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Construction and Characterization of a Bovine Bacterial Artificial Chromosome Library

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A bacterial artificial chromosome (BAC) library has been constructed for use in bovine genome mapping using the pBeloBAC11 vector. Currently, the library consists of 23,040 clones, which achieves a 70% probability (P = 0.70) of the library containing a specific unique DNA sequence. Sixty thousand clones, or about three haploid bovine genomes, will be required to achieve a 95% probability (P = 0.95) of containing a unique sequence. An average insert size of 146 kb was estimated from the analysis of 77 randomly selected BAC clones produced by one or two rounds of size selection. The bovine DNA inserts proved to be very stable for at least 100 cell generations. No chimeric clones were detected among 11 large, size-selected BAC clones using fluorescence *in situ* hybridization (FISH) on metaphase bovine chromosomes. The polymerase chain reaction (PCR) was used to screen the library for single-copy nuclear sequences. Thirty-three of 46 (72%) sequences were present in the library in at least one copy, which is consistent with the estimated 70% probability of this library containing a unique DNA sequence. A BAC clone containing the 3β-hydroxy-5-ene steroid dehydrogenase (HSD3B) gene was physically mapped to bovine chromosome 3 by FISH. Two new microsatellite markers were isolated from the HSD3B-positive BAC clone as sequence-tagged sites for genetic mapping. These markers cosegregated, and no recombinants were detected in 193 informative meioses. Plasmid end rescue and the inverse polymerase chain reaction methods were used to rescue both ends of this BAC clone, and chromosome walking was performed using PCR primers designed within the end region sequences. Based on our experimental results, the BAC system provides a very useful tool for complex genome analysis.

INTRODUCTION

Clones containing large DNA fragments are required to obtain efficiently accurate, high-resolution physical maps of eukaryote genomes. Bacteriophage λ and cosmid vectors are commonly used to construct eukaryote genomic DNA libraries and have a cloning capacity of up to 24 or 45 kb, respectively (Sambrook *et al.*, 1989). Although the cloning efficiency of these systems is high, the relatively small DNA insert size and the instability of cloned DNA in conventional cosmid vectors (Yokobata *et al.*, 1990) limit their utility for chromosome walking procedures.

Yeast artificial chromosome (YAC) technology was developed to take large DNA inserts (Burke *et al.*, 1987), and a bovine YAC library with an average insert size of 750 kb has been constructed (Libert *et al.*, 1993). YACs have been used to construct large-scale physical maps (Green and Olson, 1990a; Silverman *et al.*, 1991; Wada *et al.*, 1990; Bronson *et al.*, 1991; Coulson *et al.*, 1988; Garza *et al.*, 1989), to walk along large segments of mammalian chromosomes (Silverman *et al.*, 1989), and to examine the structure and function of large genes. However, there are several difficulties that are encountered in the construction and manipulation of a YAC library. First, cloning efficiencies are low (Smith *et al.*, 1990). Second, it is difficult to isolate cloned DNA relative to bacterial systems, because yeast cells have a rigid cell wall and insert fragments often comigrate with endogenous yeast chromosomes. Third, YAC clones are often chimeric, consisting of fragments of DNA from different regions of the target genome (Green and Olson, 1990a), especially in very large insert libraries (>500 kb) (Anderson, 1993). It is estimated that one-third of the inserts in the bovine YAC library are chimeric (Libert *et al.*, 1993). Finally, deletions are often a problem of YACs (Anderson, 1993). Both chimeras and deletions can cause serious problems when chromosome walking and jumping are performed, as walks will be led in the wrong direction by chimeras or stopped by deletions.

New cloning systems based on the *Escherichia coli* F factor have been used to construct large DNA insert libraries in human and plants: bacterial artificial chromosomes (BACs) (Shizuya *et al.*, 1992; Woo *et al.*, 1994; Wang *et al.*, 1994) and P1-derived artificial chromosomes (PACs) (Ioannou *et al.*, 1994). These systems

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promise to solve many of the problems associated with YACs. The F factor is stably maintained and inherited among the cells of a bacterial population and can maintain DNA fragments up to 1 Mb in length (Low, 1987). Data from human BAC and PAC libraries show that transformation is 10–100 times more efficient than in yeast. The manipulation of cloned DNA in BACs and PACs is easier than in YACs because supercoiled, circular plasmid DNA is resistant to shearing. Finally, human and plant DNA have been shown to be quite stable in BAC and PAC vectors after 100 generations of growth (Shizuya *et al.*, 1992; Ioannou *et al.*, 1994; Woo *et al.*, 1994). However, a disadvantage of F factor-based systems for chromosome walking is that the average size of cloned DNA inserts is somewhat smaller than in YACs.

A positional cloning study is underway at Texas A&M University to identify quantitative trait loci (QTLs) for economically important traits in cattle. Recently, two genetic maps (Barendse *et al.*, 1994; Bishop *et al.*, 1994) and an integrated cytogenetic and meiotic map (Eggen and Fries, 1994) of the bovine genome have been developed. The genetic maps span 90% of the expected 2800-cM length of the bovine haploid genome. Most of the markers in these maps are type II microsatellites; however, restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) markers for type I anchor loci are included. The bovine genetic map is now of sufficient resolution that it can be used for the positional cloning of QTLs. Markers closely linked to QTLs may be useful for marker-assisted selection to improve economically important traits. However, the ultimate goal is to identify the QTL and to understand the physiological basis for the phenotypic variation due to DNA sequence variation at the locus. A large DNA insert library for physical mapping and chromosome walking will be the key resource in positional cloning. In this paper, we present a method of construction, characterization, and screening of a bovine BAC library. Also, the 3β-hydroxy-5-ene steroid dehydrogenase gene is used as a target gene to test the feasibility of chromosome walking in this BAC system.

MATERIALS AND METHODS

Construction of a BAC Library

Preparation of high-molecular-weight DNA. Blood was drawn from a purebred Angus bull (Y6; American Angus Association Reg. No. 11519666), and anticoagulant solution (35% sodium citrate, 30% glucose, pH 6.1) was added immediately to the blood sample at 50 ml of anticoagulant solution per liter of blood. The blood sample was kept on ice until the white blood cells were isolated. Sixty milliliters of the fresh blood sample was centrifuged at 3000 rpm for 15 min at 4°C. The white blood cell layer was removed and resuspended in 10 ml Hank's balanced salt solution (HBSS, Sigma). The white blood cell suspension was carefully added on the top of 5 ml of prewarmed (37°C) histopaque-1077 (Sigma) in a 15-ml conical centrifuge tube. The white blood cell layer ("buffy coat") was removed after centrifugation at 1300 rpm for 40 min at 4°C, and the white blood cell concentration was adjusted to 2×10^7 white blood cells/ml using HBSS. High-molecular-weight DNA was prepared by the method described by Wing *et al.* (1993). Five milliliters of 1% low-melting agarose (FMC, Seaplaque) in HBSS was prewarmed to 45°C and added to 5 ml of this cell suspension. After a quick swirl, 20 ml of prewarmed mineral oil was added, and the mixture was rapidly swirled for at least 3 s. The resulting slurry was immediately poured into 120 ml of ice-cold HBSS and stirred for 5 min on ice. The microbeads were collected by centrifugation at 500*g* for 10–15 min and washed once with SDE (1% SDS, 25 m*M* Na_2EDTA, pH 8.0) at room temperature for 15 min. The embedded white blood cells were lysed and the proteins degraded by incubation for 24 h in ESP (1% sarcosyl, 25 m*M* Na_2EDTA, pH 8.0, 1 mg/ml proteinase K) at 50°C with gentle shaking. New ESP was added (0.1 mg/ml proteinase K) for 24 h further incubation, and the microbeads could be stored in ESP for up to a year at 4°C without loss of DNA quality. The beads were given six 1-h washes with TE (10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0) on ice. PMSF (phenylmethylsulfonyl fluoride, Sigma) was included in the first three washes (1 m*M*/ml for the first wash and 0.1 m*M*/ml for the second and third washes) to inhibit the proteinase K.

BAC vector preparation. pBeloBAC11 (7.4 kb) was kindly provided by Drs. H. Shizuya and M. I. Simon, Department of Biology, California Institute of Technology (Pasadena, CA). The preparation steps followed the description of Woo *et al.* (1994).

Partial digestion of genomic DNA. Fifty microliters of high-molecular-weight DNA (approximately 6 μg) embedded in the agarose beads was incubated with 50 μl *Hin*dIII (4 m*M* spermidine, 100 μg/ml BSA, 6 m*M* Tris–HCl, pH 7.5, 6 m*M* $MgCl_2$, 50 m*M* NaCl, 1 m*M* DTT) or *Bam*HI (4 m*M* spermidine, 100 μg/ml BSA, 6 m*M* Tris-HCl, pH 7.5, 6 m*M* $MgCl_2$, 100 m*M* NaCl, 1 m*M* DTT) reaction buffer on ice for 30 min. Three and one-half units of *Hin*dIII or *Bam*HI (Promega) was added and incubated on ice for another 20 min. The partial digestion reaction was performed by transferring the beads to a 37°C water bath for 15 min and stopped by adding 10 μl of 0.5 *M* EDTA, pH 8.0.

Size selection. The partially digested DNA was run on a 1% low-melting agarose gel (FMC, Seaplaque) using a CHEF apparatus (Bio-Rad). Megabase-size DNA was separated in 1× TAE buffer (50 m*M* Tris–acetate, pH 7.7, 0.5 m*M* EDTA, pH 8.0) at 11.5°C and 6 V/cm for 20 h with a 90-s pulse time. The 300- to 500-kb fraction was excised from the gel and melted at 70°C for 10 min. The melted agarose was reloaded on a 1% low-melting agarose gel for a second size selection. The selection was carried out at 4 V/cm, with a 5-s pulse time for 10 h at 11.5°C in 1× TAE buffer. The compression band was cut off for further manipulation.

Ligation and transformation. The agarose slice was melted at 70°C for 10 min, incubated at 45°C for 10 min, and digested with GELase (Epicentre, U.S.A.) (1 unit enzyme per 100-μg gel piece) at 45°C for 1 h. About 200 ng of size-selected DNA was ligated to *Hin*dIII- or *Bam*HI-digested pBeloBAC11 vector DNA (10 *M* excess of vector DNA) with 5 units of T_4 DNA ligase (USB) in 100 μl total volume (66 m*M* Tris–HCl, pH 7.6, 6.6 m*M* $MgCl_2$, 10 m*M* DTT, 66 μ*M* ATP) at 16°C overnight. One microliter of the ligation product was used to transform 20 μl ElectroMAX DH10B competent cells (BRL, U.S.A.) by electroporation. The transformation procedure followed the standard electroporation procedure (GIBCO BRL; voltage booster settings: resistance on voltage booster, 4000 Ω; cell-porator settings: voltage, 400 V; capacitance, 300 μF; impedance, low Ω; charge rate, fast). After electroporation, cells were incubated in 1 ml SOC medium (10 m*M* $MgSO_4$, 10 m*M* $MgCl_2$, 10 m*M* NaCl, 2.5 m*M* KCl, 20 m*M* glucose, 2% Bactotryptone, 0.5% yeast extract, pH 7.0) at 37°C with gentle shaking for 1 h and spread on LB plates containing chloramphenicol (12.5 μg/ml), X-gal (40 μg/ml), and IPTG (100 μg/ml). The plates were incubated at 37°C overnight.

Storage of the BAC library. Blue and white color selection was used to identify the recombinants. White positive BAC clones were picked manually to 96-well microtiter tubes containing 300 μl LB with 12.5 μg/ml chloramphenicol. The microtiter tubes were incubated at 37°C with 225 rpm shaking for 20 h. One hundred microliters of each culture was removed for the DNA pooling procedure

(see below). Two hundred microliters of 2× glycerol solution (65% glycerin, 0.1 *M* $MgSO_4$, 0.025 *M* Tris–HCl, pH 8.0) was added to the remaining 200 μl of the overnight culture and mixed by pipetting up and down several times, and 150 μl of the mixture was transferred to a 96-well microtiter plate. An additional 150 μl was transformed to a second 96-well microtiter plate, and the two copies were prepared and stored at −80°C. The remaining 100 μl in the 96-well microtiter tube was stored at −20°C as a working copy of the library.

Characterization of the BAC Library

Insert isolation. BAC clones were grown in LB medium with chloramphenicol (12.5 μg/ml) for 20 h at 37°C with shaking. DNA preparation followed the standard alkaline lysis protocol (Silhavy *et al.*, 1984). Inserts were excised using *Not*I, which has recognition sequences flanking the *Hin*dIII, *Bam*HI, and *Sph*I cloning sites in the *LacZ* gene of pBeloBAC11. Inserts were sized by CHEF gel electrophoresis.

Stability. BAC clones were incubated in 100 ml LB + chloramphenicol (12.5 μg/ml) medium at 37°C overnight. The overnight cultures were diluted 100-fold, and 0.1 ml of diluted cultures was added into 500 ml LB with chloramphenicol (12.5 μg/ml) and also grown overnight. This procedure was continued for 4 more days. By Day 5, approximately 100 cell generations had passed. The restriction fragment map from Day 1 cultures was compared with the restriction fragment map from Day 5 cultures by electrophoresis on a 1% agarose gel or 1% CHEF gel.

Chimerism. Fluorescence *in situ* hybridization (FISH) was used to evaluate the degree of chimerism in this library. The chromosomal FISH protocol follows standard protocols (Pinkel *et al.*, 1986) with slight modifications. Domestic cattle chromosome preparations were incubated in 2× SSC at 37°C for 60 min and sequentially dehydrated in 70, 80, 90, and 100% ice-cold ethanol for 2 min. The air-dried slides were then denatured in 70% formamide, 2× SSC solution at 72°C for 2 min. One more dehydration was performed in 70% ice-cold ethanol for 2 min. BAC DNA was labeled with Clontech's biotin-21–dUTP nick-translation labeling kit. Probe was purified using Clontech's Chroma Spin-30 columns + TE. About 100 ng of labeled BAC DNA, 8 μg of Cot-1 DNA, 50 μg of sonicated salmon sperm DNA, 50% formamide, 2× SSC, 10% dextran sulfate, pH 7.0 were included in the hybridization mixture. After hybridization overnight at 37°C, the cover glass was removed and the slide was washed in 50% formamide, 2× SSC three times, and 2× SSC three times at 40°C for 5 min. The probes were detected by fluoresceinated avidin and biotinylated anti-avidin (Vector Laboratories, U.S.A.). One round of signal amplification was used. Photographs of yellow probe signal against orange counterstained chromosomes were taken on Kodak Gold 400 Print Film using a Zeiss Axioskop equipped for simultaneous visualization of fluorescein and propidium iodide. In turn, QFH-bands were visualized using a filter set appropriate for excitation of Hoechst 33258, and chromosomes identified at the microscope according to the domestic cattle standard karyotype (Reading Conference, 1980; ISCNDA, 1990).

PCR-Based Screening

DNA pool. Single-plate DNA pools were prepared by combining the 100-μl overnight cell cultures of 96 BAC clones. DNA was extracted according to the standard alkaline lysis method (Silhavy *et al.*, 1984), resuspended in 200 μl TE (10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0), and stored at −80°C. Single-plate DNA pool working solutions were made by a 1:10 dilution of the stock DNA with TE. Superpools of DNA that contained DNA from 480 BAC clones were made by pooling five single plate DNA pools.

PCR reaction. PCR reactions were performed in 96-well Techne HI-TEMP 96 microplates on a MW-2 Dri-Plate cycler (Techne). The PCR reactions were carried out in a 30-μl reaction mixture: 10 m*M* Tris–HCl, pH 9.0, 1.0–2.5 m*M* $MgCl_2$, 200 μM each dNTP (dATP, dCTP, dGTP, dTTP), 0.5–1.0 μM each primer, 50–150 ng DNA, 0.5 unit of *Taq* enzyme (Promega). Samples were denatured at 94°C for 5 min and 35 cycles performed under the following conditions: 94°C denaturing for 30 s, annealing temperature dependent upon the specific primer pair from 30 s to 1 min, 72°C elongation for 30 s to 1 min. After an additional 1 cycle at 72°C for 10 min, 10- to 20-μl samples were loaded on 2% agarose gels or 8% nondenatured acrylamide gels containing 1× TBE (0.09 *M* Tris–borate, 0.002 *M* EDTA). Gels were stained with ethidium bromide, and the DNA was visualized under UV light.

Screening strategy. The PCR systematic screening strategy (Green and Olson, 1990b) was used to screen the BAC library with the following modifications. First, the DNA superpools were screened for each marker. Then, the five single DNA pools comprising the positive superpool were screened to locate the positive clone to a single 96-well microtiter plate. Eight row and 12 column DNA pools were made for the positive plate. After row–column screening, positive clones containing each marker were identified by the intersection of the positive row and column pool. PCR and cycle sequencing were performed for the positive clone to confirm the screening result. Genomic DNA of the purebred Angus bull whose DNA was used to construct the BAC library was used as a positive control. A PCR reaction mixture without any DNA was used as a negative control.

Identification of Microsatellite Markers in BAC Clones

Generation of microsatellite markers. DNA from a single BAC clone was completely digested by *Sau*3 AI in a 100-μl mixture (6 m*M* Tris–HCl, pH 7.5, 6 m*M* $MgCl_2$, 50 m*M* NaCl, 1 m*M* DTT, 1–2 μg BAC DNA, 20 units *Sau*3 AI, Promega). The digestion product was loaded on an 0.8% low-melting agarose gel (FMC, Seaplaque). After electrophoresis, the gel was stained with ethidium bromide. The DNA smear of fragments less than 600 bp was excised from the gel and smashed with a pipette tip. The resulting slurry was alternately frozen at −80°C and thawed at 55°C for 4–5 times to release the DNA. Following the freeze–thaw cycles, the agarose was pelleted by centrifugation at 13,000 rpm for 5 min. The supernatant was precipitated by adding 0.1 vol of 2 *M* NaCl and 2.5 vol of absolute ethanol and incubating at −80°C for 2 h. DNA was recovered by spinning at 10,000 rpm for 15 min. The DNA pellet was dried and resuspended in 100 μl phosphatase reaction mixture (50 μM Tris–HCl, pH 9.3, 1 m*M* $MgCl_2$, 0.1 m*M* $ZnCl_2$, 1 m*M* spermidine). Five units of CIP (calf intestinal alkaline phosphatase) enzyme (Promega) was added. The mixture was incubated at 37°C for 40 min. Proteinase K (100 μg/ml), 0.5% SDS, and 5 m*M* EDTA (pH 8.0) were added, and the sample was incubated at 55°C for 40 min to inactivate the CIP. The sample was extracted once each with equal volumes of phenol/chloroform/isoamyl alcohol (25/24/1) and chloroform, respectively. Bluescript M13- vector was completely digested with *Bam*HI (6 m*M* Tris–HCl, pH 7.5, 6 m*M* $MgCl_2$, 100 m*M* NaCl, 1 m*M* DTT, 24 units *Bam*HI) and an appropriate amount of digested vector (equal molar vector DNA and BAC DNA) and size-selected BAC DNA was coprecipitated at −80°C for 5 h. DNA was recovered by centrifuging at 10,000 rpm for 15 min and washed with 70% ethanol. The DNA pellet was resuspended in 50 μl of ligation reaction buffer (30 m*M* Tris–HCl, pH 7.8, 10 m*M* $MgCl_2$, 10 m*M* DTT, 1 m*M* ATP, 50 ng vector DNA, 50 ng BAC DNA, 4 units T_4 DNA ligase) and incubated at 16°C overnight. Twenty microliters of DH5a competent cells (GIBCO BRL) and 2 μl of ligation product were mixed, incubated on ice for 30 min, heated at 42°C for 60 s, and put back on ice for 2 min. Prewarmed SOC medium (10 m*M* $MgSO_4$, 10 m*M* $MgCl_2$, 10 m*M* NaCl, 2.5 m*M* KCl, 20 m*M* glucose, 2% Bactotryptone, 0.5% yeast extract, pH 7.0) was added to the mixture and shaken at 37°C at 225 rpm for 1 h. The transformants were plated on LB plate with ampicillin (200 mg/liter), X-gal (40 mg/liter), and IPTG (100 mg/liter) at 37°C overnight. The colony filter lift method (Sambrook *et al.*, 1989) was used to screen for microsatellites. A $(GT)_{15}$ oligomer was used as a probe to detect the microsatellites. Hybridization was performed as follows: filters were soaked in 2× SSC for 5 min and prewashed in prewashing solution (5× SSC, 0.5% SDS, 1 m*M* EDTA, pH 8.0) at 50°C for 1 h with gentle shaking, bacterial cell debris was wiped from the filters, and filters were prehybridized in hybridization

buffer (5× SSC, 0.5% SDS, 25 m*M* KPB, pH 6.5, 5× Denhardt's) at 65°C for 2 h. A $(GT)_{15}$ probe was made in 40 μl total volume: 0.15 nmol oligo primer, 4 μl 10× PNK buffer, 2 μl (20 μCi) γ-^{32}P-labeled ATP, and 10 units of T_4 polynucleotide kinase enzyme. The mixture was incubated at 37°C for 1 h and stopped by heating at 70°C for 10 min. This 40-μl probe solution was used for 20 filters. Probe was added to the prehybridized filters in hybridization solution and incubated at 37°C overnight. Filters were washed twice in 2× SSC, 0.2% SDS at room temperature for 20 min and twice in 1× SSC, 0.1% SDS at 40°C for 20 min, followed by autoradiography.

Sequencing clones containing microsatellites. DNA from the positive clones was extracted from a 1-ml LB + ampicillin (200 mg/liter) overnight culture and dissolved in 10 μl TE. Two microliters miniprepped DNA plus 7.5 μl ddH_2O was heated at 100°C for 2 min and chilled on ice for 2 min, and then 9.5 μl Terminator Premix (ABI), 1 μl reverse primer (5′ CAGGAAACAGCTATGACC 3′, 0.1 μM), and 2 drops of oil were added. Cycle sequencing was carried out on a Perkin Elmer/Cetus 480 DNA thermal cycler under the following conditions: 96°C denaturing for 30 s, 50°C annealing for 15 s, 60°C elongation for 4 min. After 25 cycles, the reaction product was cleaned through a QIAquick PCR purification column and was run on an ABI 373A automated DNA sequencer.

Microsatellite marker screening and linkage analysis. PCR primers were designed from the two flanking regions of microsatellites using the MacVector V4.1.1 program. After a cold test to optimize PCR conditions, forward primers were end-labeled with γ-^{32}P-labeled ATP, hot PCR was carried out on an MW-2 Dri-Plate Cycler (Techne), and the PCR products were electrophoresed on an 8% acrylamide gel. Twenty-one three-generation reciprocal backcross and F_2 Angus × Brahman full sib families with an average of 10 progeny per family were used to screen microsatellite markers. CRI-MAP V2.4 was used for the linkage analysis.

End Rescue

Plasmid end rescue. The strategy followed for BAC clone end rescue is represented in Fig. 1. Separate aliquots of BAC DNA were completely digested with *Sac*I, *Bam*HI, and *Sph*I, which cut the pBeloBAC11 vector only once in the *Hin*dIII insert BAC library. The digestion products were self-ligated in a 40-μl total volume at 16°C overnight under dilute conditions (Collins and Weissman, 1984), about 10–80 ng DNA per 100-μl mixture (30 m*M* Tris–HCl, pH 7.8, 10 m*M* $MgCl_2$, 10 m*M* DTT, 1 m*M* ATP, 2–3 units ligase). One microliter of ligation product was transformed into 20 μl ElectroMAX DH10B competent cells (BRL, U.S.A.) by electroporation. Positive transformants were identified by plating the transformation product on an LB plate with chloramphenicol (12.5 μg/ml). DNA was extracted from six positive colonies, digested with the same enzyme used for the subcloning experiment, and electrophoresed on a 1% agarose gel. The clone that contained the smallest insert was chosen, and the plasmid DNA from this clone was purified by CsCl density gradient centrifugation. Cycle sequencing was performed using the universal reverse primer (5′ CAGGAAACAGCTATGACC 3′, 0.1 μM). The right end sequence of the BAC clone was obtained by running the cycle sequencing product on an ABI 373A automated DNA sequencer.

Inverse polymerase chain reaction (IPCR). Separate aliquots of BAC DNA were completely digested with *Eco*RI, *Bam*HI, *Kpn*I, *Sac*I, and *Sma*I. The digested DNA was self-ligated at 16°C overnight under dilute conditions (30 m*M* Tris–HCl, pH 7.8, 10 m*M* $MgCl_2$, 10 m*M* DTT, 1 m*M* ATP, 2–3 units ligase). One to two microliters of ligation product was used for a 100-μl PCR reaction. PCR reactions were performed on a Perkin–Elmer/Cetus 480 DNA thermal cycler under the following conditions: 10 m*M* Tris–HCl (pH 9.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μM each dNTP (dATP, dCTP, dGTP, dTTP), 0.5–1.0 μM primers (IPCR1, 5′ GGATCCCCGGGTACCGAG 3′; IPCR2, 5′ CTAGAGTCGACCTGCAGG 3′; BAC1, 5′ CTGCAGGCATGCAAGC 3′; BAC2, 5′ GTCGACTCTAGAGGATC 3′) and 2.5 units *Taq* DNA polymerase. The PCR conditions for the IPCR1 and IPCR2 primer pair were 35 cycles of 94°C denaturing for 1 min, 56°C annealing for 1 min, and 72°C elongation for 2 min with a final 10-min elongation at 72°C. The conditions for the BAC1 and BAC2 primer pair followed those developed for the Sorghum BAC library (Woo *et al.*, 1994). The IPCR product was used directly for cycle sequencing, and the left end sequence of the BAC clone was obtained by running the cycle sequencing product on an ABI 373A automated DNA sequencer.

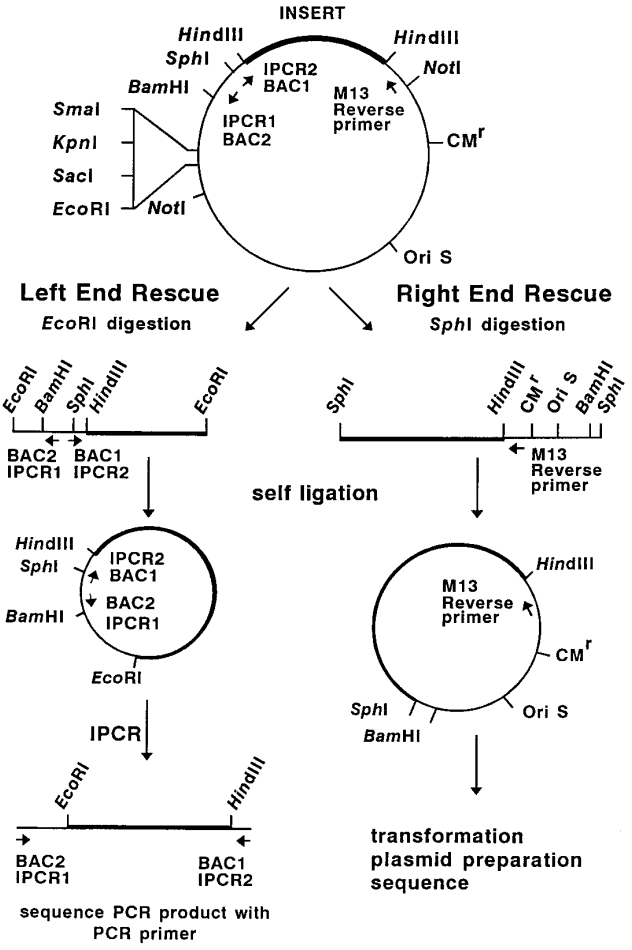


FIG. 1. Structure of the pBeloBAC11 vector and a strategy for the end rescue of BAC clones. Details are provided under Materials and Methods.

RESULTS

Construction and Characterization of the Bovine BAC Library

There are three cloning sites (*Hin*dIII, *Bam*HI, and *Sph*I) within the *LacZ* gene of the pBeloBAC11 vector, but only *Hin*dIII (A/AGCTT) and *Bam*HI (G/GATCC) produce 5′ overhangs for DNA phosphatation. These two different restriction enzymes were used to construct the bovine BAC library. Since some regions of the bovine genome are rarely cut by one enzyme or are cut too frequently, using two restriction enzymes with different base compositions of their recognition sequences provided an increased coverage of the genome represented in the digested fragment pool. The first 23,040 BAC clones that defined a library with a 70% probability of containing a unique DNA fragment were

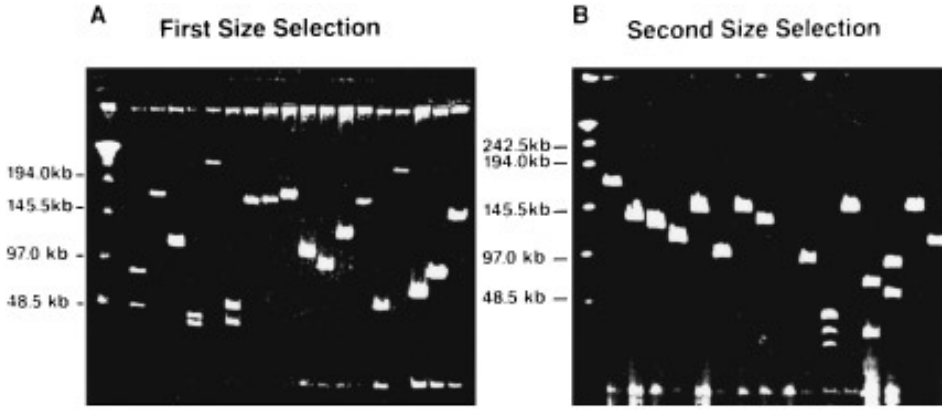


FIG. 2. PFGE analysis of insert size in the bovine BAC library following one (**A**) and two (**B**) rounds of size selection. Inserts have been removed from the pBeloBAC11 vector by digestion with *Not*I. Separation of the digested DNA was carried out on a CHEF-DRII (Bio-Rad) for 18 h; field strength was 6 V/cm in a 1% agarose gel in 0.5× TBE buffer at 11.5°C with linear pulse time ramping from 5 to 15 s. The size standard is composed of bacteriophage λ concatemers.

constructed from *Hin*dIII partial digests. The optimal partial digestion conditions were determined by wide and narrow window digestion to give the most DNA in the size range 200 to 500 kb. DNA fragments that ranged from 300 to 500 kb were excised from a low-melting CHEF gel and ligated to the pBeloBAC11 vector. Transformation was carried out by electroporation (Dower *et al.*, 1988). There is an inverse relationship between the insert size and the transformation efficiency (Albertsen *et al.*, 1990), and the transformation efficiency for BACs was 8.2×10^5 transformants/μg DNA, or about 1650 transformants from 1 μl of ligation product. The average insert size of clones produced from a single round of size selection was 142 kb (range 40–300 kb) as determined by CHEF analysis of 44 randomly chosen clones.

Inserts smaller than 100 kb are not desirable for a large DNA insert library. To remove these small inserts and increase the average insert size of the BAC library, the excised band from the first size selection gel was melted at 70°C for 10 min, reloaded onto a second 1% low-melting agarose CHEF gel, and electrophoresed in 1× TAE buffer at 4 V/cm, 11.5°C for 10 h with a 5-s pulse time. The compression band was excised and used for ligation and transformation. The transformation efficiency following this second size selection decreased to 1.2×10^5 transformants/μg DNA, or about 250 clones from 1 μl of ligation product. Based on CHEF analysis of 33 randomly chosen clones, the average insert size increased to 150.3 kb, and the insert size ranged from 100 to 270 kb. Two rounds of size selection eliminated small DNA fragments trapped by comigration with the selected DNA (Woo *et al.*, 1994), increased the average insert size, and gave a more uniform insert size distribution in the library. However, the second size selection produced a lower proportion of >300-kb clones. This might have been caused by the lower DNA concentration following the second size selection. Figure 2 shows the inserts from 18 BAC clones produced by a single round of size selection and 15 inserts from BAC clones produced by two rounds of size selection. BAC DNA was prepared from 5 ml LB + chloramphenicol (12.5 μg/ml) overnight culture, and 25% of the DNA was loaded on the gel. Bovine DNA inserts were removed from the pBeloBAC11 vector by digestion with *Not*I. The common 7.4-kb vector bands are shown in Fig. 2. Twenty-six of the 32 clones did not have a *Not*I site within the bovine DNA insert. Figure 3 shows the insert size distribution for the first and second size selection.

Stability. The ability to maintain the insert DNA stably is one of the most important features of a large DNA insert library. Insert stability affects the library's representation of the genome, the ability to find and characterize accurately specific sequences in the library, and the ability to use the library for chromosome walks and physical mapping (Kim *et al.*, 1992). Insert stability was tested for the bovine BAC library using

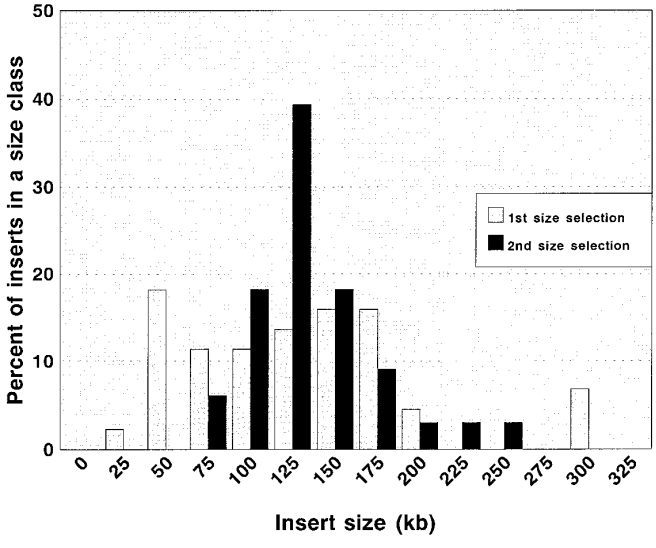


FIG. 3. Bovine BAC insert size distribution following the first and second size selection.

four randomly chosen clones with insert sizes of 280, 260, 180, and 140 kb. These clones were grown for 100 generations, and DNA was extracted from 0 and 100 generation cell populations. After digestion with the restriction enzymes *Nhe*I, *Sac*I, *Xho*I, *Hin*dIII, and *Eco*RI, the DNA was electrophoresed on a 1% agarose gel, and no detectable deletions were seen by comparing the restriction maps from generations 0 and 100. Also, when the insert DNA from 10 randomly chosen BAC clones from generations 0 and 100 was excised by *Not*I and electrophoresed on a 1% CHEF gel, no detectable changes were seen. Since no deletions have been detected in clones from human (Shizuya *et al.*, 1992), sorghum (Woo *et al.*, 1994), and bovine BAC libraries, we conclude that foreign DNA inserts are stable in the pBeloBAC11 vector.

Chimerism. Chimeric clones pose a serious problem for both physical mapping and chromosome walking. Consequently, evaluation of the proportion of chimeric clones in the BAC library is important. The five largest inserts identified by CHEF gels of 68 BACs (sizes 325, 310, 300, 285, and 275 kb) and 6 BACs known to contain single-copy nuclear sequences were examined by FISH to bovine metaphase chromosomes (Fig. 4). No chimeric clones were detected.

PCR-Based Screening of the Bovine BAC Library

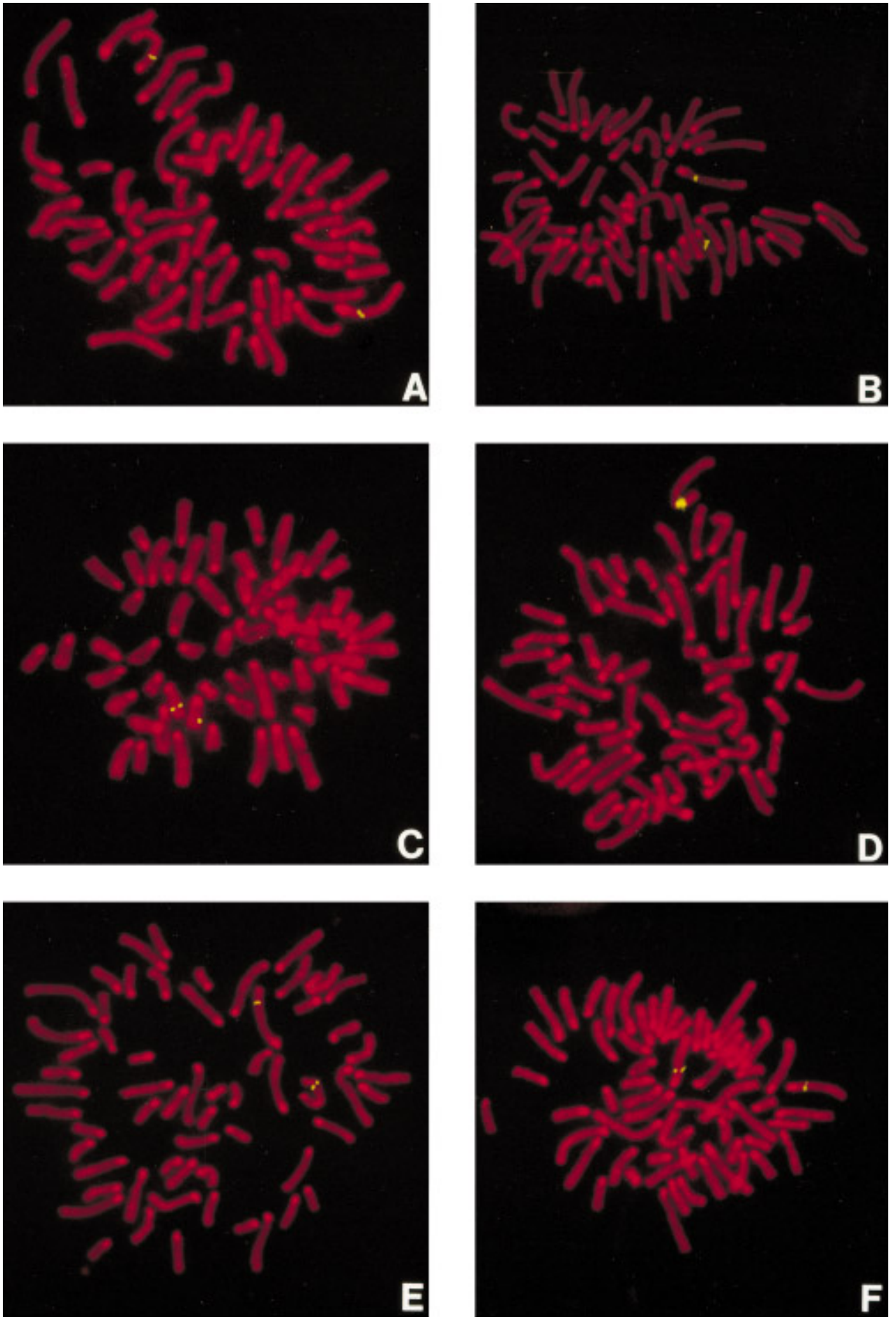
To test the degree of genomic coverage of the library, a PCR systematic screening protocol was developed (Green and Olson, 1990b). The 23,040 clones were picked into 240 96-well microtiter plates, and DNA was isolated from all of the clones on each plate to produce 240 single-plate pools. These single-plate pools were further pooled to form 48 superpools each containing DNA from 5 plates (480 BAC clones). DNA from the 48 superpools was screened by PCR for the presence of single-copy nuclear sequences. If the sequence was present in any one superpool, the five single-plate pools constituting the appropriate superpool were then screened to determine the plate containing the positive clone. Once the appropriate plate was identified, DNA was extracted from each of the 8 rows and 12 columns to produce row and column pools. A final round of amplification then located the row and column containing the clone with the target fragment. The intersection of the appropriate row and column on the microtiter plate identified the correct clone. With this system, the clone containing a desired fragment could be identified with 73 PCR reactions (48 superpools, 5 single pools/superpool, 12 row and 8 column pools/single pool). The entire library screening process requires about 3 days. An example of the isolation of BAC clones containing the microsatellite marker ETH225 is given in Fig. 5. There were occasionally some extra fragments amplified from superpool DNA that were not amplified in genomic DNA or in DNA from a single BAC clone (1 of 33 STSs screened). This was probably due to the presence of segments that could be effectively amplified when present at fairly high concentration (Green and Olson, 1990b). To verify the screening process, all PCR products were sequenced. Sequence verification was very important due to the high sensitivity of PCR, and although some PCR products were of about the expected size, they did not contain the target gene fragment. Table 1 summarizes the PCR primer pairs and the results of the library screening. Among 46 STSs used to screen the BAC library, 33 (72%) were found in the library, which is consistent with the 70% probability of finding a unique DNA fragment (Sambrook *et al.*, 1989). Among 21 single-copy genes and 11 microsatellite markers, one BAC clone was found for each of 13 sequences, and two or more BAC clones were found for each of 20 sequences. Four BAC clones were isolated for the IGF-1 receptor, and sequence data indicated that all four BAC clones contained the gene. This may reflect an increased cloning efficiency associated with this region of the genome. Three BAC clones were found to contain the MHC class I gene family. Similarly, three BAC clones were found for the prolactin gene; however, sequencing data indicated that one BAC clone contained prolactin while the other two contained prolactin-like genes.

The single-copy nuclear genes butyryl cholinesterase, glucocerebrosidase, and 3β-hydroxy-5-ene steroid dehydrogenase have been mapped in human and mouse but not in bovine. Identification of closely linked microsatellite markers within the BAC clones containing these loci would provide additional useful marker loci for candidate gene analysis. BACs containing an anchor locus can also be assigned to a specific chromosomal region by fluorescence *in situ* hybridization to align the genetic and physical maps in these chromosomal regions.

Genetic and Physical Mapping of 3β-Hydroxy-5-ene Steroid Dehydrogenase

Three β-hydroxy-5-ene steroid dehydrogenase/$\Delta^5 - \Delta^4$-isomerase catalyzes the oxidative conversion of Δ^5-3β-hydroxysteroids to the Δ^4-3-keto configuration and is essential for the biosynthesis of all classes of hormonal steroids, including progesterone, glucocorticoids, mineralocorticoids, androgens, and estrogens (Lachance *et al.*, 1990). In human, HSD3B maps to 1p13.1, which corresponds to bovine chromosome 3

FIG. 4. Fluorescence *in situ* hybridization of bovine metaphase chromosomes of DNA fragments cloned into bacterial artificial chromosomes. (**A**) 3β-Hydroxy-5-ene steroid dehydrogenase (95 kb) maps to chromosome 3. (**B**) BAC1 (325 kb) maps to chromosome 3. (**C**) BAC2 (310 kb) maps to chromosome 12. (**D**) BAC3 (300 kb) maps to the X chromosome. (**E**) BAC4 (285 kb) maps to chromosome 3. (**F**) BAC5 (275 kb) maps to chromosome 8.


A
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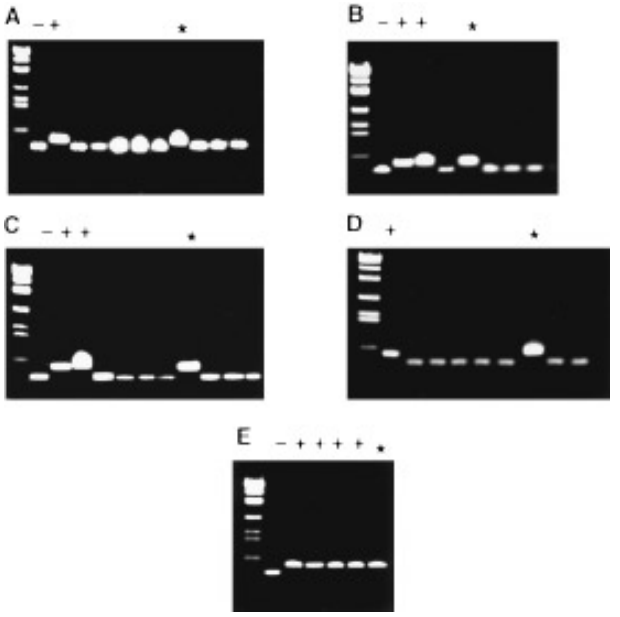

FIG. 5. PCR systematic screening of the bovine BAC library for clones containing the microsatellite marker ETH225. (−) indicates a negative control, (+) indicates a positive control, and (★) indicates a positive result from the library screening. The very small fragment found in all negative lanes is a primer-dimer formed in the absence of the true ETH225 amplification product. (**A**) Super DNA pool screening. (−) no DNA; (+) DNA from bull Y6, the source of the DNA for the BAC library; BAC superpools 1–9. Superpool 6 contains ETH225. (**B**) Single DNA pool screening. (−) no DNA; (+) Y6, superpool 6; plate 1–5 of superpool 6. Plate 2 contains ETH225. (**C**) Row DNA pool screening. (−) no DNA; (+) Y6, plate 2; row pools 1–8 from plate 2. Row 5 contains ETH225. (**D**) Column DNA pool screening. (+) plate 2; column pools 1–8 from plate 2. Column 6 contains ETH225. (**E**) Confirmation of clone identification. (−) no DNA; (+) Y6, plate 2, row 5, column 6, individual BAC clone 2.5.6.

(Fries *et al.*, 1993). Sequence data from human, rat, mouse, and bovine were used to design a pair of PCR primers (HSD3B-F, 5′ CCTCTCCATACCCATACAGCAA 3′; HSD3B-R, 5′ GGTTTCTGCTTGGCTTCCTCC 3′) within the exon IV region that amplified a 711-bp product. Reaction conditions were 93°C denaturing for 40 s, 56°C annealing for 30 s, and 72°C elongation for 1 min for 35 cycles. These PCR primers were used to screen the BAC library, and one 95-kb clone was identified and confirmed to contain the HSD3B gene by sequencing the PCR product. A small fragment library constructed from this BAC clone and containing 1400 clones (<600-bp insets) was screened with a $(GT)_{15}$ oligomer, and two microsatellites were found and were designated TEXAN23 and TEXAN27 (Table 2). There may have been more than two microsatellites in the 95-kb BAC clone since many fragments were excluded by the size selection in the construction of the microsatellite library.

The two microsatellite markers TEXAN23 and TEXAN27 were screened in the Angus × Brahman backcross and F_2 families and the data analyzed using CRI-MAP v2.4. Linkage analysis indicated that there were no recombinants for TEXAN23 and TEXAN27 in 193 informative meioses and that, as expected, they mapped toward the centromere of bovine chromosome 3 between RM19 and RM3 (Barendse *et al.*, 1994; data not shown). The zero recombination rate indicates that, as expected, these two microsatellite markers found in the 95-kb BAC clone are closely linked. Recombination studies indicate that 1 cM is about 10^6 bp for mammalian genomes (Shows *et al.*, 1980), and as TEXAN23 and TEXAN27 are separated by no more than 95 kb, or 0.095 cM, it is not surprising that no recombinants were detected in our mapping population.

To align the genetic and physical maps, fluorescence *in situ* hybridization was used to map the BAC clone containing HSD3B to bovine metaphase chromosomes. The FISH results (Fig. 4A) confirm that this BAC maps near the centromere of bovine chromosome 3.

End Rescue

To walk from genetic markers to a gene of interest, both BAC ends must be isolated to generate primers for rescreening the BAC library. Plasmid end rescue and IPCR methods were used to isolate the right and left ends of the HSD3B BAC clone. Figure 1 indicates the strategy that we followed. To isolate the right end, for a *Hin*dIII insert BAC library, *Sph*I, *Bam*HI, or *Sac*I was used to digest the insert DNA, and the resulting fragments were self-ligated under dilute conditions. After transformation, chloramphenicol resistance was used to select the clones that contained the right end of the BAC. The end clones from *Sph*I, *Bam*HI, or *Sac*I digestion were compared, and that with the smallest insert fragment was used for end sequencing. The end clone insert fragments varied from 9 to 25 kb and were sequenced with the reverse primer (5′ CAGGAAACAGCTATGACC 3′) located about 40 bp downstream of the *Hin*dIII cloning site. We attempted to use the T7 and Sp6 promoter sequences that flank the cloning sites of the pBeloBACII vector to cycle sequence the intact insert directly. However, due to the large insert size, copy number was too low in the sequencing reaction to produce reliable data. After plasmid end rescue, the smaller insert fragments (up to 25 kb) could be sequenced using the standard ABI cycle sequencing kit. Since the size of the insert was still fairly large, DNA was extracted, purified by ultracentrifugation through a CsCl gradient, and concentrated before cycle sequencing. Strong, clean signals were detected after CsCl purification. The right end sequence of the BAC clone containing the HSD3B gene is shown in Fig. 6A.

Plasmid end rescue could not be used to rescue the left end of inserts in the *Hin*dIII library due to the lack of convenient restriction sites, so IPCR was performed. Two pairs of primers were designed on the left side of the *Hin*dIII restriction site (see Fig. 1). For BAC1 and BAC2, six different restriction enzymes could be used for IPCR (*Ava*I, *Bam*HI, *Eco*RI, *Kpn*I, *Sac*I, and *Sma*I). The IPCR1 and IPCR2 primer pair was designed due to the inconsistency of the BAC1 and BAC2 primer

TABLE 1

Summary of the Results from Screening the Bovine BAC Library

STS	No. BACs	Sources
BOVIRBP[a]	0	Moore *et al.* (1991)
ETH225[b]	2	Barendse *et al.* (1994)
ETH1113[b]	0	Barendse *et al.* (1994)
Glucocerebrocidase	1	S. K. Davis and J. F. Taylor, Texas A&M Univ.
HSD3B	2	S. K. Davis and J. F. Taylor, Texas A&M Univ.
Butyrophilin	0	C. Taylor, Victorian Institute of Animal Science, Melbourne, Australia
Calpastatin	0	S. K. Davis and J. F. Taylor, Texas A&M Univ.
MHC class I gene	3	L. C. Skow, Texas A&M Univ.
BoLA class I	2	L. C. Skow, Texas A&M Univ.
MHC 21OH[b]	2	L. C. Skow, Texas A&M Univ.
Heat-shock protein 70.1	1	L. C. Skow, Texas A&M Univ.
DYα	1	L. C. Skow, Texas A&M Univ.
Leukemia inhibitor factor	4	J. Piedrahita, Texas A&M Univ.
Prolactin	1	K. L. Herring, S. K. Davis, and J. F. Taylor, Texas A&M Univ.
Growth hormone receptor	0	K. L. Herring, S. K. Davis, and J. F. Taylor, Texas A&M Univ.
IGF-1 receptor	4	K. L. Herring, S. K. Davis, and J. F. Taylor, Texas A&M Univ.
Growth hormone	1	K. L. Herring, S. K. Davis, and J. F. Taylor, Texas A&M Univ.
Placental lactogen	1	K. L. Herring, S. K. Davis, and J. F. Taylor, Texas A&M Univ.
Somatostatin	1	K. L. Herring, S. K. Davis, and J. F. Taylor, Texas A&M Univ.
BONRAMP[c]	2	J. W. Templeton, Texas A&M Univ.
NRAMP microsatellite[b]	2	J. W. Templeton, Texas A&M Univ.
Histamine H1 receptor	2	W. Barendse, CSIRO, Brisbane, Australia
Interferon-α receptor	0	W. Barendse, CSIRO, Brisbane, Australia
Glial fibrillary acid protein	3	B. W. Kirkpatrick, Univ. of Wisconsin
5-Hydroxy tryptamine receptor	2	B. W. Kirkpatrick, Univ. of Wisconsin
Retinoblastoma	0	B. W. Kirkpatrick, Univ. of Wisconsin
Coagulation factor 10	4	B. W. Kirkpatrick, Univ. of Wisconsin
Esterase D	1	B. W. Kirkpatrick, Univ. of Wisconsin
Collagen type 4α-1	1	B. W. Kirkpatrick, Univ. of Wisconsin
Fms-related tyrosine kinase 1	0	B. W. Kirkpatrick, Univ. of Wisconsin
Gastrin-releasing peptide	1	B. W. Kirkpatrick, Univ. of Wisconsin
PCCA[d]	0	B. W. Kirkpatrick, Univ. of Wisconsin
Plasmin activator inhibitor	1	B. W. Kirkpatrick, Univ. of Wisconsin
ACTH receptor	2	B. W. Kirkpatrick, Univ. of Wisconsin
TGLA49[b]	0	Barendse *et al.* (1994)
MAF46[b]	0	Bishop *et al.* (1994)
BM2113[b]	1	Bishop *et al.* (1994)
INRA006[b]	3	Bishop *et al.* (1994)
BM2924[b]	1	Bishop *et al.* (1994)
RM188[b]	2	Barendse *et al.* (1994)
CSSM14[b]	0	Moore *et al.* (1991)
BM6026[b]	1	Bishop *et al.* (1994)
BM2320[b]	2	Bishop *et al.* (1994)
SR-CRSP-5[b]	2	Arevalo *et al.* (1994)
BM47[b]	0	Bishop *et al.* (1994)
BM1443[b]	3	Bishop *et al.* (1994)

[a] Bovine interphotoreceptor retinoid binding protein.
[b] Microsatellite markers.
[c] Bovine natural resistance associated macrophage protein.
[d] Propionyl coenzyme A carboxylase.

pair. For IPCR1 and IPCR2, only *Eco*RI could be used. The IPCR product could be used directly for cycle sequencing using either primer. The IPCR product and left end sequence of the BAC clone containing the HSD3B gene are shown in Fig. 6B.

Chromosome Walking

After obtaining both end sequences for the HSD3B BAC, primers were designed for each end of the clone (Fig. 6). These PCR primers were used to rescreen the BAC library to find overlapping BAC clones. Two BAC clones were isolated for each primer pair, the original BAC containing the HSD3B gene and one new BAC that partially overlapped the original clone. Further analysis was performed on these three clones to characterize the degree of overlap. First, *Hin*dIII was used to cut the BAC DNA and examine these three clones for common restriction fragments. Second, DNA from the three BACs was amplified with five PCR primer pairs (primers for HSD3B, primers for the right and left end of BAC HSD3B, HSD3B-RW, HSD3B-LW, and primers

TABLE 2

Microsatellite Markers Closely Linked to HSD3B

Markers	Primer	Size (bp)	Annealing temperature (°C)	Alleles	Heterozygosity	PIC	Females	Males	Informative meioses
TEXAN27	F:5′ GCCCCTATTTCCTGTCTGTGGAAG 3′ R:5′ ACCCACTCCAGTATTCTTGCCCTGG 3′	216	57	2	0.4395	0.3655	99	124	193
TEXAN23	F:5′ TACTCTTAGGATAGGGACAACTCTG 3′ R:5′ TGGACACAACTGAAACAGCTTAG 3′	150	54	3	0.5931	0.5156	96	108	198

FIG. 6. (**A**) The right end sequence of BAC HSD3B. The M13 universal reverse primer was used for cycle sequencing. The positions of the HSD3B-RWF and HSD3B-RWR primers used for chromosome walking are indicated in boldface and underlined. (**B**) The left end sequence of BAC HSD3B produced by *Eco*RI digestion, self-ligation, and IPCR with the primers IPCR1 and IPCR2. The positions of the HSD3B-LWF and HSD3B-LWR primers used for chromosome walking are indicated in boldface and underlined.

for the two microsatellite markers (TEXAN23 and TEXAN27). Data are summarized in Table 3, and the resulting STS contig map is in Fig. 7. The left walking BAC clone (HSD3B-LW) contained the original HSD3B BAC clone. This clone was not in the library at the time of the first screening using the HSD3B primers. The HSD3B gene and the two microsatellite markers found in the original HSD3B BAC clone were not included in the right walking BAC clone (HSD3B-RW). This narrowed the distribution of the gene and the microsatellites to the nonoverlap region of clone HSD3B. Also, based on the restriction map, the right walking BAC clone was not completely covered by the left walking BAC clone. This model shows that chromosome walking could be rapidly performed in a BAC system, requiring about 1 week per step on both sides compared to 1 month for a YAC system.

DISCUSSION

One of the major goals of genome research in livestock species is the positional cloning and identification

TABLE 3

STSs Analysis for HSD3B and the Right and Left Overlapping BACs

STSs	HSD3B 95 kb	HSD3B-RW 120 kb	HSD3B-LW 130 kb
HSD3B F → R	+	−	+
HSD3B-RW F → R	+	+	+
HSD3B-LW F → R	+	−	+
TEXAN27	+	−	+
TEXAN23	+	−	+

of genes underlying economically important traits (economic trait loci or ETLs). The publication of two bovine genetic maps, the microsatellite/SSCP map produced by USDA-MARC at Clay Center, Nebraska (Bishop *et al.*, 1994) and the microsatellite/RFLP map produced by an international collaborative effort (Barendse *et al.*, 1994), cover approximately 90% of the bovine genome with an average map distance of 20 cM. The resolution of the integrated genetic map is now sufficient to allow the identification of chromosomal regions containing ETLs (Bishop *et al.*, 1994), and ETLs affecting Weaver disease (Georges *et al.*, 1993a), milk production (Georges *et al.*, 1993a, 1995), and horn development (Georges *et al.*, 1993b) have been localized. To move beyond this point, a library of large DNA clones must be available to bovine genome researchers for physical mapping of the corresponding regions and localization of the ETLs. A bovine YAC library of 21,500 clones with an average insert size of 750 kb (six haploid genome equivalents) has been constructed (Libert *et al.*, 1993). This library was successfully screened with six locus-specific markers; however, an analysis of nine of the resulting YACs indicated that 33% of the clones contained noncontiguous DNA. Despite the serious level of chimerism, this library would have provided an excellent resource for bovine genome research. However, this library is owned by the American Breeders' Service and is not currently publicly available (Mike D. Bishop, 1994, DeForest, WI, pers. comm.). Therefore, a bovine bacterial artificial chromosome (BAC) library was constructed.

The pure-bred Angus bull that provided the resource DNA for construction of the BAC library has produced two large full sib families by multiple ovulation and embryo transfer in matings to F_1 Brahman × Angus (21 calves) and Angus × Brahman (8 calves) cows, and DNA from the bull, his progeny, and their dams provides an important resource to accompany the BAC library. The partial bovine BAC library (23,040 clones with an average insert size of 146 kb and a 70% probability of containing a unique DNA sequence) combines 11,328 clones from the first size selection and 11,712 clones from the second size selection. DNA fragments were very stable in the BAC vector, and no chimeric clones were found among the 11 tested BAC clones. Although the average insert size was smaller than for the YAC library, the efficiency of construction (high transformation efficiency), ease of handling the cloned DNA, and very low chimerism and deletion rates make BACs an attractive complementary large DNA library to a YAC library for physical mapping by fluorescence *in situ* hybridization, for the isolation of closely linked polymorphic markers (such as microsatellites) as sequence-tagged sites for genetic mapping, and for the

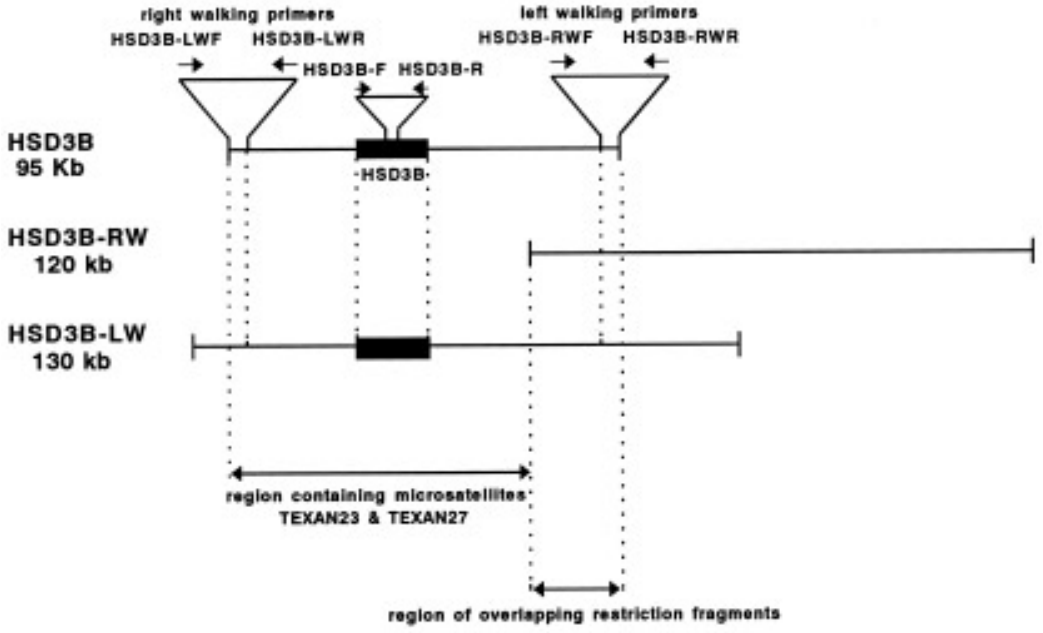

FIG. 7. The STS contig map for the chromosomal region surrounding the HSD3B gene. The right and left walking primers, the HSD3B gene primer, and two microsatellite primers were used to construct the map. The microsatellites were absent in the HSD3B right walking clone. The overlap RFLP region was determined by *Hin*dIII digestion of the three BAC clones.

isolation of regulatory sequences controlling gene expression.

A PCR systematic screening system was developed to test the coverage of the library (Green and Olson, 1990b). In comparison to traditional colony hybridization methods, this PCR-based method allows the rapid and efficient screening of an ordered large genome library due to the high sensitivity and specificity of PCR, particularly for systematic analysis employing many primer pairs. Single clones can be isolated using three PCR steps in 3 days. Also, PCR-based screening can provide sequence-tagged sites for type I anchor loci that provide unique reference points on genetic and physical maps.

The key feature of the PCR-based screening strategy is DNA pooling. The DNA pool concentration must be adjusted to ensure consistent amplification, even though differences in the growth rates of individual clones lead to their differential representation. Some DNA pools need to be purified since some samples contain higher concentrations of inhibitors of *Taq* polymerase activity (Green and Olson, 1990b). The DNA superpools in this study contained 480 BAC clones. This does not appear to be the upper limit for the maximum number of pooled clones, and preliminary results suggest that several thousand BAC clones can be combined into superpools without adversely affecting library screening (unpublished data). Fewer clones in the superpools reduces the problem of spurious PCR amplification products, but more PCR reactions are needed. Increasing the number of clones in the DNA superpools results in the dilution of the template sequence with nontarget DNA.

For 1 of 33 primers screened in the library, spurious PCR products were generated from superpools of BAC DNA. The reasons for this are not clear. The same phenomenon has been reported for YACs, and it has been speculated that these sequences are effectively amplified only when present at a relatively high concentration (Green and Olson, 1990b). The use of high-resolution nondenatured polyacrylamide gels to examine PCR products assisted in the confirmation of the PCR amplification. To confirm the identity of each PCR product, the most reliable and efficient method was to sequence the product directly.

The 3β-hydroxy-5-ene steroid dehydrogenase gene was physically and genetically mapped to bovine chromosome 3 by FISH and by linkage analysis and provides an additional anchor locus for comparative gene mapping across human, mouse, and other species to identify the boundaries of genomic conservation between evolutionarily divergent species (Schook *et al.*, 1991). Two microsatellite markers were isolated from one BAC clone containing HSD3B as sequence-tagged sites to align the genetic and physical maps. The generation of microsatellite markers closely linked to target genes by this method provides a useful tool for the detection of QTLs affecting economically important traits in segregating populations.

The current 20-cM bovine genetic map is of sufficient resolution to localize chromosomal regions containing ETLs, but not to allow chromosome walking using the BAC library. However, it is anticipated that the resolution of the bovine genetic map will be increased to 5 cM within 1–2 years. The feasibility of chromosome walking was tested in the bovine BAC library. Both ends of BAC clones could be sequenced, and new STSs could be generated to rescreen the library for overlapping clones. One-step chromosome walks in both directions were performed from the HSD3B BAC clone in the 70% bovine BAC library, and no repetitive sequences were found in the 300- to 500-bp end sequences. Three to seven successful chromosome walking steps from flanking markers using this bovine BAC library will cover an expected 584–1168 kb (0.58–1.2 cM) of the bovine genome and would represent a reasonable walking range.

The bovine BAC library is available for collaborative research. Interested scientists should contact Dr. S. K. Davis (e-mail: SKDAVIS@ZEUS.TAMU.EDU) or Dr. J. F. Taylor (e-mail: JTAYLOR@ZEUS.TAMU.EDU).

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