

Construction and characterization of an ovine bacterial artificial chromosome library

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Identification of regions of the genome that contain genes for economically important traits in livestock species and isolation of those genes so that they can be utilized in breeding programs requires high-density genome maps. Large-insert clones such as yeast artificial chromosomes (YAC), P1-derived artificial chromosomes (PAC), and bacterial artificial chromosomes (BAC) can be used to efficiently obtain accurate, high-resolution physical maps of eukaryotic genomes (Cai et al. 1995). YAC libraries containing clones with inserts up to 1 Mb in size have been constructed for humans (Albertsen et al. 1990), mice (Larin et al. 1993), rats (Cai et al. 1997), cattle (Libert et al. 1993), sheep (Broom and Hill 1994), and pigs (Rogel-Gaillard et al. 1997). However, YAC clones are often chimeric, consisting of DNA from different parts of the genome that have accidentally been combined into a single clone (Libert et al. 1993). This is a serious hindrance to physical mapping and chromosome walking because the true location of the gene of interest cannot be readily determined.

BAC libraries have been constructed for a number of plant species as well as for humans (Kim et al. 1996), mice (Research Genetics, Inc., Huntsville, AL), cattle (Cai et al. 1995), chickens (Zimmer and Gibbons 1997), goats (Schibler et al. 1998) and horses (Godard et al. 1998) with cloning systems based on the *Escherichia coli* F factor. BAC clones are stable, easy to manipulate, and are rarely chimeric (Shizuya et al. 1992; Cai et al. 1995). The only disadvantage of the BAC technology for chromosome walking is that the DNA inserts are somewhat smaller (100–300 kb) than those maintained by YAC clones. Despite this, BAC clones are becoming the vector of choice for physically mapping genes to specific chromosomal locations and for isolating genes by positional cloning.

In this paper, we present the construction and characterization of an ovine BAC library containing 59,904 clones with an average insert size of 103 kb, which corresponds to about two genome equivalents. The quality of the library was determined by PCR-based screening and fluorescence in situ hybridization (FISH). A BAC library covering the ovine genome will be a key resource for comparative gene mapping studies, for identifying quantitative trait loci (QTL) by positional cloning, and for functional studies to understand gene expression and regulation.

High-molecular-weight DNA for BAC library construction was prepared by encapsulating white blood cells from a purebred Suffolk ram in agarose microbeads which were digested with *Hind*III and separated by PFGE as described by Cai et al. (1995). To get a majority of DNA fragments in the size range of 50–250 kb, two-thirds of the microbeads used for library construction were digested with 0.75 U *Hind*III, and the remainder were digested with 1 U *Hind*III. This restriction enzyme was selected to avoid the

bulk of the repetitive centromeric DNA that is resistant to digestion with *Hind*III and remained in the compression band when the fragments were separated by CHEF gel electrophoresis.

The partial digests with 0.75 U and 1 U *Hind*III were pooled in a single lane for size selection by gel electrophoresis. The CHEF gel for size selection was electrophoresed in 1 × TAE rather than 0.5 × TBE because borate ions inhibit ligation. The pulse time was also altered to 90-s because a 50-s pulse time previously had been found to adversely affect the ligation efficiency of the fragments, presumably because the 5' overhang or phosphate group was lost. The lambda ladder, however, was not resolved when a 90-s pulse time was used. Consequently, the spread of fragments from 50 to 250 kb were excised from the low-melting-point agarose gel based on the mobility in the previous CHEF gel that used a 50-s pulse time. This problem is likely to account for the smaller (~100 kb) than expected average library insert sizes (~150 kb).

The excised gel was cut into four pieces. DNA from the three gel pieces containing the largest fragments was ligated to pBeloBAC11 and transformed by electroporation into DH10β *E. coli*. Colonies produced from each ligation were sized, and it was determined that the DNA in the second largest gel piece would be used for library construction. Many of the inserts obtained from the largest gel piece were actually smaller than those from the second largest piece and may indicate that the majority of fragments in that gel slice exceeded the cloning capability of the BAC vector. The transformation efficiency for the BAC clones was 1.5×10^6 cfu/μg or ~300 transformants from 1 μl of ligation product (i.e., ~600 colonies/plate). Two 100-μl ligations were required to produce sufficient colonies for the BAC library, and in total, 59,904 white colonies were picked.

Although a second round of size selection has been shown to give a more uniform insert size distribution across the library, it is accompanied by a 10-fold reduction in transformation efficiency (Cai et al. 1995). Consequently, the ovine BAC library was generated from DNA that was subjected only to a single size selection to maximize the efficiency of library construction.

The average insert size of the BAC clones was determined by CHEF gel electrophoresis after digestion with *Not*I. Occasionally, double bands were observed that migrated through the gel about 7 kb apart and hindered accurate sizing of the BAC clones. It was determined that the doublet was due to incomplete digestion of a *Not*I site on the vector since pBeloBAC11 is 7.4 kb, and the problem was overcome by reducing the amount of DNA used in the *Not*I digest to ~100 ng. In addition, very high molecular weight bands >500 kb were sometimes visualized. These bands were much fainter than the insert bands and were supercoiled or open-circular DNA (Wang and Lai 1995), again caused by incomplete digestion. In total 139 clones were selected at random, and the average insert size was determined to be 103 kb with a range from 25 kb to 410 kb (Fig. 1). This corresponds to two genome equiva-

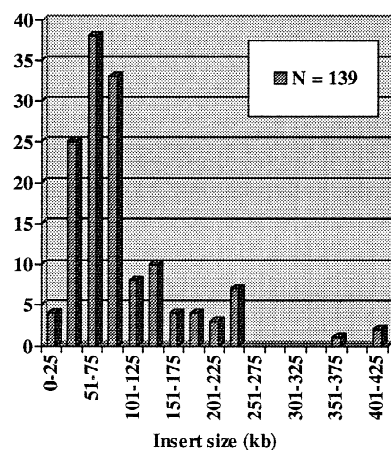


Fig. 1. Distribution of insert sizes in the ovine BAC library. The inserts were excised by digesting 100 ng BAC DNA with 10 U *NotI* (Promega, Madison, WI) at 37°C for 5 h. The whole digest was loaded onto a 1% agarose CHEF gel in 0.5 × TBE, and the fragments were separated by electrophoresis at 14°C for 16 h with an initial switch time of 1.4 s and a final switch time of 13.5 s. The insert size was determined by comparison with a lambda concatomer (Promega) and 250 ng *HindIII* lambda ladder (Promega) which were loaded in a single lane.

lents assuming that the ovine genome is 3×10^9 bp. Therefore, there is an 88% chance of finding any given sequence in the library.

Only one of the BAC clones that were selected for sizing failed to have an insert, suggesting that fewer than 1% of the clones in the library are empty clones. Many of the clones that were sized produced a single insert band. However, 32 inserts had at least one internal *NotI* site. *NotI* sites are indicative of CpG islands which are associated with all housekeeping genes and some tissue-specific genes in mammalian genomes (Aïssi and Bernardi 1991).

The BAC library was screened by PCR with primers for 69 microsatellite markers and genes (Table 1). Primers were selected so that at least one BAC clone was isolated for every sheep chromosome. To minimize the number of PCR reactions needed to

isolate an individual clone containing a sequence of interest, we pooled the BAC DNA. The first screen was of 78 superpools where each superpool contained DNA from eight plates of 96 BAC clones. In the second step, eight single pools, representing DNA from each of the eight plates in the positive superpool, were screened. Finally, the pooled row and column DNA from the positive plate were screened. The intersection of the row and column identified the location of the positive BAC clone. One or more positive superpools were found for 58 loci with an average of 2.1 positive superpools per locus (range 1–6). *IL5* and *IRF1* were both found within a single BAC clone. *DQB*, *DQA1*, and *DQA2* were not found in the library. It is expected that they are clustered within 100 kb of each other and, therefore, would represent a single missing BAC clone (J. Maddox, personal communication). If these three genes are treated as a single locus, then it can be estimated that there is an 85.5% chance of finding any sequence of interest in the ovine BAC library. This is comparable to the estimation based on average insert size.

It was important that the coverage of the ovine BAC library was evaluated by sizing inserts and by PCR-based screening. The presence of additional bands owing to incomplete digestion could lead to an over-estimation of insert size and, therefore, genome coverage if this was the only method used to characterize the BAC library. PCR-based screening of 69 markers and genes verified that the estimation made on the basis of size was accurate.

The insert sizes in at least two of the BAC libraries that have been constructed previously were smaller than expected (Cai et al. 1995; Frijters et al. 1997) with the average insert size of the clones below the size of the DNA that was selected to make the BAC libraries. The ovine BAC clones were also smaller than expected, but within the selected size range, with an average insert size of 103 kb instead of the intended 150 kb. The smaller than expected insert sizes means that at present there is an 88% chance of identifying an ovine BAC clone containing a sequence of interest. The BAC library will be expanded to ensure that there is a 99% chance of identifying any sequence. However, this is necessary only for functional studies where the exact sheep sequence is required. For physical mapping studies, if a locus is not present in the ovine library, then either the cattle (Cai et al. 1995) or goat (Schibler et al. 1998) BAC libraries could be screened and these clones used for comparative mapping.

Table 1. Summary of results from screening the ovine BAC library by PCR with primers for microsatellites and genes. The number of positive superpools for each locus (NF: not found) and the size of the individual BAC clone (ND: not done) that was isolated for a locus are listed.

Locus	No. of positive superpools	Insert size (kb)	Locus	No. of positive superpools	Size (kb)	Locus	No. of positive superpools	Size (kb)
<i>ADCYC</i>	6	100	<i>DRB1</i>	1	55	<i>MAF209</i>	2	ND
<i>BM1227</i>	2	75	<i>DRB2</i>	2	50	<i>MAF214</i>	1	25
<i>BM1303</i>	3	125	<i>DYB</i>	2	55	<i>MAF35</i>	3	150
<i>BM1329</i>	2	235	<i>FN1</i>	2	80	<i>MAF50</i>	NF	—
<i>BM1824</i>	3	250	<i>FSHB</i>	1	215	<i>MAF92</i>	3	90
<i>BM3413</i>	1	405	<i>GH1</i>	1	ND	<i>McM111</i>	2	ND
<i>BM4107</i>	2	155	<i>HUJ614</i>	1	ND	<i>MTNR1A</i>	NF	—
<i>BM415</i>	NF	—	<i>IFNG</i>	2	75	<i>OarEL01</i>	1	85
<i>BM4208</i>	4	30	<i>IGF1</i>	1	ND	<i>OarFCB48</i>	2	ND
<i>BM6438</i>	1	70	<i>IL1A</i>	NF	—	<i>OarHH64</i>	1	ND
<i>BM6526</i>	5	80	<i>IL2RA</i>	2	75	<i>OarJMP58</i>	4	55
<i>BM719</i>	2	100	<i>IL3</i>	4	60	<i>OarJMP8</i>	2	135
<i>BRN</i>	3	ND	<i>IL5</i>	3	95	<i>OarVH110</i>	2	ND
<i>CAPN2</i>	NF	—	<i>IL6</i>	1	195	<i>OarVH116</i>	1	250
<i>CD3D</i>	2	240	<i>ILSTS005</i>	3	ND	<i>OarVH72</i>	4	75
<i>CD5</i>	NF	—	<i>ILSTS008</i>	3	45	<i>OB</i>	2	ND
<i>CSR241</i>	4	30	<i>ILSTS011</i>	2	75	<i>OCAM</i>	2	125
<i>CSR270</i>	1	85	<i>ILSTS043</i>	1	130	<i>PRL</i>	1	55
<i>CSR287</i>	1	230	<i>IRF1</i>	1	95	<i>RJH1</i>	NF	—
<i>CSSM43</i>	1	40	<i>KAP1.1</i>	2	105	<i>SCYA</i>	2	170
<i>DQA1</i>	NF	—	<i>KAP6</i>	2	ND	<i>SRY</i>	4	90
<i>DQA1</i>	NF	—	<i>KRT2.13</i>	3	30	<i>TGLA122</i>	4	ND
<i>DQB</i>	NF	—	<i>LGB</i>	1	95	<i>TGLA357</i>	NF	—

Table 2. Physical map locations of ovine BAC clones localized to G-banded metaphase chromosomes by fluorescence in situ hybridization. Each BAC was isolated by PCR with a standard 25- μ l PCR reaction consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 125 μ M dCTP, 125 μ M dATP, 125 μ M dTTP, 125 μ M dGTP, 25 pmol forward primer, 25 pmol reverse primer, 1.5 mM MgCl₂, 100 ng DNA template, and 0.5U *Taq* DNA polymerase (Life Technologies). The primers, expected product size, and annealing temperatures (T_A °C) that were used in PCR are listed. The number of metaphase spreads that were scored are in parentheses.

Locus	Primers	Product size	T _A	Physical location	Locus	Primers	Product size	T _A	Physical location
<i>BM1824</i>	5' GAGCAAGGTGTTTTTCCAATC 3' 5' CATTCTCCAACGTCTCTCTTG 3'	180–192bp	55	1q4.2	<i>FSHB</i>	5' TGGGATATAGACTTAGTGCC 3' 5' CAGTTTCAAGGCTACATGGT 3'	~200bp	50	15q3.3–3.4
<i>FN1</i>	5' TCCAAACCAGAAAACAAGCGT 3' 5' GATCCCAATCCAAATCCAAAGT 3'	284bp	50 ^b	2q4.1	<i>MAF2/4</i>	5' GGGTGATCTTAGGGAGGTTTTGGAGG 3' 5' AATGCGAGGATCTGAGGCGAGGCG 3'	182–250bp	55 ^c	16q1.7
<i>IFNG</i>	5' TTGTGACTGTAGCTAGATGTGTT 3' 5' ATACACATATTTGCCCATCTTTT 3'	124–128bp	52 ^e	3q1.4	<i>OarrFCB48</i>	5' GACTCTAGAGGATCGCAAGAACCAG 3' 5' GAGTTAGTACAAGGATGACAAGAGGCCAC 3'	143–167bp	55	17q2.1–2.3
<i>IL6</i>	5' CTAAGAATGAGCGTTAGGAC 3' 5' GAGGTAAGCTACACATTTCA 3'	126bp	50	4q1.3–1.4	<i>TGLAI/22</i>	5' AATCACATGGCAAAATAGTACATAC 3' 5' CCTCCTCCAGGTAATCAGC 3'	137–173bp	55	18q1.2–1.4
<i>IL5</i>	5' GACAGTTTTCTACGATGCTTATTGTC 3' 5' TCTCCTCCAGAACTTTCCAGTACAG 3'	179bp	45	5q1.5	<i>BMI303</i>	5' CTTGGGAAAATGGCAGC 3' 5' CTCGCCGCTTCCCTCTC 3'	131–147bp	55 ^a	19q1.2–1.3
<i>BM1329</i>	5' TTGTTAGGCAAGTCAAAGTC 3' 5' AAGACCGCAGCTTCATCC 3'	145–161bp	55 ^a	6q1.6–1.8	<i>DRB1</i>	5' TGTGCGGCGGCGAGGTGAG 3' 5' CGTACCCAGAGATTGAGTGAAGTATC 3'	289bp	55	20q2.2–2.3.1
<i>ILSTS005</i>	5' GGAAGCAATGAAATCTATAGCC 3' 5' TGTCTGTGAGTTTGTAAAGC 3'	~180bp	50	7q3.6–3.8	<i>OCAM</i>	5' CTGACTATAATGTACAGATCCCTC 3' 5' GCAGAAATGACTAGGAGGATGGCA 3'	181–185bp	50	21q2.1–2.2
<i>BM4208</i>	5' TCAGTACACTGGCCACCATG 3' 5' CACTGCATGCTTTTCCAAAC 3'	154–174bp	55 ^b	8q2.3–2.5	<i>MAF92</i>	5' TAGAATGTCATGTTCTCAGCATTCCTC 3' 5' TCAAGAAATTTGGACACAAATCTGG 3'	110–116bp	60 ^f	22q2.3–2.4
<i>ILSTS011</i>	5' GCTTGCTAGTGGAAAGTGC 3' 5' CTAAGATGAGAGCCCTACC 3'	250bp	55 ^a	9q2.1	<i>MAF35</i>	5' AACCCATGAATCATCTAACTACCTC 3' 5' AGTTACAATGCAAGCATCATACCTG 3'	98–114bp	50	23q1.7–2.1
<i>CSRD287</i>	5' ACAAGGGCATGACTGAGCAACTA 3' 5' GGAGGGATTCAGTGCATCGTA 3'	145–171bp	60 ^d	10q1.3	<i>ILSTS043</i>	5' TATTCAAAGTACAGACGCC 3' 5' TAGGGAATGGCGAAATTTGCC 3'	~150bp	55 ^f	24q1.2–1.3
<i>KAP1.1</i>	5' TATGACAAAACAAGCCAGGG 3' 5' GAGTTGGGTTGAGGAGGG 3'	329bp	63 ^f	11q3.1	<i>OarrVH72</i>	5' GGCCCTCAAGGGCAAGAGCAGG 3' 5' CTCTAGAGGATCTGGAATGCAAGCTC 3'	121–135bp	55	25q1.3–1.4
<i>BM719</i>	5' TTCGCAATGGCTAGAGG 3' 5' CACACCTAGTTGTAAAGCAGC 3'	147–155bp	55 ^a	12q2.2–2.3	<i>BM6526</i>	5' CATGCCAAACAATATCCAGC 3' 5' TAGAGGTAGAGCAAGCAGC 3'	142–172bp	55	26q1.2–1.3
<i>IL2RA</i>	5' AGCAGGGTACAGGTGGTAAAGCA 3' 5' GATATGCTTTGGAAAGGTAGCGTAT 3'	172–192bp	50	13q1.3	<i>CSRD241</i>	5' TAACACGGCAACAGCCCTTTCA 3' 5' GTCTGTTTGGTAAACAGATAGCA 3'	144–164bp	50	Xq1.1–1.4
<i>CSRD270</i>	5' TTGCTGAGCCAACTCTTACAGT 3' 5' TGCCTGCAGCTGGTTGTATTAG 3'	205–233bp	55	14q1.3–1.4	<i>SRY</i>	5' TGCCAGAGGATTGAGGG 3' 5' CAGAGGAGCAGTTATTTTGG 3'	1009BP	63 ^f	Yp1.1–1.2

^a 12.5 pmol each primer.

^b 5 pmol each primer.

^c 0.5 pmol each primer.

^d 2.5 pmol each primer.

^e 3.5 mM MgCl₂.

^f 2 mM MgCl₂.

An individual BAC clone was isolated for each locus that had at least one positive superpool. The identity of every isolated BAC clone was confirmed by sequencing prior to physical mapping (Table 1). The sequences of PCR products generated from the BAC clones were compared with published sheep or cattle sequences and in all cases shared >85% sequence identity with the published data (85–100% sequence identity for microsatellites and 93–100% for gene sequences). A number of single nucleotide polymorphisms were identified in the DNA flanking microsatellites, particularly when cattle microsatellite primers were used; this explains the lower sequence identity observed for these sequences.

To estimate the number of chimeric clones in the BAC library, all the BAC clones that were isolated by PCR-based screening were physically mapped by fluorescence in situ hybridization (FISH) as described by Cai et al. (1995). The sheep metaphase chromosomes were stained with 5 µg/ml propidium iodide and mounted with 100 µl p-phenylene diamine dihydrochloride (pH 11) antifade solution, which produces G-bands (Lemieux et al. 1992). At least one BAC clone was mapped to every ovine chromosome. The BAC clones that were physically mapped by FISH to ovine metaphase chromosomes ranged in size from 25 kb to 405 kb, and no chimeric clones were detected. The physical location of one BAC clone for every sheep chromosome was determined (Table 2), and these clones can be used to unambiguously identify the chromosomes in future FISH experiments for localization of previously unmapped genes.

The human and mouse genomic maps are well developed and consist of over 10,000 and 7000 microsatellites, respectively (Dib et al. 1996; Dietrich et al. 1996). In addition, over 5000 genes have been mapped in humans, and 4000 have been mapped in mouse. By comparison, the genetic maps of livestock animals are less well developed. Second-generation genetic linkage maps have been developed for cattle (Kappes et al. 1997; Barendse et al. 1997) and sheep (de Gortari et al. 1998), and a first-generation map is available for goat (Vaiman et al. 1996).

The ovine genetic linkage map consists of 519 markers, and every chromosome has been oriented either by direct physical assignment in sheep or indirectly through the homologous bovine chromosome (de Gortari et al. 1998). In total, 91 loci have been physically assigned by in situ hybridization in sheep, with 25 acting as anchors for 16 chromosomes (SheepGBase, AgResearch). We have physically assigned an additional 28 loci, using ovine BAC clones, and every sheep chromosome is now anchored.

Since it is unlikely that the number of markers on the livestock maps will increase greatly in the immediate future (Kappes et al. 1997; de Gortari et al. 1998), it is important that the physical mapping effort continues. Markers for quantitative trait loci need to be localized to specific chromosome bands so that comparative mapping information from humans and mice can be used to identify candidate genes on the basis of location and function. The isolation and physical assignment of ovine BAC clones for these markers will greatly assist positional cloning. The ovine BAC clones will be mapped simultaneously in sheep, cattle, goat, and deer so that there are common comparative anchor loci present on each genome map.

The ovine BAC library will be a powerful tool for comparative mapping and the positional cloning of economically important traits. BAC clones containing genes of interest could be modified and expressed in mammalian cells, and they could be used in transgenics programs. The ovine BAC library will enable genome organization, gene regulation, and chromosome evolution in closely related species to be investigated and is available for collaborative research by contacting the authors.

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