Detecting inherited variation in DNA sequences and its application in animal science.

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ABSTRACT

Modern methods in molecular biology have immense practical application in many areas of agriculture and veterinary medicine. These methods include the use of enzymes (restriction endonucleases) that recognise and cleave DNA at specific sites, the cloning of specific fragments of DNA into suitable plasmid or bacteriophage vectors, separation of DNA or RNA fragments followed by fixation to a suitable membrane support, and the use of cloned radioactive DNA or RNA probes to detect matching sequences by hybridisation.

These methods have been combined to provide powerful new techniques to detect DNA polymorphisms that show Mendelian inheritance. Differences in DNA among individuals can be detected as restriction fragment length polymorphisms (RFLP) or by DNA fingerprinting. Both techniques are used extensively in medicine for the analyses of heritable genetic diseases and genetic mapping. They can also be applied to livestock to find DNA markers linked to important economic traits.

We have recently begun screening human DNA probes in a search for RFLPs in New Zealand sheep breeds. The objective of the experiments is to find markers linked with with Booroola fecundity (F) gene. Several human DNA probes show specific binding to sheep genomic DNA, demonstrating homology between sheep and humans for some DNA sequences. Techniques which identify linked markers will have immediate application in selection for important major gene traits such as genes affecting fecundity, fleece weight, and disease and parasite resistance. New Zealand is in a strong position to apply linkage studies to important production traits in existing breeding programmes.

Keywords Sheep; molecular genetics; DNA; polymorphisms; RFLP.

INTRODUCTION

Studies of the structure, function and expression of genes, made possible by recombinant DNA technology, will bring major advances in our understanding of animal physiology and biochemistry. In addition, these powerful new methods will have considerable practical application in agriculture and veterinary medicine. The potential for recombinant DNA technology has been discussed previously at meetings of this Society (Fennessy, 1983; Baker 1984), and reviews at the Animal Science Congress of the Asian-Australasian Association of Animal Production Societies last year described applications for the diagnosis of infectious agents and the production of new vaccines (Fahey, 1987) and the introduction of foreign genes to create *transgenic* animals (Wagner, 1987).

Other major applications will come from the detection of variation between individuals in DNA structure. These techniques have revolutionised human genetics through the use of DNA markers linked to human genetic diseases. DNA polymorphisms are widely used for the prenatal detection of inherited diseases, the detection of deletions and chromosomal rearrangements resulting in some forms of cancer, mapping the location of genes to individual chromosomes and measuring the order of, and relative distance between genes on the human genetic map. In contrast, animal science has been slow to apply this technology. The object of this paper is to describe the methods used in the search for DNA markers linked to economically important traits in livestock, report preliminary observations on the search for such markers in sheep and discuss their potential role in animal production.

METHODS IN MOLECULAR BIOLOGY

Recombinant DNA technology has a wide range of applications made possible through isolation of specific enzymes for cutting, joining and copying DNA molecules, and developments in cloning, sequencing and synthesising DNA and RNA. This section is intended as a brief introduction to the tools of recombinant DNA technology. Additional information including detailed methods can be obtained elsewhere (Rosenfield *et al.*, 1983; Watson *et al.*, 1983; Maniatas *et al.*, 1982).

Restriction Enzymes

The most important group of enzymes available for the manipulation of DNA are restriction endonucleases, commonly called restriction enzymes. Before their discovery the only enzymes known to cut DNA resulted in a hopeless collection of small random fragments. The first restriction enzyme was isolated from the bacterium *Haemophilus influenzae* (Smith and Wilcox, 1970). Since that time over 200 restriction enzymes have been isolated.

Restriction enzymes recognise specific sequences in DNA molecules and cut the strands of DNA within or close to the recognition site. Four common restriction enzymes with their recognition/cutting sites are given in Table 1. The number of bases in the recognition/cutting site determines the frequency of sites in the DNA and the average fragment size from digestion with the different enzymes (Table 1). Digestion of DNA from the same individual by a specific enzyme will always give the same pattern of fragments and these fragments can be separated by a variety of methods. An additional feature of restriction enzymes is that some cut directly through both strands of the DNA and are called *blunt cutters* (*Hae* III, Table 1) wjile others cut at different places in the 2 strands (*Hha*I, Table 1) leaving *sticky* ends which are very useful when rejoining pieces of DNA.

Hybridisation

DNA consists of 2 complimentary strands held together by pairing of the nucleotides (A - T and G - C). These strands can be separated by different means such as treatment with heat or alkali and then fixed to a solid support. Once the DNA is separated to single strands and fixed, small pieces of DNA can find their matching sequence by nucleotide pairing. This process is known as hybridisation. The small pieces of purified DNA are referred to as probes or clones. Probes used for hybridisation experiments are usually labelled with radio-activity. The radio-active tag makes it possible to determine the location of the probe and its matching sequence.

Cloning

The term DNA cloning refers to the isolation and amplification of a single piece of DNA, via a carrier molecule or vector, to produce many copies of that sequence. Individual clones used for the many applications are generated by creating a recombinant DNA molecule that is replicated in a suitable bacterial host. In most cases individual clones of interest are isolated by first creating *libraries* containing large numbers of DNA sequences. Two types of DNA libraries are constructed, starting either from total genomic DNA or from messenger RNA extracted from cells.

Mammalian gene sequences include coding blocks (exons) and non-coding blocks (introns) (Fig. 1) together with sequences responsible for gene regulation. During and following the transcription of the DNA, the intron sequences are removed and the exons joined to form a mature messenger RNA (mRNA). Messenger RNA can be isolated directly,

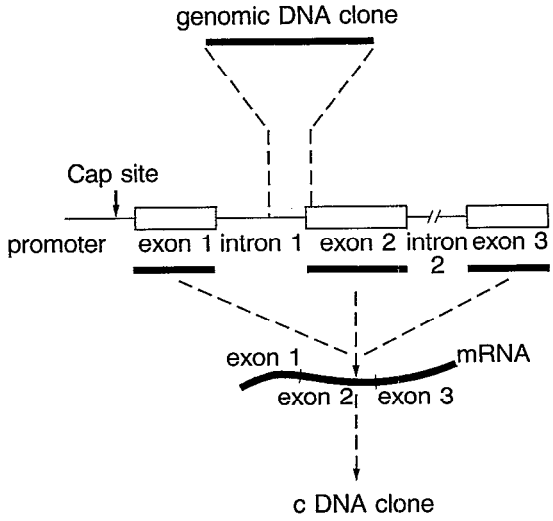

FIG. 1 Diagramatic representation of a typical mammalian gene showing the exon (coding) sequences interrupted by intron (non-coding) sequences.

TABLE 1 Recognition and cutting sites for 4 restriction endonucleases.

Enzyme	Recognition sequence	Cutting site	Average fragment size
Eco R1	–GAATTC– –CTTAAG–	G + AATTC CTTAA G	4096
Hind III	–AAGCTT– –TTCGAA–	A + AGCTT TTCGA A	4096
Hae III	—GGCC— —CCGG—	GG + CC CC GG	256
Hha I	—GCGC— —CGCG—	GCG + C C GCG	256

from an appropriate tissue. The libraries constructed by synthesising double-stranded DNA copies of mRNA populations contain only coding sequences and are referred to as copy DNA (cDNA) libraries. Libraries created from genomic DNA will contain all DNA sequences including coding, non-coding and repeat sequences and are referred to as genomic DNA libraries. Once DNA libraries are constructed specific clones of interest can be isolated making use of the technique of hybridisation. A single library can be used as the starting point for the isolation of many clones for different applications including use as probes in hybridisation experiments.

A gene may cover up to 40 kilobases (kb) of genomic DNA with the exons spread over most of the sequence. In contrast, mRNA are approximately 2 to 5 kb in length. Clones from cDNA or genomic DNA libraries will differ in the number of fragments detected by Southern blotting (see below) and in the degree of homology between species. In this discussion, the term *gene* is used loosely to refer to a DNA sequence forming at least part of the DNA sequence necessary to generate the active gene product.

DNA POLYMORPHISMS

Small inherited differences in the structure of proteins have been known for many years. They are referred to as polymorphisms, and are used increasingly in animal production (for example, the horse racing industry) to blood-type individuals and identify parents and offspring. However, the differences in protein structure must ultimately result from differences in the primary structure of genomic DNA. Moreover, the coding sequences for protein molecules represent only a small portion of the DNA. Techniques developed recently can identify polymorphisms in total genomic DNA and detect far more variation than is possible by searching for protein polymorphisms.

Restriction Fragment Length Polymorphism

Changes in the genomic DNA sequence caused by point mutations of individual bases can lead to the appearance and disappearance of restriction/cutting sequences for a given restriction enzyme. This in turn leads to changes in the sizes of fragments generated in any given region of the DNA. Variation between individuals in the patterns of fragments cut by restriction enzymes are called restriction fragment length polymorphisms or RFLPs for short.

A method described by Southern (1975) for separating and then transferring DNA fragments to a solid support is now used routinely for the detection of RFLPs (Fig. 2). The most convenient source of

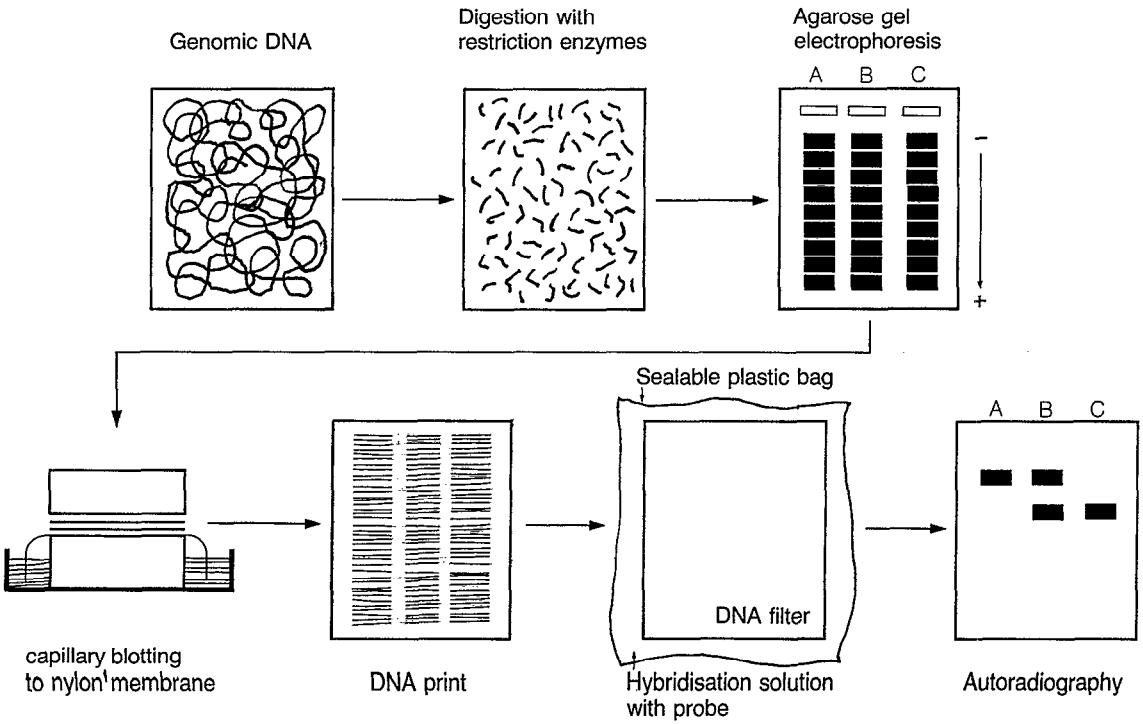

FIG. 2 Diagramatic representation of the Southern blotting procedure.

DNA is white blood cells, but DNA can be extracted from other tissue samples. The purified DNA is cut with an appropriate restriction enzyme and the fragments separated by agarose gel electrophoresis. The separated DNA is denatured then transferred and fixed to a nylon membrane. The DNA bound to the membrane support is hybridised with a DNA probe *tagged* with a radioactive label. Excess radioactive probe is removed by washing and the filter is exposed to X-ray film. The result appears as bands on the X-ray film after development.

An example (Fig. 3) shows an RFLP detected as 2 alleles of either 13 kb or 7 kb fragments after Southern blotting. The extra restriction site in the second allele results in 2 fragments instead of 1, but only 1 fragment is detected by the probe. These fragments are inherited in a simple Mendelian fashion. Members of the population can be homozygous for either the 13 kb or the 7 kb band or heterozygous so that both bands appear on an autoradiograph. Changes in the length of fragments can also result from an insertion or a deletion of a piece of DNA between the enzyme cutting sites (Wyman and White, 1980). However, the vast majority of RFLPs detected so far alter only a single restriction enzyme site and appear to be point mutations.

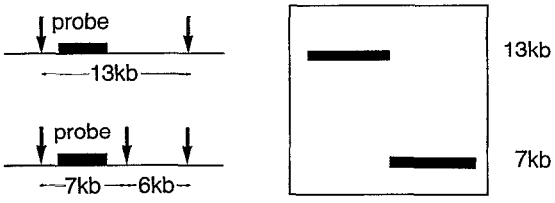

FIG. 3 Diagramatic representation of a difference in restriction fragment lengths. In the top example, the restriction enzyme sites (shown by the arrows) produce a 13 kg DNA fragment that is detected by the probe after Southern blotting. In the lower example, the additional restriction site induced by a point mutation results in 2 fragments of 7 kb and 6 kb. The probe detects only the 7 kb fragment.

DNA fingerprints

The technique of DNA fingerprinting arose from the chance observation that human DNA contains a number of highly variable regions made up of tandem repeats of a short sequence or *minisatellite*. The first such sequence was isolated by Wyman and White (1980) from a library of random segments of human DNA. Jeffreys and his colleagues at Leicester University recognised the potential for the repeat sequences (Jeffreys *et al.*, 1985 a; b) and went on to develop the method of DNA fingerprinting from basic research on a short minisatellite sequence in the human myoglobin gene.

Probes from the variable tandem repeats detect altered restriction fragment lengths due to variation between individuals in the number of multiple, short, repeated sequences of these minisatellites. The variation presumably arises from infrequent events resulting in unequal exhanges of DNA at mitosis or meiosis, or DNA slippage during replication. The resulting minisatellite length variation can be detected using any restriction enzyme that does not cut the repeat unit, providing a set of stably inherited markers that are highly polymorphic. The multiple banding patterns are in marked contrast to simple RFLP patterns derived from DNA sequences present as single copies in the genome. The extreme variability of the pattern of minisatellites detected by the repeat probes, together with the fact that bands are inherited in a Mendelian manner, provide the basis for DNA fingerprinting. The technique can identify individuals from a single blood sample and determine parents and offspring with a high degree of certainty.

SOUTHERN BLOTTING OF SHEEP DNA

RFLP analysis or DNA fingerprinting can be applied to detect DNA markers linked to any locus in the genome. In medicine, progress has been made in finding markers linked to a large number of human genetic diseases. The same techniques can be applied to livestock. DNA markers linked to important economic traits would greatly assist selection programmes where the traits are difficult to measure directly. We have recently begun a search for RFLPs in New Zealand sheep breeds with the objective of finding a marker linked to the Booroola fecundity (F) gene. Several hundred RFLPs have been published for the human genome. In contrast, only a small number of RFLPs have so far been reported for sheep (Chardon 1986).

In the initial experiments we have screened human DNA probes for homology with sheep DNA sequences. Results from a typical Southern blot from 2 human and 2 sheep samples digested with different restriction enzymes are shown in Fig. 4. The filters were hybridised with a radioactively labelled human proto-oncogene cDNA probe. The number of bands or fragments detected by any probe will depend on the nature of the probe (whether the probe is derived from a cDNA or genomic DNA clone) and on the exact location of enzyme cutting sites. In this example, there are clear bands for both human samples. The probe reveals 4 bands in the digestions with *Eco* R1 and 2 bands in the digestions with *Bam* H1.

The probe also detects sequences in sheep DNA. The hybridisation signal is not as strong for

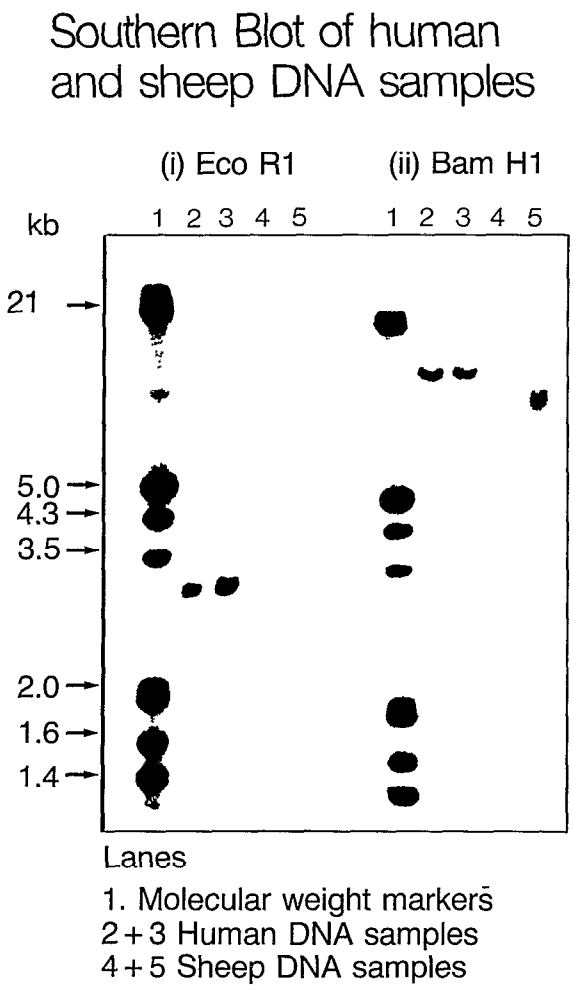

FIG. 4 Southern blots of human and sheep DNA samples.
DNA was extracted from white blood cells and digested with the appropriate restriction enzymes.
DNA fragments were separated on 0.8% agarose gels and transfered to Hybond-N (Amersham, UK) nylon membranes by capilary blotting. The membranes were hybrdidised with a ^{32}P-labelled human proto-oncogene probe, washed and exposed to X-ray film.
(i) Two human DNA samples (lanes 2 and 3) and 2 sheep DNA samples (lanes 4 and 5) were digested with the restriction enzyme *Eco* R1.
(ii) The same human samples (lanes 2 and 3) and 2 sheep DNA samples (lanes 4 and 5) were digested with the restriction enzyme *Bam* H1.
In each case land 1 shows molecular weight markers.

the sheep samples when compared to the major band with the human samples. However, the results clearly show that the sequence for the proto-oncogene c-raf is conserved between sheep and humans. We have now screened a number of human probes that show homology with sheep genomic DNA. Detecting homology is essential for the probes to be useful in our experiments, but it also provides information that can immediately be used for studies to map the sheep genome. Our objective is to find at least 1 informative probe from each of the human chromosomes to help ensure wide coverage of the sheep genome.

The c-raf proto-oncegene probe was hybridised with a filter containing samples from 9 Romney sheep and 9 Booroola Merino sheep digested with *Eco* R1. The results show a single band with a good hybridisation signal for all 18 sheep samples (Fig. 5). This probe is not *informative* for DNA samples digested with *Eco* R1 since there is no variation between the individual sheep or between the 2 breeds.

In addition to the detection of single copy genes by Southern blotting, a number of genes are members of gene families. The genes in these families may include copies of related genes that are expressed at different times during development or have evolved with different functions. Some gene families also contain *pseudo* genes; gene sequences present in the genome, but not expressed. An example of a gene family with a number of pseudo-genes and a single functional gene is cytoskeletal beta-actin. This is one of the most abundant proteins found in mammalian non-muscle cells. There are at least 20 different beta-actin sequences in the human genome (Ng *et al.*, 1985). However, there is only 1 functional beta-actin gene located on chromosome 7. The pseudo-genes are dispersed throughout the genome and 5 of the sequences have been mapped to chromosomes 5, 7, 18 and X.

Probes that detect common sequences in both the functional and pseudo-genes will give multiple bands following Southern blotting (Fig. 6). It is likely that the sequences are spread over several chromosomes in sheep and therefore, more than 1 chromosome can be screened in a single experiment with such a probe.

An example of a probe detecting polymorphic fragments with 2 alleles in Romney sheep is shown in Fig. 7. The sheep samples were digested with *Bgl* II and fragments of approximately 13.5 kb and 8.5 kb in length were detected. The example includes sheep that are both homozygous and heterozygous for either the 13.5 or 8.5 kb bands.

THE APPLICATION OF RFLPS AND DNA FINGERPRINTING

The use of RFLPs in linkage studies for mapping and

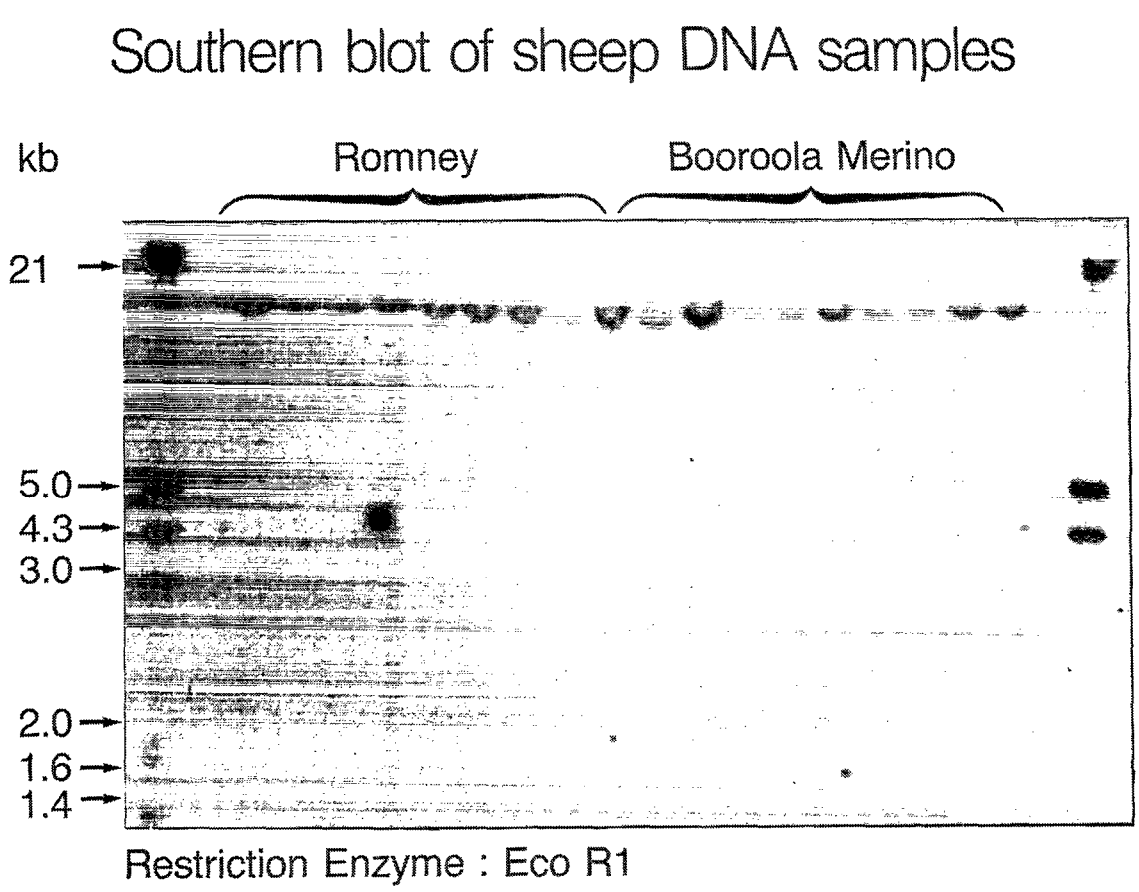

FIG. 5 A Southern blot of 18 sheep samples. Samples from 9 Romney and 9 Booroola Merino sheep (lanes 3-20) were digested with the restriction enzyme *Eco* R1. Experimental conditions and the probe used were the same as for Fig. 4.

selection for quantitative traits in livestock has been discussed now for some time (see Beckman and Soller, 1987; Kashi *et al.*, 1986). It has been estimated that rates of genetic progress in selection for milk production could be increased by 25 to 50% (Kashi *et al.*, 1986). The costs of typing sires and establishing linkage with quantitative traits would be high. The use of highly polymorphic repeat sequence probes (Jeffreys *et al.*, 1985b) linked to such traits may markedly reduce the costs and bring prospects for use of DNA markers in breeding for quantitative traits closer to realisation. However, no linkage relationships with any productive traits have yet been established. Indeed adequate evaluation and cost/benefit analyses must await more information on the frequencies of RFLPs in livestock (see below).

Distances along the chromosome are measured in units of recombination as centiMorgans (cM) representing 1% recombination. It is estimated (Gusella, 1986) that an informative DNA marker can be expected to show linkage with the disease gene if the defect lies within 10cM of the RFLP locus. Each DNA marker tested will exclude (or include) the disease gene from 20 cM. Since the entire human genome consists of approximately 3000 cM, the probability of linkage to an automsomal locus with a randomly chosen probe is 1 in 150. In humans, it has been estimated that approximately 1 base in 250 to 500 differs between any 2 chromosomes taken at random (Gusella, 1986). Information on the variation in DNA sequence between individuals in domestic animal species is not known. It may be lower than in humans, at least in some populations (Georges *et al.*, 1987), but adequate assessment must await additional information with wide coverage of the genome. RFLPs have been detected that can be applied to linkage studies in domestic species (Chardon *et al.*, 1986; Georges *et al.*, 1987).

The use of RFLPs will have most immediate application for animal breeding in finding markers for single genes that are important in animal production. The N gene, causing hairiness in Romney sheep, has

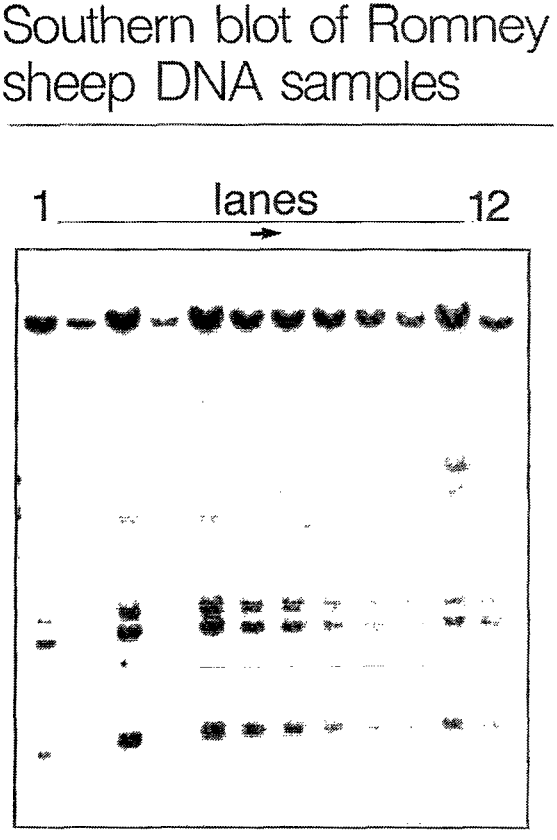

FIG. 6 A Southern blot of 12 romney samples. Samples from Romney sheep (lanes 1 - 12) were digested with the restriction enzyme *pvu* II. Experimental conditions were similar to those described in Fig. 4. The membrane was hybridised with a ^{32}P-labelled human probe detecting sequences related to cytoskeletal beta-actin.

been recognised for a long time (Dry, 1955) and exploited in the Drysdale line. In recent years the search for major genes influencing animal production has intensified. Ewes of the Booroola strain of Merino have higher ovulation rates and litter sizes due to the presence of a major gene influencing ovulation rate (Piper and Bindon, 1982; Davis *et al.*, 1982). Other major genes influencing ovulation rate may be segregating in Javanese sheep (Bradford *et al.*, 1986) and the Ruakura twinning cattle herd (C.A. Morris and A.M. Day pers. comm.). Recently, it has been suggested that there may be major genes segregating in a flock selected for high fleece weight (Hawker *et al.*, 1988) and for ovulation rate in a specially selected Romeny line at Woodlands Research Stration (Davis *et al.*, 1987). Gentic variation in milk proteins in cattle affects the processing properties of milk (Aleandri *et al.*, 1986).

Linked DNA markers are not required when the desired trait can be measured directly on individual animals at a young age. However, in the case of the Booroola F gene, measurements of ovulation rate and litter size are the only practical way of detecting carriers of the gene. The genotypes of males cannot be determined directly and the costs of progeny testing males is in the order of $20,000. A linked DNA marker would offer a far cheaper alternative for determining the genotype of carriers. RFLP analysis requires only a small blood sample and can be carried-out on newborn animals. Consequently, if a marker was located, the genotype of animals could be determined without the very long delay required waiting for females to mature or for the results of progeny tests.

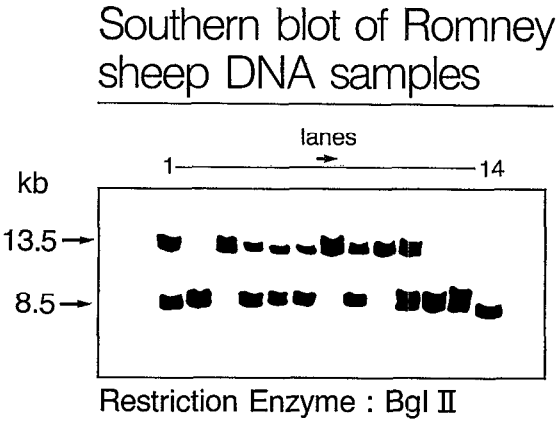

FIG. 7 A Southern blot of 14 romney samples. Samples from Romney sheep (lanes 1 - 14) were digested with the restriction enzyme *Bgl* II. Experimental conditions were similar to those described in Fig. 4. This figure shows an RFLP with the sheep being homozygous or heterozygous for a 13.5 or 8.5 kb band.

DNA polymorphisms may have an important role in the gentics of resistance to disease and parasitism. Studies in Australia (Albers *et al.*, 1987) have detected progeny from a ram with very high resistance to *H. contortus* and segregation experiments have been set-up to test for a putative major gene. Genetic variation has been demonstrated for resistance to facial eczema (C.A. Morris, pers. comm.) and footrot (Skerman, 1986) in sheep and in bloat resistance in cattle (Cockrem *et al.*, 1983). Attempts have been made to look for markers linked to disease resistance. For example, higher susceptibility to facial eczema has been demonstrated in lambs carrying a particular transferin

morph (C.A. Morris, pers. comm.). Associations have been reported between certain ovine lymphocyte antigen markers and resistance to both footrot and parasites (Outridge *et al.*, 1987).

The detection of markers linked to disease resistance would allow direct measurements on individuals without the necessity for challenging the animals with the disease in question. Closely linked markers would lower the costs of breeding programmes for disease resistance and provide benefits for animal welfare. Analysis for DNA polymorphisms should be applied to these problems because far more polymorphic sites can be detected by the use of RFLPs and repeat probes than with blood-bourne proteins.

In addition to the detection of linked markers, DNA fingerprinting can uniquely identify individuals. This has extensive application in forensic science, since sufficient DNA can be recovered from blood stains, semen, and hair roots (Gill *et al.*, 1985). DNA fingerprints can be used to assist in the identification of suspects and they have been used to confirm the relationships between mother and son in a disputed immigration case (Jeffreys *et al.*, 1985c). Fingerprinting has been used in ecology studies to identify parents and offspring providing valuable additional information for population studies (Burke and Bruford, 1987; Wetton *et al.*, 1987). There are a number of applications in animal science including direct commercial application to identify individuals and for determining parents and offspring. Fingerprinting is unlikely to replace traditional blood-typing methods for simple cases because the technique is complicated and therefore expensive. However, it will provide a very important complimentary method that can give definitive answers when traditional blood-typing cannot determine the parents. Fingerprinting can be used to confirm that twins are identical and distinguish between tissue of different origin. Examining the origin of tissue may be important for determining the origin of tissues after transplantation or in experiments on foetal and placental development and for controlling the integrity of cell lines.

CONCLUSION

The field of recominant DNA technology is moving very rapidly. The use of the techniques is firmly established in medicine and the development of new methods and commercial systems are improving the speed and reducing the costs of diagnosis. Considerable progress has been made in the integration and expression of foreign genetic material to produce *transgenic* animals. Nevertheless, the development and use of transgenic animals faces some difficult technical and commercial questions over the next few years. The use of DNA markers for the detection of important economic traits combined with traditional animal breeding does not face the same problems. In addition, it is essential to identify genes with major effects on production if the animal industries choose to use transgenic animals to increase production.

New Zealand is particularly well positioned to apply linkage studies to important production traits. The large populations of livestock are extensively recorded and there are a number of breeding programmes to help identify traits of particular interest. The challenge is to identify the most important traits for study and to find the linked markers. Once the markers are found, the genes can be identified and the physiological basis of the genetic differences determined. The Booroola F gene offers the best opportunity to look for DNA markers linked to an important economic trait at present, since the segregation of the gene is well documented and there are several independant flocks in which the gene is segregating. However, once the initial polymorphisms are described for our flocks in New Zealand these polymorphic systems can be applied to other flocks where genes of major interest are thought to be segregating.

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