of wool quality traits and, if so, develop PCR tests which could be used to select rams for breeding to produce high quality wool. This paper presents the results of our search for polymorphism using candidate gene clones as labelled probes.

MATERIALS AND METHODS

Creation of flock and collection of phenotypic data

Half-sibling families (approximately 100 offspring in each) were created by crossing two superfine wool Merino rams (average fibre diameter <18 micron) with strong wool Merino, medium wool Merino or Border Leicester ewes. Five of the progeny were then backcrossed to strong and medium wool Merino ewes. Pedigree records, sex, birth type (twin, single, reared or not reared) and wool growth were recorded. Wool production data including average fibre diameter, coefficient of variation in fibre diameter and percentage of fibres greater than 30 micron was collected over two years for each animal in the flock. Samples were taken from the shoulder, midside and flank of each animal. Total fleece weight was recorded at shearing.

Search for polymorphisms

DNA was extracted from blood samples by phenol/chloroform extraction (Sambrook *et al.*, 1989). DNA (10 microgram per digest) was digested with one of twenty different restriction enzymes (*Acc* I, *Alu* I, *Bam* HI, *Bgl* II, *Eco* RI, *Eco* RV, *Hae* III, *Hinc* II, *Hind* III, *Hinf* I, *Kpn* I, *Msp* I, *Pst* I, *Pvu* II, *Rsa* I, *Sal* I, *Sph* I, *Taq* I, *Xba* I and *Xho* I). Each of the candidate gene loci was surveyed for genetic variation (as restriction fragment length polymorphisms) with each of the twenty restriction enzymes using DNA samples from at least four sheep from a total panel of 98 unrelated medium wool Merino ewes. DNA was loaded and electrophoresed in 0.8% agarose for 24-48 hours at 25 volts to size separate the restriction fragments. Lanes containing 2 micrograms of lambda phage digested with *Hind* III were included on each gel as molecular weight standards. Restriction fragments were transferred by vacuum to nylon membrane for hybridisation with radioactively labelled candidate gene probes.

The following clones were used as labelled probes in this study. 1. phEGF121 and pmEGF26-F12 for EGF (Murray *et al.*, 1986; Bell *et al.*, 1986; Gray *et al.*, 1983); 2. pHER-A64-1 and pE7 for the EGF receptor (Ullrich *et al.*, 1984; Xu *et al.*, 1984); 3. WEHI-1, pDH14 and pDH15 for aFGF (Halley *et al.*, 1988; Jaye *et al.*, 1986); 4. WEHI-2 for bFGF (O. Bernard, pers. com.); 5. WEHI-3, phTGF1-10-925 and phTGF1-10-3350 for TGFα (Derynck *et al.*, 1984; Tricoli *et al.*, 1986); 6. WEHI-4 and phTGFB-2 for TGF-β1 (Derynck *et al.*, 1985); 7. WEHI-5 and

pCMCBam for c-*myc* (Stanton *et al.*, 1983; Vennstrom *et al.*, 1982); 8. WEHI-6 for N-*myc* (DePhino *et al.*, 1986). 100 ng of probe was used for each labelling with [alpha^{32}P]-dCTP using the random priming method (Feinberg and Vogelstein, 1983). Membranes were prehybridized for more than two hours and hybridized overnight at 50°C in 20 ml of hybridization solution (Church and Gilbert, 1984). Membranes were then washed first at room temperature (2 X SSC, 0.1% SDS for 10 minutes) and then twice at 50°C (2 X SSC, 0.1% SDS for 20 minutes) and exposed to X-ray film with intensifying screens for approximately one week. Each membrane was used up to three times with different gene probes. Membranes were stripped of DNA probes by immersion in a boiling solution of 0.5% SDS.

Polymorphism was also investigated at a microsatellite locus 5' to the insulin-like growth factor-I gene by the methods of Kirkpatrick (1992).

RESULTS

Search for polymorphisms

Polymorphism was detected using each of the candidate gene clones except for basic fibroblast growth factor cDNA clone WEHI-2. Epidermal growth factor cDNA clone phEGF121 detected polymorphism with *Pst* I and *Sph* I restriction enzymes as did pmEGF26-F12 with *Bgl* II, *Pvu* II and *Sph* I. Epidermal growth factor receptor cDNA clone pHER-A64-1 detected polymorphism with *Pst* I as did pE7 with *Hinf* I and *Pst* I restriction digests. Acidic fibroblast growth factor cDNA clone pDH14 detected polymorphism with *Bgl* II as did pDH15 with *Acc* I, *Bgl* II, *Pst* I and *Pvu* II restriction digests. Transforming growth factor-alpha cDNA clones phTGF1-10-925 and phTGF1-10-3350 detected polymorphism with *Hinf* I and *Taq* I restriction enzymes respectively. Transforming growth factor-beta 1 cDNA clone WEHI-4 detected polymorphism with *Pst* I as did phTGFB-2 with *Hinf* I and *Pvu* II restriction digests. c-*myc* proto-oncogene clone WEHI-5 detected polymorphism with *Pst* I and *Eco* RI as did pCMCBam with *Bgl* II, *Kpn* I, *Pst* I and *Pvu* II restriction digests. N-*myc* proto-oncogene clone WEHI-6 detected polymorphism with *Eco* RI restriction digests.

Three alleles (124, 126 and 128 base pairs size) were detected at the 5' IGF-1 microsatellite locus in 154 sheep.

DISCUSSION

Prior studies have identified growth factor genes and proto-oncogenes as likely candidates affecting the development of the wool follicle and wool quality. The clones of growth factor and proto-oncogene loci used as labelled probes in this study have revealed a number of polymorphisms. Our work is currently concentrating on genotyping existing animals in the flock at the Victorian Institute of Animal Science for the polymorphisms identified in this paper.

Some problems were encountered with the use of heterologous gene probes. A number of gene probes revealed the same patterns of polymorphism for particular restriction enzymes. This could be because of close linkage between these loci or could reflect cross hybridisation of probes with the homologous DNA sequences of other candidate genes at low stringency hybridisations. Current work also involves the cloning of sheep growth factor genes from a genomic cosmid library. These clones will allow us to better resolve some of the polymorphisms identified in this paper and to identify further variation.

REFERENCES

ATKINS, K. D. *et al.* (1992) In Wool Technology and Sheep Breeding, 106-113.
BAKER, J. *et al.* (1993) Cell, 75: 73-82.
BELL, I. G. *et al.* (1986) Nucleic Acids Research, 14: 7873-7882.
BLISTER, K. and JANSEN, H. W. (1986) Advances in Cancer Research, 47: 99-188.
CAM, G. R. and SUTTON, R. (1989) Proc. of Workshop on Wool Biology (in press).
CHOI , Y. AND FUCHS, E. (1990) Cell Reg., 1: 791-809.
CHURCH, G. M. and GILBERT, W. (1984) Proc. Natl. Acad. Sci. USA, 81: 1991-1995.
DEPHINO, R. A. *et al.* (1986) Proc. Natl. Acad. Sci. USA, 83: 1827-1831.
DERYNCK, R. A. *et al.* (1984) Cell, 38: 287-297.
DERYNCK, R. A. *et al.* (1985) Nature, 316: 701-705.
FEINBERG, A. P. and VOGELSTEIN, B. (1983) Addendum Annals Biochemistry, 137: 266-267.
GRAY, A. *et al.* (1983) Nature, 303: 722-725.
GUO, I. *et al.* (1993) EMBO J., 12: 973-986.
HALLEY, C. *et al.* (1988) Nucleic Acids Research, 16: 10913.
HARDY, M. H. and LYNE, A. G. (1956) Australian J. Biological Science, 9: 423-433.
JAMES, P. J. *et al.* (1990) Australian J. Agricultural Research, 41: 583-594.
JAYE, M. *et al.* (1986) Science, 233: 541-545.
KIRKPATRICK, B. W. (1992) Animal Genetics, 23: 543-548.
LIU, J-P. *et al.* (1993) Cell, 75: 59-72.
LYONS, K. M., PELTON, R. W. and HOGAN, B. L. M. (1989) Genes and Development, 3: 1657-1688.
MANN, B. G. *et al.* (1993) Cell 73, 249-261.
MCAVOY, J. W. and CHAMBERLAIN, C. G. (1989) Development, 107: 221-223.
MOORE, G. P. M. *et al.* (1991) Annals New York Acad. Sci., 642: 308-325.
MOORE, G. P. M. (1988) In The Biology of Wool and Hair. eds. Rogers G. E. *et al.*, 351-364.
MORTIMER, S. I. and ATKINS K.D. (1989) Australian J. Agricultural Research, 40: 433-443.
MURRAY, J. C. *et al.* (1986) Nucleic Acids Research, 14: 5117.
NAGORCKA, B. N. (1989) J. Theoretical Biology, 137: 127-162.
PISANSARAKIT, P. *et al.* (1989) In Proc. of Worshop on Wool Biology (in press).
SAMBROOK, J. *et al.* (eds) (1989) Molecular Cloning: A Laboratory Approach.
SELLHEYER, K. *et al.* (1993) Proc. Natl. Acad. Sci. USA, 90: 5237-5241.
STANTON, L. W. *et al.* (1983) Nature, 303: 401-406.
SUTTON, R. and WARD, W. (1990) Proc. of Workshop on Wool Biology (in press).
SUTTON, R. *et al.* (1990) In Proc. of Workshop on Wool Biology (in press).
SUTTON, R. (1989) Proc. Australian Biochemical Society, 21: 19.
TRICOLI, J. V. *et al.* (1986) Cytogenetics and Cell Research, 42: 94-98.
ULLRICH, A. *et al.* (1984) Nature, 309: 418-425.
VENNSTROM, B. *et al.* (1982) J. Virology, 42: 773-779.
WERNER, S. *et al.* (1993) EMBO J., 12: 2635-2643.
XU, Y. *et al.* (1984) Nature, 309: 806-810.

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