

CHARACTERIZATION OF A PORCINE CHROMOSOME 6 SPECIFIC LIBRARY

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SUMMARY

A swine chromosome 6 specific library was generated using size fractionated DNA isolated from chromosomes sorted by flow cytometry. Procedures have been established in our laboratory to reproducibly prepare high quality chromosomes from PHA stimulated swine peripheral blood lymphocytes and to sort individual chromosomes after staining with Hoechst 33258 and chromomycin A3. DNA from 1,000 isolated chromosome 6 was then *Sau3A* digested, cloned into pBluescript SK II+, and PCR amplified; 300-600bp DNA fragments were isolated from the amplified DNA and cloned to produce the chromosome 6 specific library. Our chromosome 6 library has already yielded 17 clones containing (CA)_n repeats and is now being screened for more (CA)_n microsatellite repeats as well as other repeat sequences. New microsatellite markers on chromosome 6 will be used to develop a more detailed map of the region where the genes that encode positive carcass traits and Porcine Stress Syndrome (PSS) are found.

INTRODUCTION

Because of the extensive use of pigs both as a food animal and as a model in biomedical research, it has moved into the forefront for developing genome maps in domestic animals. Coordinated efforts are now aimed at isolating new genetic markers to measure the inheritance of quantitative trait loci (QTL) associated with major production traits. One type of genetic marker that has recently enhanced these efforts is microsatellite sequences, since microsatellite sequences are distributed randomly and abundantly throughout the porcine genome (Johansson *et al.*, 1992; Wintero *et al.*, 1992; Andersson *et al.*, 1993; Echard *et al.*, 1993; Ellergen *et al.*, 1993; Fredholm *et al.*, 1993; Rohrer *et al.*, 1994).

The gene responsible for Porcine Stress Syndrome (PSS), *ryr1*, the ryanodine receptor (or CRC, calcium release channel), is one locus that is associated with quantitative traits in swine (Fujii *et al.*, 1991; Otsu *et al.*, 1991). In addition to an increased likelihood of sudden death in response to stress, the inheritance of PSS is also known to affect a number of production traits such as superior lean meat content, ham content, loin eye muscle area, and decreased back fat depth compared to other breeds (Webb *et al.*, 1982, 1985; Jensen and Barton-Gade, 1985). The nature of the genes responsible for the positive carcass traits demonstrated in PSS positive swine remains to be identified, but it seems likely that the favorable carcass traits are the result of one or more different genes closely linked to the PSS gene. The PSS gene has been physically mapped to chromosome 6 (6p11->q21) (Davies *et al.*, 1988; Harbitz *et al.*, 1990). The linkage map of this region is not fully defined (Andersson *et al.*, 1993), although many new markers have recently been added (Rohrer *et al.*, 1994). Therefore, the development of a more detailed genetic linkage map of the region around the PSS gene will aid in the separation of the negative traits associated with the PSS gene from the gene(s) responsible for the positive carcass traits.

A method that has contributed to the pig genome mapping efforts is flow cytogenetics (Schmitz *et al.*, 1992; Langford *et al.*, 1992, 1993; Yerle *et al.*, 1993). The flow sorting of specific chromosomes has proven useful for the production of chromosome specific libraries, and such libraries have now been produced in pigs (Miller *et al.*, 1992). We report here the production of a chromosome 6 library using flow sorted chromosome 6 material to use for the detection of new microsatellite markers and to develop a more detailed linkage map of the chromosome 6 region around the PSS gene.

MATERIALS AND METHODS

Porcine peripheral blood lymphocytes are isolated from blood drawn aseptically from our NIH minipigs. Lymphocytes are separated by Dextran sedimentation followed by the density gradient, Ficoll/Hypaque, and cultured at 2.5×10^6 cells/ml in RPMI containing 5ug/ml PHA-M, 10% fetal bovine serum, 2mM L-glutamine, 1mM non-essential amino acids, 1mM sodium pyruvate, and 2×10^{-5} M beta-mercaptoethanol. The cells are harvested for chromosome isolation according to the polyamine protocol (Cram *et al.*, 1990). Briefly, blastogenic cells grown for 72 hours are blocked in metaphase by incubating in 0.1 ug/ml Colcemid for 20 hours. The cells are harvested and resuspended in 1 ml of 75mM KCl with 100uM spermine and 250 uM spermidine and incubated at room temperature for 45 minutes to allow for swelling. Following centrifugation, the pellet is resuspended in 1 ml of Chromosome Isolation Buffer (CIB) (75mM KCl, 7.5mM Tris-Cl, 1mM EDTA, 10mM NaCl, 250uM EGTA, 100uM spermine, 250uM spermidine, and 1mM digitonin) and the chromosomes released by vortexing for 20-50 seconds.

The chromosomes are stained for bivariate flow cytometry by incubating in chromomycin A3 (75 ug/ml) overnight at 4°C. Hoechst 33258 is then added (14 ug/ml) and the chromosomes are incubated on ice for over 1 hour before sorting. Dual color flow cytometry is performed on the Coulter Epics V FCM with guidance from Mr. Glenn Welch and Dr. Larry Johnson (Gemplasm and Gamete Physiology Laboratory, USDA, ARS, Beltsville, MD).

Following sorting of the swine chromosomes, the purity of each preparation is analyzed by primer-extension preamplification (PEP) of aliquots of 1,000 chromosomes from each sort (Zhang *et al.*, 1992). The equivalent of 100 chromosomes from the PEP reaction mixture is then tested for chromosome purity using a panel of PCR based microsatellite markers (Johansson *et al.*, 1992; Wintero *et al.*, 1992; Fredholm *et al.*, 1993; Rohrer *et al.*, 1994).

DNA from 1,000 flow sorted chromosome 6 was extracted in Tris:NaCl:EDTA (TNE) (10:80:10 mM) in the presence of 1% sarcosyl and 100 ug/ml Proteinase K at 55°C for 5 hours. The DNA was precipitated in ethanol in the presence of glycogen, and then digested with *Sau3A* in a total volume of 10 ul. The use of small volumes for the extraction step (<100 ul) and the presence of glycogen in the precipitation step proved to be vital for good DNA recovery. Following digestion, the DNA was ligated into *Bam*HI digested, alkaline phosphatase treated pBluescript SK II+ (Stratagene). The cloned DNA was then amplified by PCR using the T7 and T3 primers. The amplified DNA was digested with *Xba*I and *Pst*I and 300-600bp fragments were isolated from a low melt agarose gel. These DNA fragments were then ligated into *Xba*I/*Pst*I digested pBluescript SK II+ and used to transform DH5-alpha *E. coli*. Colony blots were screened with a [α - 32 P]dATP (ICN) labelled poly-d(AC) DNA probe (Pharmacia) and developed by autoradiography.

RESULTS

A typical flow karyotype of our preparation of swine chromosomes is shown in Figure 1. The largest swine chromosomes, 1 and 13, are readily separable from the smaller chromosomes, and chromosome 6 is also easily identifiable for sorting. Several sorts of chromosome 6 have been performed, along with sorts of almost every other swine chromosome.

Several chromosome 6 specific libraries were developed as outlined in Materials and Methods; our best results were obtained following the down-scaling of reaction volumes. These libraries were then screened for (CA)_n repeats. On average, the libraries contained a total of 10,000 recombinant clones. To date, approximately 5,000 clones have been screened, and of these, 17 were detected as positives with the poly-d(AC) probe and verified by a secondary screening procedure. These clones are currently being sequenced for analysis of their repeats. Further rounds of screening are being performed on the libraries for the detection of more (CA)_n repeat containing clones, in addition to several other repeat sequences including (AT)₈, (GTG)₅,

(TCC)₆, and (GGAT)₄ (gifts from Dr. Charles Louis, U. of Minnesota). After comparison of the sequences of these new microsatellites with published data, unique microsatellites will be analyzed by one of the current computer programs so that specific primers can be designed to flank the repeat sequence. They will then be tested by PCR and mapped for chromosome specificity using reference population DNA.

All of the (CA)_n repeat analyses were performed by two different screening techniques. The 17 positive clones were detected only after using ³²P labelled probes. Other rounds of library screening were also performed using a biotinylated (GT)₁₅ oligonucleotide probe. Side-by-side comparisons of the chemiluminescence development versus autoradiography showed that only the ³²P labelled probe was sensitive enough for detection of the positive clones.

DISCUSSION

Our laboratory is currently analyzing a swine chromosome 6 specific library for the presence of various microsatellite repeat sequences. To date, our efforts have isolated 17 (CA)_n repeat containing clones. Following the verification of new microsatellite repeat sequences, these microsatellites will be checked for chromosome 6 specificity by PCR analysis. The detection of any new microsatellites will allow for the swine chromosome 6 linkage map to be further defined. The majority of the markers that have been mapped to chromosome 6 in the pig are within one linkage group (L6) which maps to 6p11->q25 (Andersson *et al.*, 1993; Echard *et al.*, 1993; Rohrer *et al.*, 1994). Our goal is to generate more chromosome 6 markers for the regions of this chromosome that have associated QTL, namely around the PSS gene.

The development of new genetic markers will allow us to perform detailed mapping studies. Resource families of pigs are being developed at the University of Minnesota that have been characterized for their carcass traits as well as their inheritance of the PSS defective allele. Genomic DNA is being saved from every animal so that these families can be analyzed in respect to their inheritance of positive carcass traits and the PSS allele.

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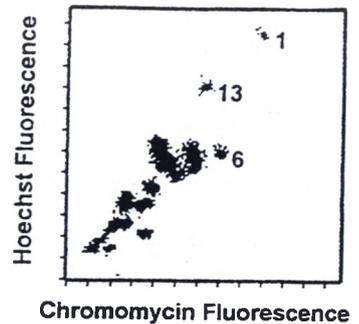


FIG. 1. Bivariate flow karyotype of the male swine chromosomes. The location of chromosome 6 allows it to be easily gated for sorting.

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