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sheep, 113 in cattle (Tate *et al.* 1992; Tate *et al.* 1995; Broom *et al.* 1996; Montgomery *et al.* 1996; ML Tate, J Slate, L Skow unpublished data). Comparison of the relative order these markers in the different species has identified very few rearrangements between sheep cattle and deer. Comparison of the deer map to humans suggests that while loci from one human chromosome are often found to be adjacent in livestock the relative chromosomal order of the markers is often different. In many genome areas, these differences in gene order make it difficult to accurately translate data. However, the interspecies hybrid panel provides a resource to rapidly make further links to improve translation of data.

The concept of linking genome maps to combine information by position has been used very successfully in translating information between mice and humans (Copeland *et al.* 1993). The human and mouse gene maps are positionally linked by over 1900 genes such that when a gene is localised accurately in one species its location can be accurately translated to the other species (MGD 1997). The resource used to map most of the linking genes in mouse is an interspecies hybrid panel involving *Mus spretus* and laboratory strains. In ruminants, the deer panel is the only interspecies hybrid mapping resource currently available. Efforts to produce a hybrid cattle panel between *Bos taurus* and *Bos gaurus* and have had some success but

as yet only a low number of useful progeny have been produced (Riggs *et al.* 1997). In other animals, for example cats, interspecies hybrid resources are also being developed (Lyons and O'Brien 1997). The difficulty in generating such resources is that suitable wide interspecies hybrids are usually either non-viable or infertile (Tate *et al.* 1992; Riggs *et al.* 1997).

CONCLUSION

In humans, a very wide range of other means have been developed to physically locate genes on the chromosomes, while initially the resolution of order was not as good as the mouse interspecies hybrid map, new resources such as radiation cell hybrids (Lawrence *et al.* 1991), and yeast artificial chromosome (YAC) contigs (Chumakov *et al.* 1995) now provide the means resolve the order of conserved genes accurately in humans. These techniques offer considerable promise for use in livestock mapping, but as has been demonstrated in the mouse, are complementary rather than a substitute for an interspecies hybrid panel. Our aim is to construct a detailed comparative map of livestock genome using the deer interspecies hybrid panel, and to integrate this with the sheep linkage map and physical mapping resources such as sheep YAC contigs.

Positional cloning to identify genes

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ABSTRACT

Target genes, identified by their phenotypic effect, can be located by positional cloning. Current genetic maps in livestock species lack the necessary detail for direct application in these projects. Fine maps of each target region must be developed using a variety of strategies. These include chromosome specific libraries and comparative mapping. A physical map of the region is generated by identifying overlapping clones containing large DNA inserts. All genes from the region are sought and candidate genes identified. The resources being developed in livestock projects, including the search for the Booroola (*FecB*) locus, and complete expression maps for the human genome will greatly facilitate future positional cloning projects in livestock.

Keywords: positional cloning; sheep; Booroola; mapping.

INTRODUCTION

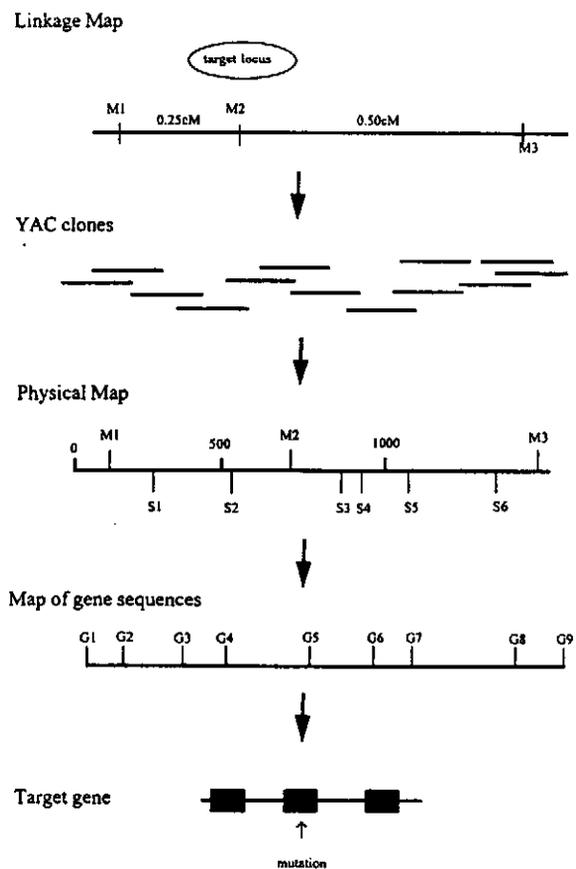
Molecular methods provide tools to identify the genetic basis of phenotypic traits. The first step is to map a trait to a chromosomal region. Using this positional

information, the next step is to identify the gene responsible for the trait. This process is known as positional cloning.

The precise location of the target locus must be defined using detailed linkage and physical maps (Fig. 1). The target locus should be less than 1 cM from the linked

markers and ideally a positional cloning project would begin from markers that show absolute linkage. The general relationship between linkage and physical distance for livestock species has not been determined, but 1 cM probably represents 1-2 million base pairs. To identify the gene and mutation causing the phenotypic difference, expressed genes from within this region must be sought and tested (Fig. 1).

FIGURE 1: Schematic diagram of the steps in positional cloning.



The genetic maps in livestock species lack the necessary detail for direct application in positional cloning projects. Instead, maps of specific regions must be developed for the traits of interest. In practice, refining the location of the target locus is an iterative process. New markers are developed using a variety of methods and the linkage and physical maps of the region refined. Any potential candidate genes in the area are immediately tested. This paper describes approaches to positional cloning in livestock species including examples from our search for the Booroola fecundity gene (*FecB*) located on sheep chromosome 6 (Montgomery *et al.*, 1994).

Developing Fine Maps of Specific Chromosomal Regions

Fine maps of highly polymorphic markers must be developed around the target region. Methods are available for developing chromosome specific markers (see Silver, 1995). We have applied a number of these in our search

for the Booroola locus. One approach is the development of genomic libraries from somatic cell hybrids containing specific chromosomes (Lumsden *et al.*, 1996). Somatic cell hybrids provide good quantities of starting material for generating libraries. However, the libraries include the genome of the host cell line and may include other chromosomal fragments in addition to the target chromosome (Lumsden *et al.*, 1996). Chromosome specific libraries can also be developed from flow sorted chromosomes. Recently, sheep chromosomes were isolated using a fluorescence activated cell sorter (FACS; Burkin *et al.*, 1997) and these are suitable for many applications including chromosome specific libraries.

Both approaches have limitations. Only a small subset of clones will yield polymorphic markers. A rapid method for screening clones to the target region is essential for both polymorphic markers and monomorphic sequence tagged sites (STS). A common method is the use of radiation hybrid panels with the genome broken into small fragments contained within somatic cell hybrids. However, radiation hybrid panels are not available for the sheep.

Comparative Genome Mapping

Comparative genome mapping across species provides a rich source of information and reagents for positional cloning projects in livestock. Mammalian species essentially have the same genes and differences between species result from differences in the timing and pattern of gene expression. There is now good evidence for conservation of the location and the general order of genes in sheep when compared with a wide range of mammalian species (Broom *et al.*, 1996; Crawford *et al.*, 1995; Lord *et al.*, 1996; Montgomery *et al.*, 1995^b; O'Brien *et al.*, 1993). The greatest degree of conservation occurs between closely related species. Anonymous microsatellite (Type II) markers from cattle amplify polymorphic loci in sheep and extensive use was made of cattle markers to develop the sheep linkage map (Crawford *et al.*, 1995). The maps show a high degree of conservation of both marker order and distance (Crawford *et al.*, 1995).

High density genetic maps have been developed in both the human and the mouse. These allow rapid and precise location of phenotypic traits. Anonymous DNA (Type II) markers from the human and the mouse do not amplify polymorphic sequences in sheep. However, actual gene sequences are conserved and provide the basis for the comparison of genetic maps from more distantly related species. Gene sequences can be used to develop genetic markers directly as restriction fragment length polymorphisms (RFLPs; Montgomery *et al.*, 1995^a), single stranded conformational polymorphisms (SSCP; Penty *et al.*, 1995), or conserved microsatellite sequences (Moore *et al.*, 1991). Alternatively, genes in the region can be used as the starting point for physical mapping using large insert libraries (see below) generating further linked markers.

Comparative approaches offer rapid and cost effective methods to develop fine maps in a particular chromosomal region. Markers from closely related species can

often be used directly. Maps from more distantly related species provide information on genes within the target region. These can be assessed as possible candidate genes for the phenotypic effect and can be used as the starting point to provide additional markers in the region.

Large Insert Genomic Libraries

Large insert genomic libraries are useful at a number of stages in positional cloning projects and are essential for building the physical map of the target region. Arrayed libraries can be easily screened by the polymerase chain reaction (PCR). Clones containing candidate genes together with the immediate flanking regions can be readily identified. Once a marker has been tightly linked to the phenotypic trait, the entire region can be covered by a series of overlapping clones known as a contig. New methods such as amplified fragment length polymorphisms (AFLP; Vos *et al.*, 1995) provide powerful ways of building the contig without the need for a complete set of STS markers in the region and are being applied in sheep. The contig is the physical map of the region and provides physical distances between markers. In addition, the contig provides the starting material for positional cloning of the gene responsible for a particular trait.

The first vector developed for cloning large DNA fragments was the yeast artificial chromosome (YAC; Burke *et al.*, 1987). More recently, cloning systems based on the bacteriophage P1 (PAC; Pierce & Sternberg, 1992) and bacterial artificial chromosomes (BAC; Shizuyu *et al.*, 1992) have been developed. Compared with YACs, smaller DNA fragments are carried in the PAC and BAC vectors, but these are more stable with fewer rearrangements.

A YAC library was developed for sheep in 1994 (Broom & Hill, 1994) and we have used this extensively in our studies. The library is gridded into microtitre plates and clones pooled in 3 dimensions to provide a rapid method for screening the library by PCR. A proportion of clones are chimeric (contain DNA from two or more chromosomes). This can be tested by direct hybridisation of YAC clones to sheep chromosome spreads using fluorescence *in situ* hybridisation (FISH; Tebbutt *et al.*, 1996).

YAC clones identified from genes, thought to be within the target region from comparative mapping, can be used to identify microsatellite markers in the region. The yeast genome does contain microsatellites. Consequently, one method that has been useful is based on PCR of common fragments using species specific repeats (eg SINE sequences). Consensus PCR primers from SINE sequences amplify sheep specific DNA from the YACs.

A Genetic Map of Expressed Sequences

The purposes of detailed physical and linkage maps are to precisely locate the target locus and identify all the expressed genes in the region so the target gene can be identified. Starting from the YAC contig, there are a number of approaches that can be used to find expressed genes. These include direct selection of cDNA clones

from libraries by hybridisation, exon trapping, and cloning of fragments around CpG islands. Good summaries of these approaches are available (see Silver, 1995). All of the methods are technically difficult and time consuming. However, improved methods are making these approaches easier.

In practice, few genes have been cloned using these methods and most positional cloning projects have succeeded by finding mutations within candidate genes in the defined region, or using rare chromosomal translocations. Detailed maps of expressed genes are emerging from the large cDNA sequencing projects. Companies and publicly funded laboratories have sequenced large numbers cDNAs from the human and other species (Hillier *et al.*, 1996; Schuler *et al.*, 1996). These are known as expressed sequence tags (ESTs) and more than 600,000 sequences are now deposited in public databases. The next step of mapping these ESTs is facilitated by the radiation hybrid and YAC contig mapping resources available for the human. This powerful resource will make candidate positional cloning far easier in the future.

We are fortunate that there is a complete YAC map for human chromosome 4. This is the region syntenic to sheep chromosome 6 carrying the Booroola locus. We have identified the equivalent region from comparative mapping analysis (Lord *et al.*, 1996). Human ESTs from the region were screened using sheep genomic DNA. Based on sequence information, 13% of PCR primers amplified the orthologous gene from sheep suggesting that alternative methods will be required to clone all the sheep genes within a target region. A large number of ESTs are being mapped into the region and orthologous sheep genes will be cloned from the critical region.

CONCLUSION

Target genes, identified by their phenotypic effect, can be located by positional cloning. The steps are difficult, and in livestock species, current mapping resources mean that positional cloning projects proceed by iterative steps refining the linkage and physical maps before proceeding to the direct search for the gene. The techniques and resources being developed in current searches for mutations responsible for a number of phenotypic effects in both sheep and cattle will make the task easier in the future. Most successful projects in other species have employed the positional candidate approach. Once the location of the gene is known, obvious candidate genes can be identified and screened at any stage of the project. The development of complete expression maps for the human genome will greatly facilitate this approach in the future. Genome conservation means that these maps will also benefit livestock positional cloning projects. Genes responsible for quantitative trait loci will be more difficult because the location can not be accurately defined. These will most likely be identified through positional candidate gene approaches.