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Development of a bovine X chromosome linkage group and painting probes to assess cattle, sheep, and goat X chromosome segment homologies

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ABSTRACT **The X chromosome linkage group is conserved in placental mammals. However, X chromosome morphological differences, due to internal chromosome rearrangements, exist among mammalian species. We have developed bovine chromosome painting probes for Xp and Xq to assess segment homologies between the submetacentric bovine X chromosome and the acrocentric sheep and goat X chromosomes. These painting probes and their corresponding DNA libraries were developed by chromosome micromanipulation, DNA micropurification, microcloning, and PCR amplification. The bovine Xp painting probe identified an interstitially located homologous segment in the sheep and goat Xq region, most probably resulting from chromosome inversion. Ten type II (microsatellite) markers obtained from the bovine Xq library and five other X chromosome assigned, but unlinked, markers were used to generate a linkage map for Xq spanning 89.4 centimorgans. The chromosome painting probes and molecular markers generated in this study would be useful for comparative mapping and tracing of internal X chromosome rearrangements in all ruminant species and would contribute to the understanding of mammalian sex chromosome evolution.**

The human gene mapping effort is accelerating the development of genetic maps for domestic animals in general (1) and for farm animals in particular (2–9). Development of genetic maps for farm animals pursues two important objectives: production of a high-resolution linkage map of highly polymorphic markers to identify regions of the genome containing loci of economic importance (10) and provide information for comparative mapping and evolutionary analysis (11). The first objective focuses on generation of a map based on identification of highly polymorphic DNA sites (type II loci; minisatellites and microsatellites), while the second focuses on mapping coding sequences or type I loci, whose sequences are more conserved across mammalian species (1, 11, 12).

The most current bovine genetic map lists >500 genetic markers ordered in 30 linkage groups and assigned to 24 autosomes and the X and Y chromosomes (3, 4, 9). Over 100 loci have been assigned by either *in situ* hybridization to specific chromosomes or somatic cell hybridization (3).

Generation of a high-resolution, comprehensive bovine map would be accelerated if chromosome-specific DNA libraries were available. Comparative mapping and evolutionary analysis would also be facilitated by generation of chromosome-specific painting probes. However, the generation of such libraries is hampered by the lack of detailed cytogenetic characterizations of bovine somatic cell hybrid panels and the relatively small DNA content differences among successive bovine chromosomes, which limits flow sorting (13). We therefore investigated the usefulness of generating chromosome-specific libraries and chromosome painting probes based on micromanipulation, microcloning, and PCR amplification of individual chromosomes or chromosome arms in contrast to an original strategy directed at cloning specific bands or chromosomal regions (14, 15).

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MATERIALS AND METHODS

Preparation of Metaphase Spreads. Metaphase spreads were prepared from lymphoid cells as described (16). Harvested cells for fluorescence *in situ* hybridization (FISH) analysis were fixed according to DiBerardino *et al.* (16). Metaphase spreads used for chromosome scraping were fixed with a series of methanol/acetic acid solutions at ratios of 9:1, 5:1, and 3:1 for 5 min each before mounting on ice-cold wet coverslips. After air drying, coverslips were stored at −20°C until used.

X Chromosome Cloning. Ten copies of bovine chromosome X, Xp, or Xq were scraped from dry Giemsa-stained metaphases using microneedles. Our estimated yield for X, Xp, and Xq was 1.0, 0.3, and 0.7 pg of DNA, respectively. The estimated yield was based on 3.2 pg of DNA per haploid mammalian genome, size of the X chromosome, and the Xp/Xq ratio (13). All DNA handling and cloning procedures were carried out in nanoliter volumes as described (15). Chromosomal DNA was cut with *Sau*3AI and ligated to adaptor molecules that provide a priming site 24 nucleotides long for PCR amplification.

***Sau*3AI Adaptor.** The strategy outlined by Saunders *et al.* (15) was used. However, our adaptor molecule was prepared by constructing two synthetic oligonucleotides: a 28-mer (5′-GATCTCCTGTGTGATATTGAATTCCGCT-3′) and a 24-mer (5′-AGCGGAATTCAATATCACACAGGA-3′). After phosphorylation of the 28-mer oligonucleotide, equimolar amounts of both oligonucleotides were annealed, ethanol precipitated, vacuum dried, resuspended in 2× ligase buffer (Promega), aliquoted, and stored at −20°C until used. The PCR amplification primer is complementary to the 28-mer oligo and extends from the 5′ *Sau*3AI sequence site to an internal 3′ *Eco*RI site (5′-GAATTCAATATCACACAG-GAGATC-3′).

This construct offers the advantage of efficient sticky end ligation of *Sau*3AI chromosomal fragments to the *Sau*3AI adaptor sites. Therefore, an adaptor molecule flanks each of the chromosomal fragments. The PCR primer sequence does

Abbreviations: FISH, fluorescence *in situ* hybridization; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine B isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; lod, logarithm of odds; cM, centimorgans.

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not recognize any specific target sequence in the bovine genome and under our PCR cycling conditions does not self amplify. After ligation and before PCR amplification, adaptor dimers were cleaved with *Bgl* II, a restriction site that is generated by the ligated products.

Chromosome Painting. Chromosomal specificity and extent of chromosome coverage of the resulting amplified chromosomal inserts was demonstrated by FISH (17, 18). Chromosome mixtures were labeled with either biotin-16-dUTP or digoxigenin-11-dUTP by PCR. Before hybridization, DNA repetitive sequences were blocked by annealing the denatured chromosome mixture probe to an excess of bovine competitor DNA (17).

Microscopy. Slides were screened under a microscope (Zeiss, Axioskop) equipped for epifluorescence microscopy. Propidium iodide/tetramethylrhodamine B isothiocyanate (TRITC) staining, 4′,6-diamidino-2-phenylindole (DAPI), and fluorescein isothiocyanate (FITC) signals were detected with 546/590, 365/480, and 450/520 excitation/barrier filter combinations, respectively. A SIT 66 video camera, attached to the microscope, was utilized to capture images digitized by an Image 1/AT software (Universal Imaging, Media, PA). Images were stored in 44-MB Bernoulli disk cartridges (Iomega, South Roy, UT). Photomicrographs of digitized images were prepared with a color video printer (Sony, model UP5000).

X Chromosome Region-Specific DNA Libraries. The original PCR amplification of the chromosomal fragments generated ≈3 μg of amplified DNA. Fifty percent of the original amplification was ethanol precipitated in the presence of glycogen and digested with *Sau*3AI to completion. After digestion, adaptor molecules were separated by passing the reaction mixture through a Microcon-30 column according to the manufacturer's instructions (Amicon). Chromosomal inserts were ligated to the dephosphorylated *Bam*HI site of the λ ZAP express vector (Stratagene). Equimolar insert/vector amounts were used to reduce the proportion of plaques with insert concatemers. Library cloning efficiencies were 1.8×10^7 and 1.2×10^8 plaque-forming units (pfu)·μg^{-1} for Xp and Xq, respectively. These libraries represent approximately three chromosome arm equivalents.

Screening of λ ZAP Express Library and Excision of Phagemid. Approximately 3×10^4 plaques of the Xq-specific DNA library were plated and screened with radiolabeled $(GT)_8$ probe at 60°C. Strongly positive clones from this primary screen were selected for secondary screening at higher stringency (65°C). Positive λ clones containing a CA/GT microsatellite(s) were plaque purified and a high titer stock was prepared for each clone. *Escherichia coli* cells (XL1-Blue-MRF′) were coinfected with bacteriophage containing a positive clone and the ExAssist helper phage (Stratagene) according to the manufacturer's instructions. Phagemids were used to infect *E. coli* XLOLR and plated directly onto LB/kanamycin plates. Colonies were selected and plasmid DNA was isolated and used directly in double-stranded sequencing (19). The presence of T7 and T3 promoters flanking opposite sides of the multipurpose cloning site allowed rapid sequencing of small (300–700 bp) clones from opposite directions using T7 and T3 sequencing primers.

Linkage Mapping. Linkage data were obtained on the USDA–Meat Animal Research Center reference population (4, 20). All genotypes were independently scored, checked for errors, and entered into an interactive data base as described (21). Markers were placed into linkage groups based on two point logarithm of odds (lod) (>3.0) scores and ordered within groups by multipoint linkage analysis (CRI-MAP 2.4; ref. 22) following a procedure similar to that of Lander *et al.* (23) and Bishop *et al.* (4). The single interval >20 centimorgans (cM) (20.9 cM) was tested for significance by comparing the lod of the initial analysis with the lod obtained holding the recombination rate of the larger interval to 0.5. The number of coinformative meioses was calculated using the Chrompic option of CRI-MAP 2.4. Genetic distances were estimated using the Haldane mapping function and then converted to Kosambi units (cM). Marker order was considered to have significant support when log likelihoods were >3.0.

RESULTS

Ten copies of X, Xp, and Xq were scraped and PCR amplified generating fragments from 100 to 1500 bp (Fig. 1). The *Sau*3AI-digested chromosomal mixtures were cloned into the *Bam*HI/λ ZAP II vector with an average cloning efficiency of 1×10^8 pfu/μg. When the whole X chromosome-specific probe was used to paint metaphase spreads of a cow (Fig. 2*A*), the entire length of the X chromosomes was painted. This demonstrates the specific nature of the probe while showing that highly repetitive sequences were efficiently blocked by the competitor DNA and that sufficient nonrepetitive and/or moderately repetitive DNA sequences were PCR amplified. As expected, heterologous chromosome painting of sheep and goat metaphase spreads demonstrated significant X chromosome conservation among bovine and these two closely related species (Fig. 2*A*). Morphological differences between the bovine submetacentric X chromosome and sheep and goat acrocentric X chromosomes were analyzed by simultaneous two-color short (Xp) and long (Xq) arm chromosome paintings. Bovine Xp DNA homologous sequences are clearly located interstitially (Fig. 2 *B–H*) in the sheep and goat X chromosomes.

Library Screening for Type II Markers. A fraction (30,000 plaques) of the Xq plaque library was screened at high density and stringency (60°C) with a radiolabeled $(GT)_8$ probe to identify clones containing microsatellites (CA > 12) as described (5). The first screen yielded 536 positive clones. Eighty-six clones were picked for a secondary round of screening at higher stringency (65°C). Sixty-one remained positive and were sequenced. Twenty (32.8%) unique microsatellites were found. Fifteen (24.6%) clones did not contain microsatellite sequences and two (3.2%) had unreadable flanking regions. Twenty-four (39.3%) clones represented five distinct repetitive classes of flanking sequences and were classified as duplicates (data not shown).

Linkage Map. The range of female coinformative meioses, among marker pairs, varied from 18 to 102, and the two-point lod scores varied from 3.0 to 15.6 (data not shown). Ten markers, designated XBM7, -11, -16, -19, -24, -25, -31, -38, -77, and -84, were developed from the Xq specific library. The remaining markers, TGLA325 (3, 24), BM4604 (4), BMS 397

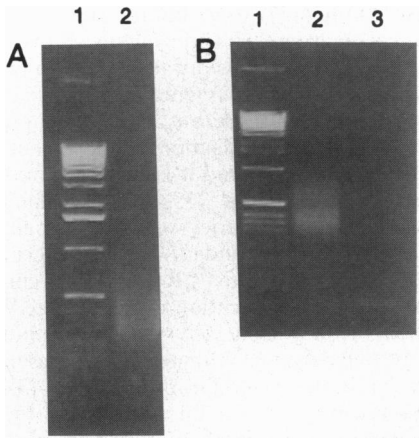

FIG. 1. PCR amplification of chromosome-specific inserts. Lanes: 1, 1-kb ladder molecular weight marker; 2, PCR amplification products for the whole X chromosome (*A*) and Xq (*B*); 3, PCR amplification reaction mixture without chromosomal DNA.

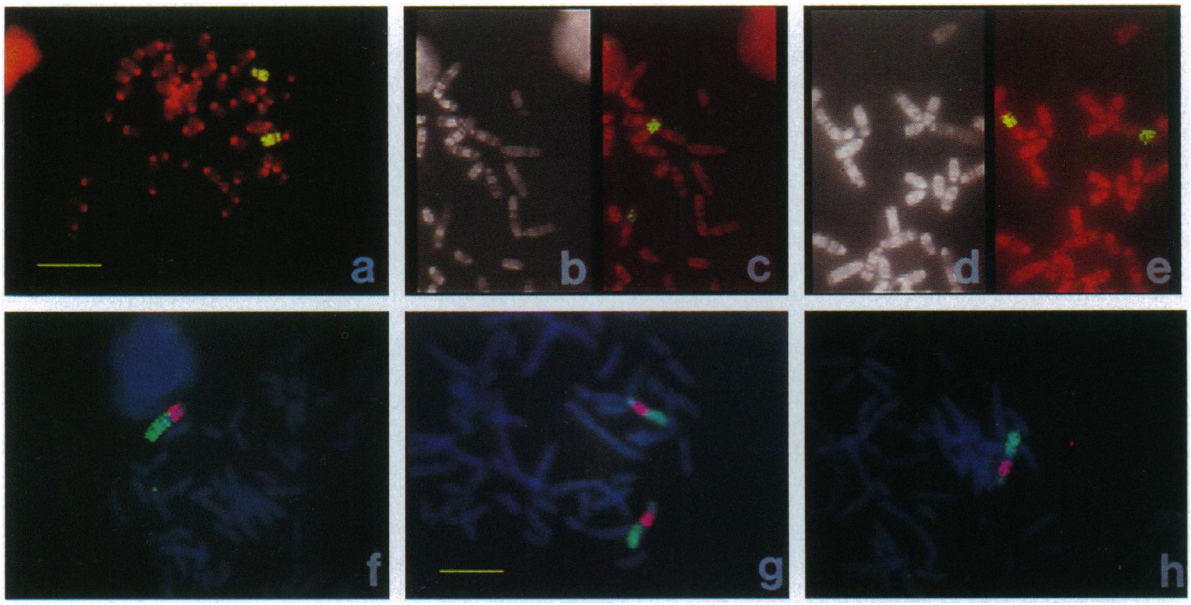

FIG. 2. FISH of X, Xp, and Xq chromosome-painting probes. (*A*) Bovine female metaphase showing FITC hybridization signals obtained with the whole X chromosome painting probe. (*B* and *C*) Same partial ovine female metaphase showing chromosome R-banding patterns (*B*) and FITC signals of the Xp painting probe (*C*). (*D* and *E*) Same partial caprine female metaphase showing chromosome R-banding patterns (*D*) and FITC signals of the Xp painting probe (*E*). (*F*) Bovine male metaphase showing simultaneous double-color TRITC/FITC FISH of Xp (red) and Xq (green), respectively. (*G*) Ovine female metaphase showing the Xp (TRITC; red) signal located interstitially in the ovine X chromosome. Chromosomes are were stained with DAPI (blue). (*H*) Caprine male metaphase but otherwise the same as in *G*. (Bars = 10 μm.)

(25), and INRA30 (26), were previously assigned to the X chromosome but not linked or are recent additions to the map (S.M.K., unpublished data). Primer sequences, annealing temperatures, and number of alleles are shown in Table 1. Two of these markers (BMC6021, contained in a cosmid clone; INRA30, contained in a phage clone) have been assigned by FISH to Xq27 (S. Salinas-Toldo and R. Fries, personal communication) and Xq43, respectively (F.A.P.d.L. *et al.*, unpublished data). This provides additional support for assigning the linkage group to bovine Xq. The generated Xq linkage group covers a total of 89.4 cM (Fig. 3).

DISCUSSION

We have generated the first bovine chromosome-painting probe and the first two X chromosome region-specific DNA libraries. Assuming an average chromosomal fragment size of 300 bp, an X chromosome length equivalent to 5.3% of the haploid genome, and the Xp arm representing 27% of the X chromosome length (13), each library represents approximately three chromosome arm equivalents. However, for our approach, a chromosome arm equivalent maximally represents only 75% of all possible DNA fragments available in a specific chromosome arm or region. Because approximately 1/4 of all adaptor-chromosomal insert ligations are lost when the adaptor dimers are digested with *Bgl* II, a chromosome equivalent may represent much less than 75% of all available chromosomal DNA. Incomplete libraries would also result if *Sau*3AI sites are not sufficiently abundant at centromeric and telomeric DNA regions to generate PCR amplifiable chromosomal fragments and if the amplification of large *Sau*3AI chromosomal fragments is inefficient. However, this is not currently limiting as the incidence of unique X chromosome-specific microsatellite clones with unique primer pairs [3.7% in this study vs. 1% bovine microsatellites identified by screening libraries composed of randomly cloned genomic DNA (4)] provide a significant number of new, chromosome region-specific informative markers (25).

Our work offers the first evidence for Xq-specific type II bovine markers linked and ordered with high local support on the X chromosome. Initial microsatellite assignments to bovine X (4, 9, 24, 25) were not linked. Barendse *et al.* (3)

Table 1. Type II loci (microsatellite) generated from clones obtained from Xq chromosome-specific DNA libraries

Locus	Forward (above) and reverse (below) primer sequences	Annealing temperature, °C
XMB7	CTGTATTAGAGTTCCCTGGAGAAA GCCAACATGCCCTGTAGAAT	56 (7)
XBM11	TACTGATGGGAGGTTTCTGAGA CCCAGAGTCTTTGTGTCAAGG	58 (5)
XBM16	AGGATAATTTGCTCTGTGCCC ATGGCAATATGAGGAGTTGC	56 (9)
XBM19	AAGAATCGGACATGACTGAATG CCTCCTTCATAATCCATTAAGCT	58 (4)
XBM24	TTACCACTGAGCCACCTGG ATGATGCTTCTGTCAAGAGGTT	60 (3)
XBM25	GGGGTCGCAAAGAGTCAGAT AATTAGAGTTTCCTGACAGCCA	58 (6)
XBM31	GATCCAACGGATGTTAGCAA GCCACACAGTCAAATGAATCA	58 (5)
XBM38	TTGGACACAACTGAGAGACTCA TGAACATCTGCTGGAATCATG	58 (5)
XBM73	CGATGTCAGCAACAATCCC TTGGTGTGTGCTTCTGTTCTG	58 (4)
XBM84	TCAGGTGAATACTTTCCCACG TCCTTGTGTCCCTTTAGTTTTG	58 (4)
BM4604*	TCTATACTGACACAAGCCCAGG AAAGTCCTTCAGGCAGAAAAG	56 (4)
BMC6021*	AGGCCAAGTGAAGAGGTCAC CTGCTCCAGCTTTCCAGTA	58 (4)

Number of alleles is in parentheses.
*Not obtained from the Xq chromosome-specific library.

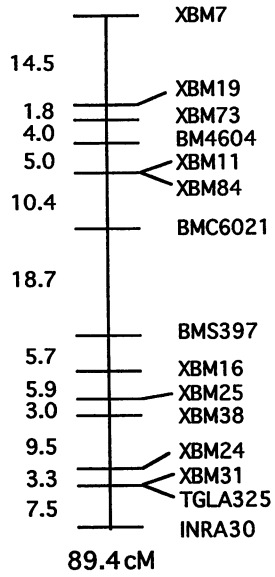

FIG. 3. An 89.4-cM linkage map for bovine Xq.

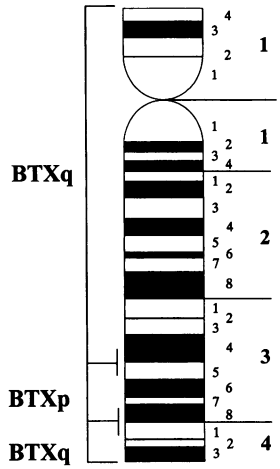

FIG. 4. G-banded X chromosome ideogram for caprinae. Brackets define the bovine Xp (BTXp) and Xq (BTXq) arm homologies as detected by FISH with the BTXp and BTXq chromosome painting probes. BTXp is homologous to the Xq34–q41 region of the caprinae X chromosome.

reported the linkage of two microsatellites, MAF45 and TGLA325, on chromosome Xp at an interval of 5 cM. Our observations appear to be in disagreement since TGLA325 and XBM31 (Xq marker) have 0% female meiotic recombination frequency (Fig. 3), suggesting that they are located in the same region and that TGLA325 is located on Xq. However, a 3% recombination frequency was detected between these markers for male meioses. Further evidence supporting the notion that TGLA325 and XBM31 are located in different regions of the X chromosome is based on the fact that TGLA325 has two alleles in males, while XBM31 has only one. Therefore, TGLA325 resides in the pseudoautosomal region, while XBM31 resides in the X chromosome-specific region of the X chromosome. This observation and our assignment of INRA30 to Xq43 by FISH (data not presented) suggest that the pseudoautosomal region is located distally at Xq4 and not distally at Xp2 as is conventionally accepted (3). Therefore, the positioning of TGLA325 and MAF45 on bovine Xp (3) will have to be revised.

Our chromosome painting results indicate that the purity of the preparation is near optimal, since no FITC background signals were observed on any other chromosome. Furthermore, the heterologous FISH carried out on sheep and goat chromosomes reinforce the specificity of the painting probes. Earlier comparisons attempting to reconstruct the ancestral bovine, sheep, and goat X chromosomes based on G-banding pattern homologies have postulated that a fraction of the bovine Xp segment is homologous to sheep and goat Xq35–q38 (27–28). It has also been suggested that the very short Xp observed in sheep and goat prometaphase chromosomes is homologous to bovine Xp12–p14 and that no homologous regions exist in bovine for the sheep and goat Xq42–43 distal region. Our results clearly indicate that, at the level of resolution afforded by FISH (Fig. 2 *B–H*) analysis, morphological differences between bovine and caprinae X chromosomes are due to rearrangements in chromatin distribution. The chromosome painting probes showed that the bovine Xp arm is homologous to a region of the sheep and goat Xq that approximately expands from the middle of band Xq34 to the middle of band Xq41. The remainder of the sheep and goat X chromosome was painted with the bovine Xq paint and therefore shows extensive homology with the bovine Xq arm. Since FISH signals could not define the exact chromosomal breakpoints, we have diagrammatically represented our observations for caprinae in Fig. 4. Based on our observations, it would appear that the lack of banding homology at Xq42–q43 between cattle, sheep, and goat chromosomes as reported earlier (28) does not imply that this region is absent in cattle but rather that the rearrangements that have occurred have altered the structure of the region so banding homology has been lost. These X chromosome painting probes should be useful in assessing chromosomal conservation among bovids and possibly between families within the order.

In sum, our observations suggest that PCR-generated small insert chromosome-specific libraries are an efficient approach for generating type II genetic markers. It further offers the opportunity to concentrate efforts in marker-deficient areas of the bovine genome and increases the possibility of rapidly saturating those areas with informative marker loci. The strategy described is applicable to development of chromosome painting probes and chromosome-specific libraries for other livestock species. Chromosome painting probes should also prove useful for analysis of somatic cell hybrid panels, ordering large insert library clones (YAC, P1, BAC) by chromosome, and allow comparative cytogenetic studies to define rearrangements of the ancestral ruminant X chromosome.

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