MOLECULAR APPROACHES FOR EMBRYO SEXING

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SUMMARY

Molecular biology has revolutionized the ability to detect genetic differences between individuals, including their sex. We have been analysing a 20 kb region of the Y chromosome from cattle. Within this segment are a number of targets that can be amplified in a PCR reaction, thereby revealing the sex of the cells from which the DNA was extracted. Routinely, cells biopsied from embryos are sexed using an assay that includes in one reaction mixture a set of Y-specific oligonucleotide probes and a set of autosomal probes. These autosomal probes generate a PCR product from both male and female DNA, therefore providing an internal control to verify that the assay was successful. This region of the Y chromosome contains male-specific repetitive elements which are interspersed with a highly repetitive SINE sequence (short interspersed elements) that is scattered throughout the genome and typically found in the 5' and 3' regions flanking expressed genes. DNA analyses, whether for embryo sexing or for characterisation of specific alleles, have the potential to aid in selection of individual animals, although the cost of the manipulations required may still be too high for routine applications.

INTRODUCTION

For centuries genetic differences between individuals in a population have been identified by unique heritable phenotypic traits. During the past two decades advances in molecular biology, notably the discovery of restriction enzymes, development of nucleic acid hybridization and sequencing techniques, and the automated polymerase chain reaction (PCR), have permitted these differences to be detected at the molecular level. Mutations in the DNA that lead to a novel phenotype can now be pin-pointed. In addition, more subtle variations in the genome occurring in non-expressed spacer regions can be detected using appropriate markers, and their correlation with phenotypic variation measured. Messenger RNA and proteins, the products of genes, can also be detected with increasing sensitivity, permitting assessment of individual differences in gene activity. For the breeder, molecular markers and probes that target specific DNA sequences expand the amount of genetic data about an individual in a population. For those interested in developmental or later events also important to livestock production and utilization, molecular analyses provide insights into the role of specific genes, and increasingly to understanding the action of mutations in those genes.

One conspicuous genetic difference between individuals is sex. The ability to know in advance the sex of an embryo, or to select the desired sex, can have consequences for herd management or breeding programs. One of the best examples is in the dairy industry, where replacement females are always needed. Since only 50% of conceptions yield female calves, there may be unnecessary costs incurred in maintaining recipient animals that are carrying male calves. There has therefore been considerable motivation to develop methods for identifying the sex of cattle embryos.

Several methods have been used successfully for embryo sexing. One direct approach is a cytogenetic test. Embryo cells are obtained by biopsy, cultured in colcemid to reveal the chromosomes, and examined microscopically for the presence of a Y chromosome. This has been successful, particularly with older embryos (day 12 to 15) from which larger numbers of cells can be removed (Betteridge et al., 1981). The procedure must be completed quickly

because embryos at that stage cannot be frozen. Younger embryos (morula/blastocyst) that can be frozen can also be biopsied, but because fewer cells are obtained it is more difficult to detect mitoses useful for cytogenetic analysis. Generally, about two-thirds of the embryos biopsied can be sexed cytogenetically.

A second method is based on the use of sperm that has been fractionated by flow cytometry into X-bearing and Y-bearing fractions. The desired sex is then obtained by routine artificial insemination or *in vitro* fertilization with the appropriate fraction. This has been successful with rabbits, swine, sheep, and recently cattle (Johnson, 1992; Johnson et al., 1994). This method has the advantage in that it is non-invasive to the embryo, and if the procedures for fractionating sperm can be simplified for application to many donor bulls the method might become the most economical because it could be linked directly with AI.

Another non-invasive procedure that requires further research but holds promise is based on immunological detection of sex-specific antigens on or within the embryonal cells. The prototypical example is the male-predominant H-Y antigen. Antibodies directed against this antigen have been used to distinguish male mice, porcine, ovine, and bovine embryos (White et al., 1982; Wachtel, 1984; Anderson, 1987). The method is currently somewhat subjective, relying upon the trained eye to interpret the immunofluorescence involved, and the accuracy rates for embryos that survive the treatment with the antibody and necessary detection reagents are in the range of 80%. Methods that do not require fluorescence, and rely simply on the ability of the antibody to retard the develoment of male embryos until the antibody is removed, have been used successfully for a number of species including cattle (Utsumi et al., 1993).

Finally, embryos from a number of species have been sexed using DNA probes to detect the Y chromosome. These probes are typically used in a PCR assay, and can be carried out with only a few cells obtained by embryo biopsy (e.g. Herr et al., 1989; Miller, 1991). Many different probes have been isolated, and of those biopsies that can be sexed the success rates are typically very high, in excess of 95%. This high success rate, the minimal biopsy required, and the speed of PCR assays has enabled this method to achieve some commercial success.

We have been interested in developing a reliable PCR-based assay for sexing cattle embryos, and, in a broader context, in the organization of DNA sequences on the bovine Y chromosome. This paper describes the approach that we have taken to sex embryos, and presents information about a region of the Y chromosome that we have cloned and are currently analysing. This region contains both male-specific and ubiquitous repetitive elements, and sequence data show that these ubiquitous elements are related to repetitive sequences frequently found associated with transcribed genes on autosomes.

MATERIALS AND METHODS

Many laboratories have isolated short DNA fragments from the bovine Y chromosome. We have taken one of these and used it as a starting point to examine a larger region flanking its location in the chromosome. Miller and Koopman (1990) isolated two male-specific fragments. One of these, BOV97M, was 157 bp long, and shown by Southern blot analysis to lie within a 5 kbp *Eco*RI fragment. There was also evidence that the sequence was present twice, within a 1 kbp sub-region, and that the two copies could be separated by digestion with the enzyme *Hinc*II.

Primers were made that allowed us to amplify the BOV97M region from genomic DNA, and this fragment was then radioactively labelled and used to screen a bovine male genomic library constructed in the cloning vector λZAP. A clone was identified that carried a 20 kb BamHI fragment. Restriction enzyme mapping identified, as expected from the work of Miller and Koopman, a 5 kbp *Eco*RI fragment spanning the BOV97M sequence. From within the 20

kb piece we isolated several *Bgl*II restriction fragments, subcloned them into plasmids and obtained complete or partial sequence data for each of them.

The sequence data enabled us to design a number of oligonucleotide probes that could be used in PCR assays to amplify DNA from male cells. Typically, pairs of 25- or 26-mers were synthesized and used in PCR reactions consisting of 45 amplification cycles, and annealing temperatures of 62 to 65 °C.

A number of probes for autosomal sequences were also selected. Male genomic DNA was digested with *Bam*HI and cloned into the plasmid vector pTZ18U. Clones were randomly picked, and the inserts in them radioactively labelled and hybridized to male and female genomic DNA. Probes that hybridized equivalently to both were assumed to be autosomal sequences, and several were sequenced. Oligonucleotide probes specific for these sequences were then made for use in PCR assays.

All Y-specific and autosomal sequences were compared to the Genbank and EMBL nucleic acid databases, to identify homologies to previously reported sequences.

RESULTS AND DISCUSSION

Isolation and testing of Y-specific probes

The segment of the Y chromosome that we have isolated is shown in Figure 1. Three different regions, Y1, Y2 and Y3, within this segment were sequenced. Oligonucleotide

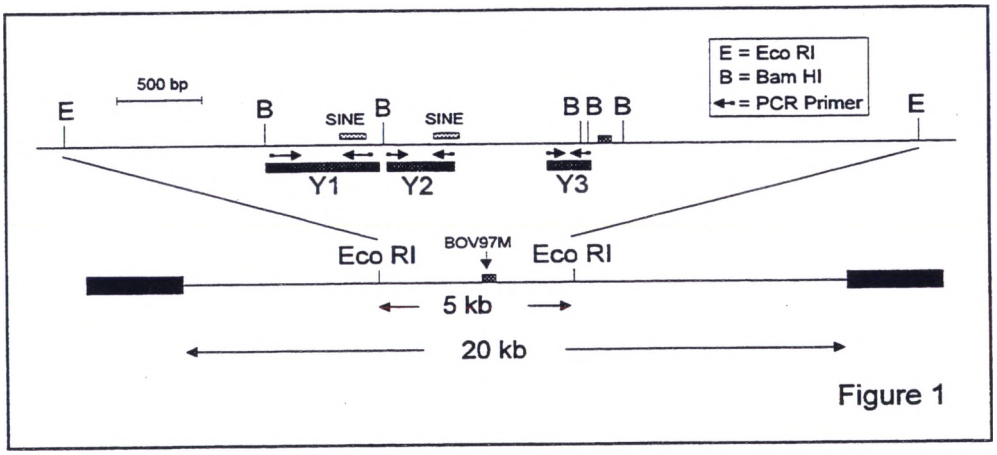

Figure 1

probes specific for sequences in each of them were used in PCR assays to test their ability to serve as male-specific probes. Several autosomal probes were also used. The PCR reactions were initially carried out using DNA purified from known male and female tissues. In each PCR reaction two sets of primers were included, one specific for the Y chromosome and one for an autosomal site. The probes were selected so that the size of the amplified autosomal DNA fragment was less than that of the male specific product. After the PCR cycles, the samples were run on an agarose gel and stained with ethidium bromide. The presence of two bands was diagnostic of male DNA, while one band from the autosomal probes indicated a female (Figure 2). All three of the Y chromosome regions successfully identified male DNA, as did oligonucleotides specific for the original BOV97M sequence. It was found that for different Y chromosome probes the optimal autosomal probe was different, perhaps because of differences in the efficiency with which different sequences amplify.

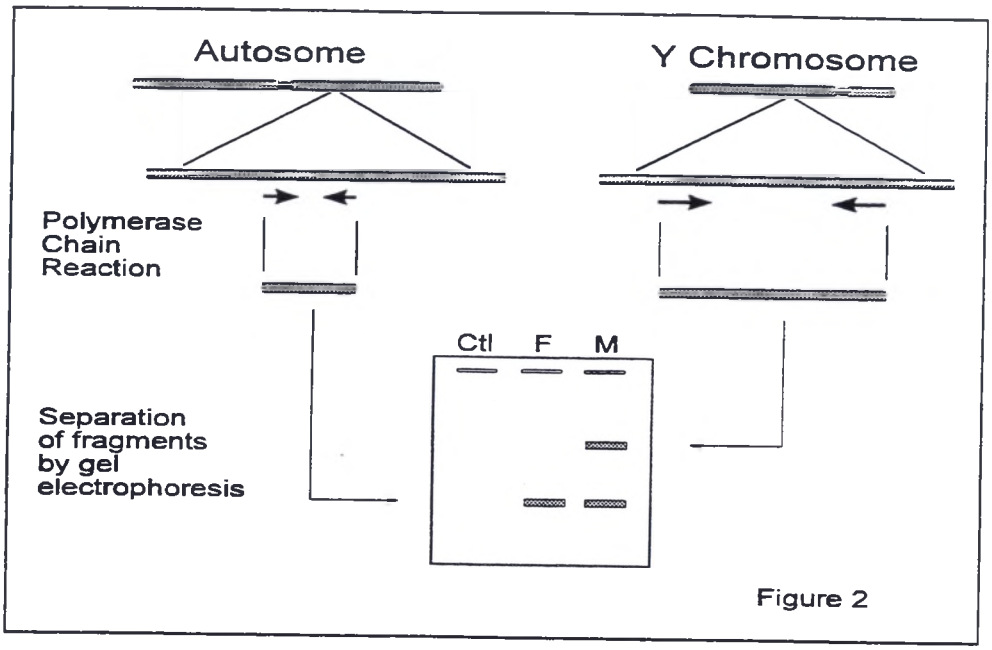

Figure 2

The probes were then used in several field trials with biopsied and transferred embryos. This work was carried out in collaboration with Dr. Keith Betteridge, Dr. Stanley Leibo and Dr. Walter Johnson of the University of Guelph and Dr. Bob Stubbings of Semex Canada. Embryos and recipient animals were obtained from either the Elora Dairy Research Center or the United Breeders of Canada. A total of 142 biopsies were taken, and of these 137 could be sexed with the DNA probes. Of these 137, 19 yielded faint signals and so were identified as male or female with caution. Thus, 118/142 (83%) were confidently sexed, but in total 137/142 (96%) received a diagnosis. From the group of 142 embryos, 95 were transferred to recipient females, yielding 33 pregnancies (33/95, or 35%) that resulted in 33 calves. Of these calves, one from the group of 19 difficult samples was incorrectly diagnosed, while the sex of the remainder was as predicted from the PCR assay. Included in these 33 calves were 5 sets of identical twins.

Thus, the assay was found to be highly reliable. In each of the field trials, the biopsies were taken, their sex determined, and the transfer of the embryo to a recipient carried out in one day wherever possible. There were some instances, however, when embryos had to be transported over long distances or the number of biopsies to be taken was large, when the PCR assays were run in the afternoon and the embryos transferred early the following morning.

Sequence analysis of the Y specific and autosomal probes

The regions of the Y chromosome that were sequenced, as indicated in Figure 1, were compared to known databases. They were not identical to any published sequences, but did contain stretches of homology to highly repetitive SINE sequences in the bovine genome. The locations of SINE sequences we identified in the sequenced region are also shown in Figure

1. SINE sequences are generally 120 to 150 base pairs long, and are thought to be derived from genes that are transcribed by RNA polymerase III, such as the transfer RNA genes. Presumably they arise when transcripts from these genes become reverse transcribed into DNA and then integrate back into the genome. It is estimated that as much as 10% of the bovine genome may be made up of these elements. They do not encode proteins, but show up frequently in introns or untranslated flanking regions of genes. Their function, if any, is not known. Mammalian Y chromosomes do not contain large numbers of expressed genes, and SINE elements may be allowed to accumulate on that chromosome because there is not strong selective pressure against them. Although the bovine SINE element is not related in sequence to the primate *Alu*I repeat, its origin and dispersion throughout the genome are thought to be similar.

The SINE elements in the region of the Y chromosome we are analysing appear to have inserted into Y-specific regions. This was shown with the PCR primers specific for Y2. One of them lies within a SINE element, and could presumably therefore have a large number of targets in the genome. The other flanks this SINE sequence and confers male specificity to the amplification.

The sequences in the vicinity of Y2 and Y3 appear to be unique, since PCR amplifications generate primarily one band. Y1 on the other hand is amplified by probes that have multiple male-specific targets, since PCR reactions generate a ladder of bands varying in length from several hundred base pairs to 2 kb. The size of the sequence amplified from Y1 specific primers shown in Figure 1 is 695 bp. In support of the observation that the Y1 PCR primers target repetitive elements, we find that a single primer, the one specific for the right-hand side of Y1 in Figure 1, can generate a male-specific band in a PCR reaction. The utility of a single primer is that the reagent components of the PCR reaction are simplified. The repetitive element within Y1 is probably unrelated to the original BOV97M sequence, since sequence comparisons reveal no obvious homology between the two. Perret et al (1990) have described a polymorphic repetitive element on the bovine Y chromosome, but like BOV97M it shows no obvious sequence similarity to any of the sequences within Y1 either.

We have also sequenced different autosomal probes that we are using. Of three randomly picked autosomal clones, two contain SINE elements and one has no similarity to known sequences. One of the clones with a SINE element was fortuitously found to be a fragment from the 3' flanking region of the α-S1-casein gene. It is likely that during the construction of a library many randomly picked clones with an insert size of several kb or more will contain a SINE sequence. In designing primers for the autosomal sequences care was taken to avoid using primers that target the SINE.

We are currently assessing whether the repetitive element contained within Y1 is polymorphic between breeds or among individuals of the same breed.

Cost of PCR based diagnoses in relation to embryo transfer

Embryo sexing by PCR is currently gaining some momentum commercially, through groups such as AB Technology in the United States, for example, which markets a probe developed in Australia. Embryo sexing is, however, only one of many potential applications of PCR-based genetic diagnosis. For any application that requires assessment of an embryo, there are many additional technical problems; embryo flushing, biopsy, culture, and transfer, and usually cryopreservation, are essential components of the procedure. Such problems do not exist if PCR is being used to study DNA polymorphisms in semen or blood samples.

Much of the limitation in using embryos comes from the fact that only 60 to 70 % survival rates are observed following embryo transfer (ET), and this can drop to 40% if the embryo has been biopsied or biopsied and frozen. For embryo sexing by PCR to be commercially valuable to the average producer, the cost of ET must be factored in. Figure 3

shows the effects of embryo survival on a producer's costs to obtain a female calf. As embryo survival rates increase, fewer embryos are needed. Embryo sexing can reduce by one half the number of embryos needed, but at a cost. The upper graph shows some estimates, based on a cost of $200 for embryo flush and transfer and $75 for embryo sexing. The dotted line indicates the current market value of a female calf, estimated at $250.

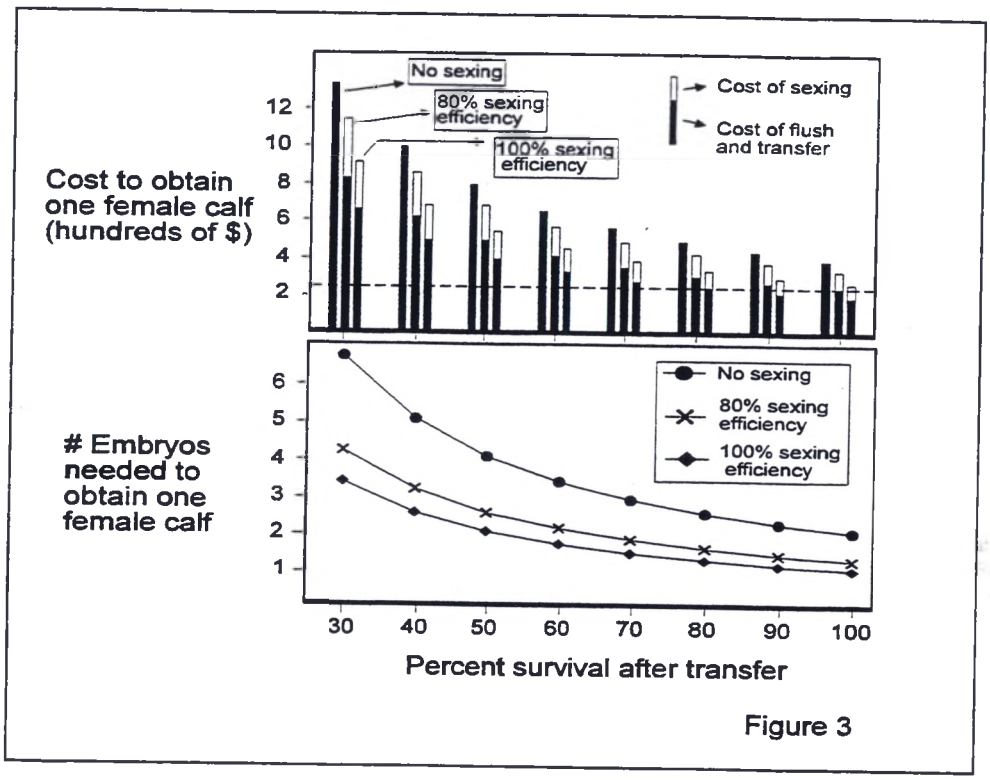

Figure 3

The most apparent conclusion from these graphs is that until we can reduce costs and/or boost survival rates the cost of ET exceeds the value of the product. The second conclusion is that the cost savings gained by embryo sexing are lost when the survival rate drops from 60%. As seen in the lower panel, with current survival rates the number of embryos required to produce a female calf when sexing is not used approximates four, the same number required when sexing is used and resulting survival rates are lower. There are of course some qualifications to this simplified cost comparison, such as the fact that a male calf is not without some value (ca. $100), and different farmers may make different uses of their recipient animals. Our experience is that while PCR sexing is accurate, approaching 100% of diagnosed embryos, the real efficiency may be between 80 and 90%, since some biopsies may be lost, or for some other reason not respond to PCR amplification. If one applies a conservative figure of 80%, the cost curves are pushed further up. Depending upon the operation, the costs of recipient animals must of course also be considered. Nevertheless, cursory assessment of the costs demonstrates that ET and embryo sexing are still expensive.

There should be room for lower costs. Both the cost of ET and the cost of sexing are variables that might be optimized. Improvements in methods of cryopreservation and embryo handling might enhance survival rates, particularly among manipulated embryos. Second, the cost of sexing drops as the number of samples to be assayed increases. One individual can process twenty or thirty samples by PCR almost as quickly as five or ten samples, so the cost/embryo could be lowered with increased demand. Third, non-invasive methods of sexing, either with sex-sorted sperm or immunological methods, could remove the problem of lower survival rates after ET. Finally, even at existing costs the procedures become worthwhile if the value of the calf is greater, as with elite breeding stocks.

CONCLUSION

Cattle embryos can be reliably sexed using Y chromosome-specific DNA probes in a PCR reaction. Broader applications of PCR diagnostics of embryos may be limited by the cost considerations, but might nevertheless have an important niche in animal breeding, particularly if the cost and efficiency of ET can be lowered.

Molecular techniques should continue to yield other types of information about the genomes of important species, particularly about polymorphisms that might be of use to quantitative geneticists as markers. In addition, current work on the molecular biology of domestic species adds to existing fundamental knowledge about mammalian genome structure and gene function. This knowledge will certainly be of importance if the molecular events regulating economically important traits are to be fully understood.

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