THE BOVINE GENE MAP:
A TOOL FOR COMPARATIVE CANDIDATE POSITIONAL CLONING

EL MAPA GÉNICO BOVINO:
UNA HERRAMIENTA PARA EL CLONAJE POSICIONAL COMPARATIVO

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SUMMARY

The bovine genome map includes a synteny map, a linkage map and at least one *in situ* hybridization for each chromosome. Well over 1000 markers are placed on at least three published linkage maps. These markers are now being used to generate maps of economic trail *loci* (ETL) at an accelerating pace. The question of how to identify genes responsible for mapped ETL in cattle (and other livestock species) is a formidable one. One proposed approach is through comparative candidate positional cloning, in which candidate genes from the human and mouse maps are obtained from the bovine comparative map. It has become apparent that identification of conserved synteny between species is insufficient for comparative candidate positional cloning due to rearrangements of gene order that are abundant within conserved syntenic groups. To this end, our laboratory and others are ordering comparative mapping markers (Type I *loci*) on the bovine map. Examples of gene order on bovine chromosomes 1, 7 and 19 are presented to illustrate the orientation of and rearrangement within regions of conserved synteny in cattle, humans and mice. Ongoing experiments to further resolve gene order include an interspecific hybrid backcross and radiation hybrid somatic cell analysis.

RESUMEN

El mapa del genoma bovino incluye un mapa sinténico, un mapa de ligamiento y al menos uno de hibridación *in situ* para cada cromosoma. Bastante más de 1000 marcadores han sido localizados en al menos tres mapas de ligamiento publicados. Esos marcadores están siendo empleados ahora a un ritmo acelerado para generar mapas de *loci* de trascendencia económica (ETL). La cuestión de identificar los genes responsables de los ETL incluidos en el mapa del bovino (y de otras especies ganaderas) es formidable. Una propuesta para abordarla consiste en el clonaje de los candidatos posicionales comparativos, mediante el cual, se obtienen genes candidatos de los mapas del hombre y el ratón, a partir del mapa comparativo bovino. Esto ha puesto de manifiesto que la identificación de la sintenia conservada entre especies, es insuficiente para el clonaje del candidato posicional comparativo, a causa de los reordenamientos del orden de los genes que son abundantes dentro de los grupos de sintenia conservados. A este fin, nuestro laboratorio, y otros, se encuentran ordenando los marcadores para la elaboración de mapas comparativos (*loci* tipo I) en el mapa bovino. Para ilustrar la orientación y el reordenamiento dentro de regiones de sintenia conservada en bovinos, humanos y ratón, se presen-

tan ejemplos del orden de los genes en los cromosomas bovinos 1, 7 y 19. Los experimentos en marcha, para finalmente aclarar el orden de los genes, incluyen un retrocruzamiento híbrido interespecífico y análisis por radiación de células somáticas híbridas.

INTRODUCTION

The rapid development of genome maps in cattle and other livestock species has been driven by a variety of motivating factors. My personal interest in mapping cattle genes was initially to better understand chromosomal evolution. The organization of extant genomes and their relationship to each other provides valuable insight into how ancestral genomes may have been organized and how changes in chromosome structure may have accompanied mammalian evolution. Comparative gene mapping, which is simply the mapping of homologous genes in multiple species, provides important information about chromosomal evolution between distant species that is not available even from today's most advanced cytogenetic technologies. All mammals have essentially the same amount of DNA in their genomes and a very similar complement of structural genes. Chromosome numbers, on the other hand, differ greatly even within some mammalian orders, and banding pattern divergence suggests that this homologous mammalian genome has undergone a variety of reciprocal chromosomal exchanges and internal rearrangements as taxa have diverged. Consequently, a motivating force in animal genome mapping is to address questions regarding chromosomal rearrangements in

animal evolution and to thereby advance the fundamental biological knowledge of the species of interest and animals in general. Comparative gene mapping has therefore become a major tool for the study of chromosomal evolution in animals. The internationally funded and highly organized human genome initiative has already provided the standard mammalian genomic map to which all others will ultimately be compared. The genome map of the laboratory mouse continues to develop rapidly and will undoubtedly be the first measure of comparison for evaluating mammalian chromosomal conservation and genomic evolution. Other mammals, including the livestock species in general and catde in particular, have extremely important roles to play, however, in helping to understand the pathways of chromosomal rearrangement that have accompanied mammalian evolution. It has become obvious through comparative mapping to date that some mammals such as cattle and cats have genomes that are more highly conserved relative to the human genome than is the most often compared mouse genome. These more highly conserved genomes probably most accurately reflect the chromosomal arrangement of the ancestral mammal. At the minimum they demonstrate that the total picture of mammalian chromosome evolution cannot be fully represented by differences in the genomes of humans and mice. Continued comparative mapping including the genomes of cattle and other domestic animals will continue to make a valuable contribution to understanding mammalian chromosomal evolution in a universal context.

The potential for marker-assisted

selection (MAS) of desirable and marketable traits drives gene mapping in many laboratories and provides the motivation for funding of animal genomic research in several countries including the U.S. Genetic markers of advantageous alleles for economic trait *loci* (ETL), including quantitative trait *loci* (QTL), have the potential to enhance the rate and efficiency of genetic gain through selective breeding, a concept advanced long before the technical tools become available for its implementation (Soller and Beckmann, 1982; Weller *et al.*, 1990; Smith and Simpson, 1986). The best markers for use in selective breeding programs are obviously the genes actually responsible for the important traits. While such markers are still rare, a few have been identified by thorough searches for variation in genes believed to be involved in the physiological pathways leading to the phenotype of interest. This so-called *candidate gene* approach to marker identification requires a sound funda-mental knowledge of physiology underlying the trait followed by extensive and usually expensive screening for variation in candidate ge-nes related to these processes. Ideally, sequence variation related to the trait can ultimately be incorporated directly into the marker assay. An excellent example of a successful candidate gene search is provided by Shuster *et al.* (1992) who identified the genetic defect responsible for leukocyte adhesion deficiency (LAD) in Holstein cattle as a missense mutation coding amino acid 128 in CD18. The mutant and normal alleles could then be distinguished by the polymerase chain reaction (PCR)

providing the ideal genetic marker of this economic trait *locus*.

Unfortunately, physiological bases for many economic traits remain unresolved and candidate genes are not obvious. Or, even when the physiology is understood, the complexity of the trait may present a long and cumbersome list of candidate genes. ETL, even QTL, can be mapped by linkage analysis, however (Lander and Botstein, 1989; Paterson *et al.*, 1989; Georges *et al.*, 1993a; Georges *et al.*, 1993b; Andersson, *et al.*, 1994; Georges *et al.*, 1995). Markers mapped in close proximity to ETL can be used to assist in selection for the ETL if the recombination frequency (map distance) is sufficiently small and the chromosomal phase of marker and ETL alleles is known. Efficiency of MAS can be increased by identifying markers on either side of the ETL since recombination in the region spanned by two markers can be detected. A major early goal of cattle gene mapping was therefore to produce maps of highly polymorphic markers spaced at intervals of approximately 20 cM (lcM = 1 p. cent recombination) or less across every chromosome. These markers could then be available for mapping studies in families segregating QTL, hopefully resulting the linkage associations of the QTL with one or more markers. Under appropriate breeding protocols, linked markers can then be used for MAS.

A third, and probably the most important, goal of gene mapping is to identify and clone genes responsible for ETL. It is obvious from the discussion above that MAS is much more efficient when done with variation in the gene actually responsible for the ETL. More

importantly, a complete understanding of the potential interaction of the trait with other physiological processes is possible only when the genes involved are known. The term *reverse genetics* has been replaced in common usage by *positional cloning* or *map based cloning* to describe the process whereby the application of map information is used to clone a gene responsible for a specific trait in the absence of information about the biochemical or molecular basis of the trait. Success of positional cloning has been experienced a number of times now in searches for human disease genes, perhaps best exemplified by the cloning of the gene for cystic fibrosis (Rommens *et al.*, 1989). While the task of positionally cloning genes in any species is a formidable one, cloning genes for ETL in livestock is almost prohibitive. Animal maps will most certainly never be as dense as those of the human. Large insert libraries for livestock species are only beginning to be developed and used. Chromosomal deletions of economically important genes, important tools in many of the human and mouse successes, have not been identified and propagated in livestock. The task is further complicated by the quantitative nature of most of traits of economic interest in farm animals and the paucity of research support worldwide for animal agriculture relative to the human genome initiative. Alternative strategies to conventional positional cloning must be planned and developed. One proposed approach is *comparative candidate positional cloning* which takes advantage of knowledge of the evolutionary history of chromosomes and rapid advances in the human and mouse maps.

METHODS USED IN BOVINE GENOME MAPPING

A. SYNTENY MAPPING

Synteny simply means on the same strand, or in genetic terminology, on the same chromosome. A synteny map is nothing more than a list of genes that are known to reside on the same chromosome in a particular species. *Conserved synteny* was used by Nadeau (1989) to describe the location of two or more homologous genes on the same chromosome in different species. *Synteny* should not be substituted for *conserved synteny* in our comparison of maps between species. Synteny mapping is probably associated with comparative mapping because the only maps available for comparison between most animal species have heretofore been synteny maps.

Somatic cell genetics is still the most common method for building synteny maps. Hybrid somatic cells can be constructed such that the chromosomes of practically any progenitor species are preferentially lost. Each hybrid clone will retain a partial genome of that species along with the complete genome of the other which is usually a transformed rodent cell line. Since chromosome loss is more or less random, each clone will retain a different subset of chromosomes from the species being mapped. Just as in human gene mapping, analysis of pairs of genes in a panel of hybrid cell lines will reveal concordance or discordance of their retention. Concordance of retention is evidence for the location of two genes on the same chromosomes. Conversely, discordance of retention is evidence for asynteny, their location on different chromosomes.

Gene products or DNA sequences may be mapped by synteny analysis in any species so long as the presence or absence of the gene or gene product of the targeted species can be ascertained against the fully retained rodent genomic background. Enzyme electrophoresis, Southern blotting with unique sequence probes, and PCR amplification with species discriminating primers have all been effective analytical tools for synteny mapping.

Somatic cell genetics does not typically result in the assignment of markers to specific chromosomal sites or even to chromosomal sub-regions. Consequently, genes on a synteny map are usually not ordered. Somatic cell methods employing rearranged chromosomes are an exception to this generalization and have been used very effectively to order genes in the human map.

Radiation hybrid mapping (Cox *et al.*, 1990; Walter *et al.*, 1994) has recently become an important tool for constructing high resolution maps of human chromosomes. The techniques employed are variations of basic somatic cell genetics in which the donor cells have been irradiated to achieve chromosome fragmentation. Statistical analysis is based on the principles of linkage analysis, i.e. the closer two *loci* are to each other the less likely they are to be separated by random chromosomal rearrangement. First used by Goss and Harris (1975), the technique can be used with single chromosome hybrids as the irradiated donor (Cox *et al.*, 1990) or with total genome irradiation in a diploid donor cell (Walter *et al.*, 1994). Whether used in mapping a single chromosome or a whole genome, the technology is effective for constructing contiguous maps of mammalian chromosomes at a 500 kb level of resolution. This method may prove to be the ideal approach to comparative gene mapping since it provides an ordered map without the requirement of segregating polymorphisms in breeding populations.

B. *IN SITU* HYBRIDIZATION

Unique DNA sequences, repetitive elements, and whole genomes have all been effectively localized to chromosomal sites by *in situ* hybridization. This technique employs the attachment of a microscopically detectable marker to a DNA probe followed by hybridization of the probe to denatured DNA of an otherwise intact chromosome. The specificity of hybridization is determined by the uniqueness of the probe. While radioactive probes dominated the early application of this technology, fluorescent probes are now generally used. In her review of fluorescence *in situ* hybridization (FISH) Trask (1991) notes the following advantages of FISH over isotopic labeling.

It provides superior spatial resolution and usually requires the visualization of fewer labeled chromosomes. It is faster and the probe employed is generally more stable. The sensitivities are similar, each requiring a few kilobase (kb) pairs of uninterrupted sequence as the hybridizing probe. Schemes have been developed which allow multiple probes with different color signals to be used on the same chromosomes. The latter is particularly important for gene mapping because it provides the potential for ordering *loci* within the limits of

approximately 100 kb resolution.

Cosmids, cloning vectors that accommodate large DNA sequences (up to 45 kb), have been effectively utilized as probes for FISH. Since these large inserts often contain repetitive DNA, the target DNA must first be pre-hybridized with unlabeled total genomic DNA. This method has been effectively used to anchor linkage maps to chromosomes by hybridizing cosmids that contain highly polymorphic markers used in linkage mapping.

The above technologies result in physical maps which describe the physical relationships of *loci* to the chromosomes on which they reside. Higher resolution physical maps which define markers in contiguous clones (contig maps) are forthcoming in cattle but will most likely span small genomic regions of special interest rather than whole chromosomes as is targeted in the human genome initiative and is prerequisite to total genome sequencing.

C. LINKAGE MAPPING

Linkage maps are defined in biological rather than physical terms, a map unit representing one percent recombination in meiosis. Since linkage is measurable only in gametic products, linkage mapping requires detection of maternal and paternal alleles in gametes of hetero-zygous individuals; thus, polymorphism is a requirement for linkage mapping. A map is then made from the percentage of recombinants in the parental arrange-ments of alleles of two *loci* on a chromosome. The segregation of three or more *loci* permits ordering of genes on the map since double recombinants are rare relative to single recombinants.

Markers for mapping purposes have been catagorized as Type I or II by O'Brien (1982). Type I markers are expressed sequences (genes) and are usually conserved from one mammalian species to another. Thus, they are the favored material for comparative gene mapping.

Unfortunately they are usually not highly polymorphic and therefore difficult to incorporate into linkage maps. Type II markers are highly polymorphic and are more widely used for linkage mapping. The value of these markers lies in their high level of polymorphism. They are not necessarily expressed ge-nes but often anonymous stretches of DNA.

STATUS OF THE BOVINE GENOME MAP

A. PHYSICAL MAPPING

More than 400 Type I *loci* have been mapped in cattle (Fries *et al.*, 1993; O'Brien *et al.*, 1993) primarily through somatic cell genetics (Womack and Moll, 1986; Arruga *et al.*, 1992). While these synteny mapped markers indicate the boundaries of chromosomal conserva-tion relative to the map rich genomes of mice and humans, they provide an incomplete comparative map. Conser-vation or rearrangement of gene order is not addressed by synteny maps.

In situ hybridization, especially FISH has been used effectively to address the order of Type I *loci*, to assign syntenic groups to specific chromosomes, and to anchor the rapidly growing linkage map to chromosomes (Fries *et al.*, 1993; Solinas-Toldo *et al.*, 1993; Iannuzzi *et*

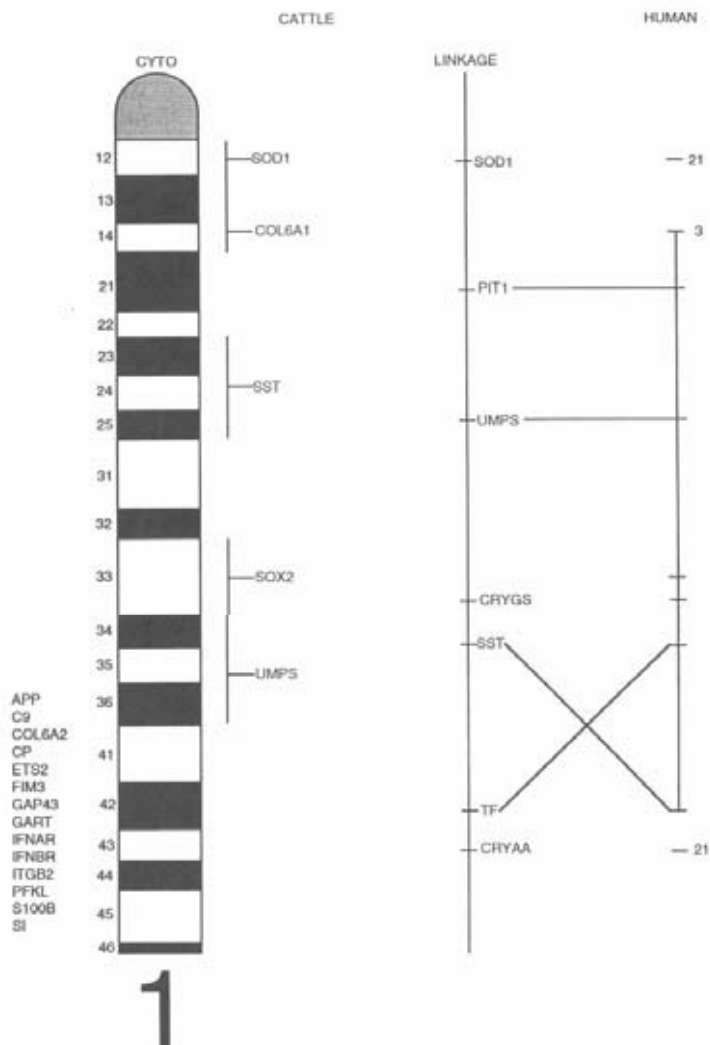# BOVINE GENE MAP: COMPARATIVE CANDIDATE POSITIONAL CLONING

Figure 1. Cytogenetic and linkage map of Type I loci on cattle chromosome 1. Rearrangement of gene order is illustrated in the region of conserved synteny with human chromosome 3. (Mapa citogenético y de ligamiento de los loci de tipo I en el cromosoma bovino 1. La redisposición del orden de los genes se ilustra en la región de sintenia conservada con el cromosoma humano 3).

al., 1993; Gallagher et al., 1993). There are presently more than 50 in situ localizations of unique sequences on cattle chromosomes. All bovine syntenic groups are now assigned to specific chromosomes and the bovine linkage map is physically anchored at 35 sites on 26 chromosomes.

B. LINKAGE MAPPING

The published bovine linkage map of 200 markers (Barendse et al., 1994) has
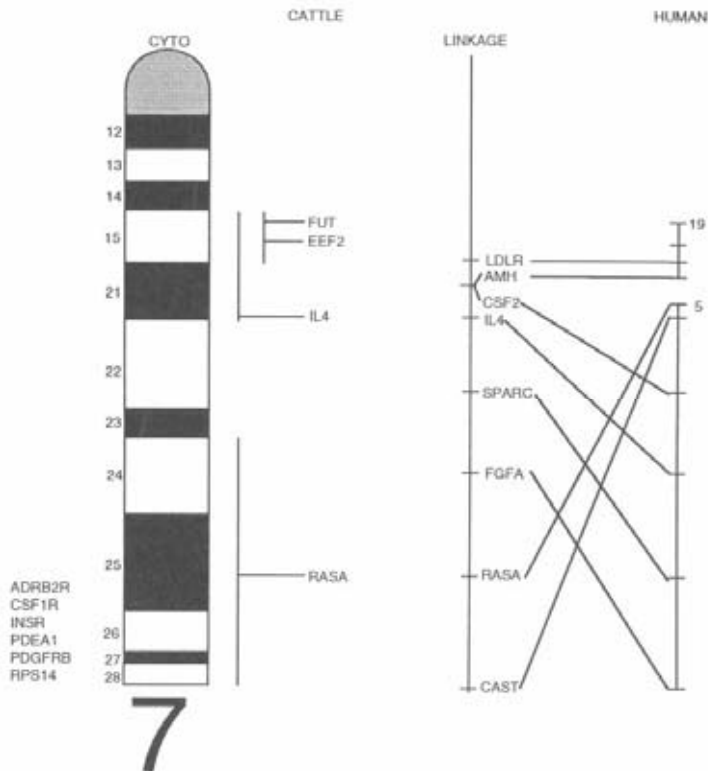

Figure 2. Cytogenetic and linkage map of Type I loci on cattle chromosome 7, including data from a bovine interspecific backcross (Gao and Womack, submitted). Rearrangement within conserved synteny on human chromosome 5 is illustrated. (Mapas citogenético y de ligamiento de los loci de tipo I en el cromosoma bovino 7, incluyendo datos de un retrocruzamiento bovino interespecífico (Gao and Womack, enviado para publicación). Se ilustra el reordenamiento dentro de la sintenia conservada del cromosoma humano 5).

grown to almost 1000 markers since its initial publication (Barendse, personal communication). This was made possible in large measure by international cooperation and the use of a common set of reference families. Combined with the independent development of other maps (Bishop *et al.*, 1994) there are probably more than 1200 markers presently assigned to cattle linkage groups. The goal of 20 cM resolution has clearly been achieved over at least 95 p. cent of the total genome. The international map includes 120 type I markers.

C. COMPARATIVE MAPPING

Approximately 400 *loci* have been mapped in both cattle and humans. Most of these have also been mapped in mice. While extensive conservation of synteny has been observed between cattle and humans (Womack and Moll, 1986; Threadgill *et al.*, 1991), conservation of linkage (conservation of gene order) may not be so prevalent. Barendse *et al.* (1994) incorporated a sufficient number of Type I *loci* into the linkage map to demonstrate the presence of several rearrangements of gene order within conserved syntenies. Such rearrangements are illustrated in a comparative map of bovine (BTA) chromosome 1 **(figure 1)**. Segments of human (HSA) 21 homology have been identified at either end of the bovine linkage map and the order of CRYGS, SST and TF appears to be rearranged. It must also be noted, however, that there is not yet agreement as to gene order on the BTA 1 linkage and cytological maps. An interspecific hybrid backcross was used to generate the maps of bovine chromosomes 7 and 19 illustrated as **figures 2** and **3**. Gene

order rearrangement within conserved synteny on BTA 7/HSA 5 was demonstrated (Gao and Womack, submitted) and the breakpoint in segment homology was identified to the small region between bovine AMH and CSF2. A similar pattern was revealed on bovine chromosome 19 (Yang and Womack, submitted). HSA 17 and BTA 19 are completely conserved syntenic groups yet the linear order of genes has been rearranged. These data support the need for ordered comparative maps to facilitate the extrapolation of candidate genes for bovine traits from the human gene map.

To make the process of comparative gene mapping in a variety of mammals a more efficient process, O'Brien *et al.* (1993) proposed a list of comparative mapping anchor *loci* (CMAL). These 321 Type I markers include the designated anchor *loci* for human and mouse maps and span the human genome at approximately 10 cM intervals. Concerted efforts to map homologues of these *loci*, by synteny, cytogenetic and linkage approaches, will rapidly advance comparative mapping in *map poor* species and facilitate the use of the growing human and mouse database for animal improvement.

A recent major breakthrough in comparative gene mapping is heterologous chromosomal painting or ZOO-FISH painting. Solinas-Toldo *et al.* (1995) and Hayes (1995) have *painted* cattle chromosomes with human chromosome specific libraries to delineate segments of homology. These studies define the boundaries of chromosomal conservation at the *human on cattle* cytogenetic level and are thus far highly consistent with the results of

comparative synteny mapping which defines *cattle on human* homology. Like synteny mapping they do not address conservation of gene order in homologous segments.

D. ECONOMIC TRAIT MAPPING

A growing number of traits of economic significance are beginning to appear on the bovine genome map. In addition to the previously discussed bovine LAD trait (Schuster *et al.*, 1992; Threadgill *et al.*, 1991), uridine monophosphate synthetase deficiency (UMPS) has also been mapped to a specific site on chromosome 1 (Schwenger *et al.*, 1993; Ryan *et al.*, 1994). BoLA was shown to be associated with susceptibility to leukemia virus infection (Lewin *et al.*, 1988). Georges *et al.* (1993a) have found linkage of the polled *locus* to microsatellites on
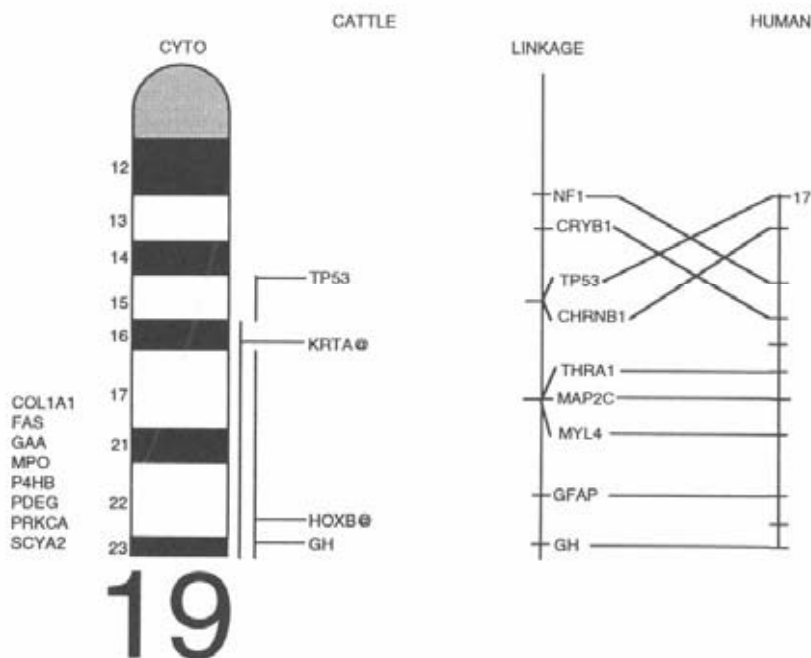

Figure 3. Cytogenetic and linkage map of Type I loci in cattle chromosome 19, including data from a bovine interspecific backcross (Yang and Womack, submitted). Rearrangement within conserved synteny on human chromosome 17 is illustrated. (Mapas citogenético y de ligamiento de los *loci* de tipo 1 en el cromosoma bovino 19, incluyendo datos de un retrocruzamiento bovino interespecífico (Yang and Womack, enviado para publicación). Se ilustra el reordenamiento dentro del grupo de sintenia conservada del cromosoma humano 17).

chromosome 1. The weaver disease maps to markers on chromosome 4 (Georges *et al.*, 1993b) and has the added interest of being associated with a quantitative trait for improved milk production. Variation around the prolatin gene on chromosome 23 (Cowan *et al.*, 1990) is related to milk production in some Holstein sire families and Georges *et al.* (1995) have used mapped microsatellites to locate an additional five QTL for milk production. The number of ETL on the cattle genome map is rapidly expanding.

FUTURE DIRECTIONS

The large number of mapped markers which now exist in cattle provide extensive genome coverage for mapping ETL in families segregating the traits. Unfortunately, each ETL usually requires a unique segregating family. Such resource families are generally expensive to develop and maintain. Nonetheless, the families are an integral and necessary step in the ultimate application of the gene map to economic improvement and must be developed. The mapping of ETL to 10-20 cM regions of a chromosome will likely be followed by high resolution mapping in an effort to ultimately identify and clone the responsible genes. Chromosome specific libraries are being developed to aid this process. Animal breeders should not and will not be satisfied with ETL-marker distance of ten or so centimorgans.

The next major step, identifying and cloning ETL, is a formidable one. The high density linkage maps, numerous chromosomal deletions, and large insert contigs that have contributed greatly to

positional cloning of human disease *loci* are simply not available for animal ETL cloning. It is unlikely that this wealth of resources will ever be available for cattle. Positional cloning of human genes is rapidly shifting toward the positional candidate (Collins, 1995) approach, however, which relies more on the availability of a pool of expressed genes mapped to the same chromosomal regions as the disease gene and less on walking and jumping from a linked marker. In cattle as in humans, the three step process for positional candidate cloning of the gene for an important trait will be

1) to localize the trait *locus* to a chromosomal subregion,

2) to search available databases for reasonable candidate genes, and

3) to test candidate genes for variation correlated with phenotype.

Obviously, step 2 is unrealistic in cattle where currently only 400 of the 70,000 or so genes are even assigned to chromosomes. This step was almost as unrealistic in humans until very recently when several international initiatives targeted the large scale mapping of expressed sequence tags (ESTs). The success of these efforts suggest that more than half of the human transcripts could possibly be mapped by the end of 1996 (Collins, 1995). Thus, the key to bovine ETL cloning may be through comparative genome databases which translate a 10 centimorgan bovine segment into its human counterpart, then search for human ESTs in that segment with attractive features relative to the bovine phenotype. Assuming 20 or so potential candidate genes per centimorgan, 200 genes or ESTs will comprise the total candidate pool. Such a

comparative positional candidate cloning strategy provides hope for ETL cloning that is not apparent with the conventional strategies of map based cloning of human disease genes.

Systematic use of human EST databases for identification of animal genes responsible for ETL will require comparative maps with greater precision than those currently available.

Identification of the boundaries of conserved synteny is not sufficient. We must continue to identify the internal rearrangements that have accompanied mammalian chromosomal evolution and have resulted in rearrangement of gene order within these boundaries of conserved synteny.

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