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Transfer of an expression YAC into goat fetal fibroblasts by cell fusion for mammary gland bioreactor

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Abstract

Yeast artificial chromosomes (YACs) as transgenes in transgenic animals are likely to ensure optimal expression levels. Microinjection of YACs is the exclusive technique used to produce YACs transgenic livestock so far. However, low efficiency and high cost are its critical restrictive factors. In this study, we presented a novel procedure to produce YACs transgenic livestock as mammary gland bioreactor. A targeting vector, containing the gene of interest—a human serum albumin minigene (intron 1, 2), yeast selectable marker (G418R), and mammalian cell resistance marker (neo^r), replaced the α-lactalbumin gene in a 210 kb human α-lactalbumin YAC by homogeneous recombination in yeasts. The chimeric YAC was introduced into goat fetal fibroblasts using polyethylene glycol-mediated spheroplast fusion. PCR and Southern analysis showed that intact YAC was integrated in the genome of resistant cells. Perhaps, it may offer a cell-based route by nuclear transfer to produce YACs transgenic livestock.

Keywords: Yeast artificial chromosome; Gene targeting; Cell fusion; Transgenic livestock

For conventional transgenic construction, the unpredictability of expression because of position effects has proved problematic [1]. Yeast artificial chromosomes (YACs) can overcome position effects in most cases by providing all regulatory sequences needed for the establishment and maintenance of the expression domain to ensure position-independent, copy-number-dependent, and optimal expression levels in the transgenic animals [2]. However, they were mainly applied on mice and rats because of the low efficiency of YACs microinjection. Transgenic livestock as bioreactor are essential to produce recombinant proteins [3]. Although lipofection [4], microinjection [5], and spheroplast fusion [6] have been developed to produce YACs transgenic mice or rats, microinjection is the only practical technique producing YACs transgenic livestock because ES cells from them have not yet been isolated successfully. In addition to intensive labor and tiresome manipulation on the isolation of YACs from yeasts, the low integration efficiency (<5%) and few intact YACs (20–70%) [2] in the genome of organism by microinjection are large obstacles to produce YACs transgenic livestock. They critically increased the cost.

With the development of gene targeting and cloning technologies by nuclear transfer (NT) from cultured somatic cells, they have been applied to express some foreign proteins in transgenic animals. However, expression level has not been high enough (600 μg/ml) [7]. Therefore, efforts have been taken for gene targeting in the mammary-specific loci [8]. But, up to now, it has not been reported that gene targeting at any mammary-specific expression locus in livestock has been completed

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yet. To circumvent the elusive gene targeting in somatic cell and to use the gland-specific expression domain to express foreign proteins, we designed this novel procedure. In this report, to express foreign protein in the α-lactalbumin locus, we sought to combine high efficient gene targeting in yeasts with the somatic cell NT technique to produce YACs transgenic goat. In other literatures, the majority of YACs were integrated in intact form after fusion, and when yeast DNA along with YACs was introduced into mice by ES cells, adverse effects were not observed [6,9]. Moreover, the potential breakage and intensive labor are avoided on the manipulation in vitro. So, we selected yeast spheroplast fusion with somatic cell as the introduction of YACs into cells. A 210 kb human α-lactalbumin YAC vector (hLA-YAC) used in this study has been confirmed the characteristics of position-independent and high-level expression with precise tissue specificity for α-lactalbumin (1.4–4.9 mg/ml) [10] and human growth hormone (1.7–8.9 mg/ml) [11] in transgenic rats. The introduced YAC into goat fetal fibroblasts showed complete and intact transfer by PCR and Southern analysis of different regions of the YAC.

Materials and methods

Preparation of primary goat fetal fibroblasts. A 35-day female fetus was surgically removed from a female goat's uterus, cut into small pieces, and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1 mmol/L sodium pyruvate (Sigma), 1× non-essential amino acids (Gibco), 20 mg/L basic fibroblast growth factor (Sigma), and 10 mg/L insulin (Sigma) at 37 °C in 75 cm^2 tissue culture flasks (Falcon) in a humidified atmosphere of 5% CO_2. When cells reached confluency, they were collected by trypsinization and frozen in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO, Sigma).

Culture of yeasts. Saccharomyces cerevisiae strain carrying hLA-YAC (724C3), AB1380 (*MAT*a *ura*3 *lys*2-10*c ade*2-101*c trp1 his5am can*1-100*oc*$^-$) was isolated from the CEPH library. Yeasts were grown on synthetic standard selective medium without uracil (Ura$^-$) and tryptophan (Trp$^-$) (AHC, Clontech) or complete medium (YPD, Clontech). YPD agar plate was supplemented with 250 mg/L G418 (Gibco).

Analysis and identification of hLA-YAC. Preparation of high molecular weight DNA plugs from yeast spheroplasts for pulsed-field gel electrophoresis (PFGE) was accomplished according to the manufacturer's protocol (Bio-Rad). PFGE was carried out on CHEF-DR III (Bio-Rad) using 1% agarose in 0.5× TBE at 6 V/cm and 14 °C for 25 h with a switch time of 60–120 s. The primer pairs p1-p2 (GC CTAGATGCTTTCATAC and AAGGCTCAGAGACAGATAAG) were designed according to the reported human α-lactalbumin gene sequences (GenBank Accession No. X05153) for PCR.

Construction and transformation of targeting vector. The replacement-targeting vector pLAhSAneoG418 comprised a 958 bp region of the 5′ end of the human α-lactalbumin gene from the translational initiation codon, a 4.2 kb human serum albumin minigene containing intron 1, 2 (minihSA), a portion of the bovine growth hormone gene containing the polyadenylation site and a mammary resistance cassette (neo^r) from *Apa*I–*Sal*I 2284 bp fragment of pcDNA3 (Invitrogen), a 1.5 kb *Sac*II region of yeast resistance cassette (G418R) from pNK-G418, a 6.1 kb of the vector pRS403 (Stratagene), and a 702 bp region of the 3′ end of the human α-lactalbumin gene from the translational stop site (Fig. 2). These two homologous regions were amplified by two primer pairs: UpLa5 and DownLa5 (CCGGAATTCGCCTA GATGCTTTCATACAGG, *Eco*RI, and TCCCCCGGGTTTGGCTA CCCCCAAGAACCT, *Sma*I), UpLa3 and DownLa3 (ATTGGGCCC ATCCCAGGGAAATGAAGGAAG, *Apa*I, and CCGCTCGAGAA GGCTCAGAGAATAAG, *Xho*I), respectively.

The *Xho*I linearized pLAhSAneoG418 transformed AB1380 containing the hLA-YAC by the lithium acetate method [12]. The transformed yeasts were plated onto YPD agar plates and incubated at 30 °C for 4 days. Single colonies transferred on AHC plates were picked and amplified to analyze further whether to accomplish homologous recombination between targeting vector and hLA-YAC or not.

Identification of homologous recombination. Four primers were designed to verify proper homologous recombination. Primers TA1 (CTCCTGGGCTCAAGTGAT) and TA2 (CATCCCTGGAAAA TAGTCT) were located in the adjacent upstream or downstream of left or right targeting arms of α-lactalbumin gene, and primers hSA1 (TGGAATAAGCCGAGCTAA) and RS1 (AGTCACGACGTTG TAAAA) were located in the minihSA and pRS403 vector in the targeting vector (Fig. 2). Fresh single transformed colonies were treated as PCR templates by quick SDS extraction protocol [13]. Primer pairs TA1 and hSA1, RS1 and TA2 were amplified, respectively.

High molecular weight yeast DNA was extracted for Southern analysis according to the protocol described by Cocchia et al. [14].

Fusion of yeasts and goat fetal fibroblasts. Yeasts were fused to goat fetal fibroblasts by a modification of the procedures described by Pachnis and Pavan [15,16]. Yeasts were grown in AHC medium to stationary phase and then diluted in 50 ml YPD medium with 1 ml for overnight. Yeasts were pelleted, washed once in 20 ml ddH_2O and SPE (1 mol/L sorbitol (Sigma), 10 mmol/L Tris–HCl, pH 7.5, 10 mmol/L EDTA, and 14 mmol/L of 2-mercaptoethanol), respectively, and suspended in 20 ml SPE, and the cell walls were digested by adding 400 μl of a 10 mg/ml zymolyase 20T (ICN) stock and the yeasts were incubated at 30 °C until 95% of the yeasts became spheroplasts (approximately 10 min). Spheroplasts were pelleted at 100*g* for 5 min, washed twice in 10 ml SPE, and counted. During spheroplast washes, fetal fibroblasts at 90% confluence were trypsinized, harvested, and washed three times in DMEM without FBS, a total of 2×10^6 fibroblasts were pelleted at 100*g* in a 15 ml polystyrene tube, the medium was decanted, and 1×10^8 yeasts in SPE were layered on top and pelleted at 100*g* for 5 min. The pellet was gently suspended in 50 μl DMEM supplemented with 1 mol/L sorbitol, pelleted, and the supernatant was decanted. Five hundred microliters of PEG (50% polyethylene glycol 1500 (Boehringer–Mannheim), 50 μmol/L of 2-mercaptoethanol, 5 mmol/L $CaCl_2$, and 5% dimethyl sulfoxide) was added in 1 min and the pellet was gently resuspended. After keeping for 1, 2, or 3 min at room temperature, DMEM without FBS was added very slowly, approximately 1 ml per 1 min in the first 3 min, then diluted PEG with another 7 ml and mixed by inversion. The tube was placed in an incubator at 37 °C for 30 min and the fusion mixture was collected by centrifugation at 100*g* for 5 min. The pellets were suspended in normal cell culture media DMEM and then plated onto ten 10 cm dishes (Falcon). Twenty-four hours later, plates were washed three times with phosphate-buffered saline (PBS), and 800 mg/L G418 was added to medium at 48 h post fusion. Cells were refed every 3 days, and cell clones typically appeared under the microscope after 8 days. Well-separated cell clones were isolated with cloning cylinders, expanded, and cryopreserved by standard procedures.

Identification of the integrity of the modified YAC in the cell genome. We designed eight primer pairs to amplify different regions for analysis of the integrity of the hLA (hSA)-YAC introduced into the genome of fetal fibroblasts. Primer pairs for detecting the right arm (CGATAAGCTTTAATGCGGTAG and TGCGCCTTAAACC AACTTG) and the left arm (CTTCCATTCACTTCCCAGAC and

CTCTCCCTTATGCGACTCCT) were designed according to the GenBank database (Accession No. XXU01086). Primers of −20 kb region (CATGCAAGACTCTCTCACTG and CTCTGTTGTTCTT CTTGGG), −50 kb region (CTTTCTCTGCTCCTTTCTCC and CA TAATGTTCTTCCAATCCG), upstream from the translational start site of the α-lactalbumin gene, were based on the reported sequences from GenBank database (Accession No. AC079951). Primers of +20 kb region (AGGAGTTCCAGTTAGGGTTG and AAGGCA CCTATTGTGTCTCC), and +50 kb region (GTTCTAGGGTCAA CTGCAGG and GGCAACAACCAACACAGAC), downstream from the transcription start site of the α-lactalbumin gene, were designed according to the GenBank database (Accession No. AC089987). Primer pairs of the part fragment of neo^r (ATGATT GAACAAGATGGATT and CTCAGAAGAACTCGTCAAGA) and minihSA (ACGCAAAGAGAATGGTAG and CACTAAGGAAA GTGCAAAG) were designed according to the sequences of pcDNA3 (Invitrogen) and GenBank database (Accession No. M12523).

Genomic DNA was extracted from cells for PCR and hybridization according to standard protocol [15].

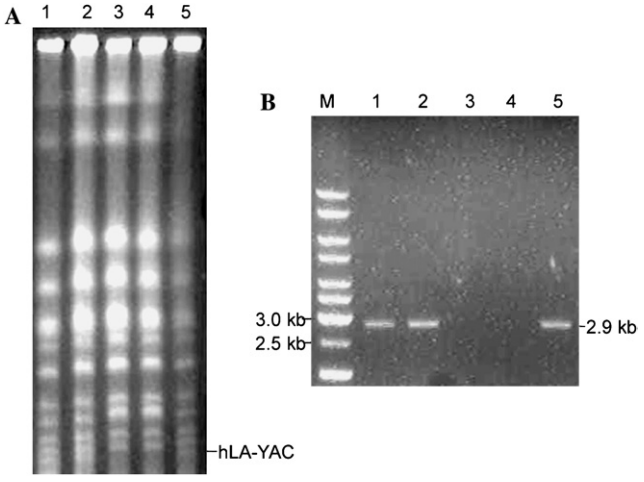

Fig. 1. Identification of the hLA-YAC. Analysis of PFGE karyotypes for the hLA-YAC in yeasts at 6 V/cm, 14 °C, and 25 h with a switch time of 60–120 s (A). Colony PCR analysis of hLA-YAC (B). AB1380 containing 210 kb hLA-YAC (lanes 1, 2, and 5); parental AB1380 (lanes 3 and 4); M, 1 kb DNA ladder (MBI Fermentas).

Results

Identification of the hLA-YAC in yeasts

To identify whether the hLA-YAC existed in yeasts, PFGE was completed. After ethidium bromide staining, there was an additional band in the yeasts compared to the parental yeast AB1380 (Fig. 1A). Colony PCR using primers p1-p2 was performed and the existence of human α-lactalbumin gene was verified (Fig. 1B). The results showed that the YAC in our experiment was the same as yLALBA (210) used in the experiments of Fujiwara et al. [10,11] from the same library.

Modification of the hLA-YAC

To use regulatory elements of α-lactalbumin locus in the hLA-YAC to control minihSA expression, we expected that the yeast integrating vector pLAhSA-neoG418 replaced the α-lactalbumin gene. Proper homologous recombination was first verified using the amplified fragments by the PCR method using primer pairs TA1 and hSA1, TA2 and RS1, respectively (data not shown). Two primer pairs spanned the 5′ or 3′ homologous arms, respectively.

Thereafter, random 10 colonies identified as positive by PCR were selected for further hybridization analysis (Fig. 3). The Southern analysis result showed that there were expectant homologous recombination by the 11 kb fragment, multiple copies of targeting vector fragment in a head-to-tail arrangement by the 13.8 kb, and more than one hLA-YAC in parental AB1380 by the 7 kb.

Fusion of yeasts and fibroblasts

The G418^r colony 1 (Fig. 3) was selected for fusion to fetal fibroblasts. In a specific experiment, the same samples of 1×10^8 yeast spheroplasts and 2×10^6 fibroblasts

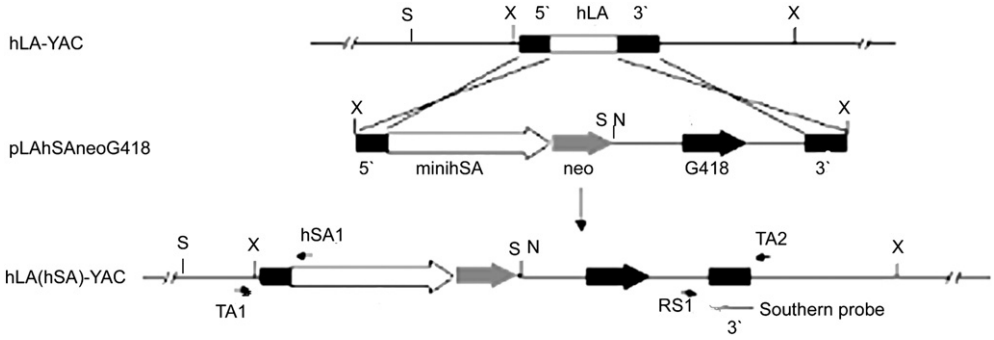

Fig. 2. Site-specific integration of the targeting vector pLAhSAneoG418 into the hLA-YAC. The pLAhSAneoG418 was linearized with *Xho*I and transformed into yeasts carrying the hLA-YAC. The α-lactalbumin gene was replaced with the mini-hSA by homologous recombination. The resulting YAC is denoted as hLA(hSA)-YAC. PCR primers, restrictive sites, and Southern hybridization probe are indicated. N, *Not*I; S, *Sal*I; and X, *Xho*I.

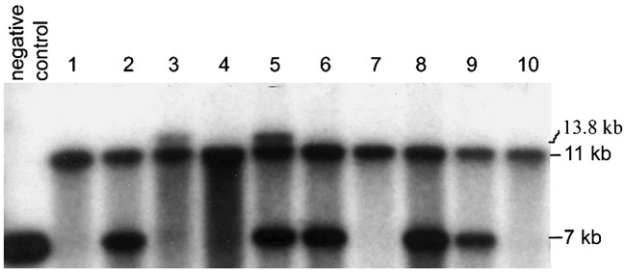

Fig. 3. Southern analysis of the modified YACs. Genomic DNA samples after digestion by *Not*I–*Xho*I hybridized with 3′ targeting arm probe. Arrows indicate the 7 kb fragment from the non-targeted α-lactalbumin locus, the 11 kb fragment from the homologous recombination in this locus, and the 13.8 kb fragment from multiple copies of targeting vector in a head-to-tail arrangement in this locus.

were fused in three different PEG incubation time-points: 1, 2, or 3 min. Thirty-five cell clones were seen at an exposure time of 1 min, 24 at 2 min, and 6 at 3 min on the twelfth day after fusion. At 1 min, we obtained 56, 7, and 43 cell clones in other three experiments. The average number was approximately 36 per 2×10^6 cells at 1 min.

Identification of cell clones

To verify the intact YAC transfer into the genome of cell clones, PCR analysis from eight different primer pairs showed that all the cell clones appeared positive signal except for +50 kb region. Moreover, when parental yeast genomic DNA carrying hLA-YAC was used as template, PCR using primer pairs of +50 kb region did not also give any positive signal. This is consistent with the chimeric explanation of the hLA-YAC described by Fujiwara et al. [17]. Southern analysis using different probes showed that the four cell clones contained an intact copy of the chimeric YAC (Fig. 4).

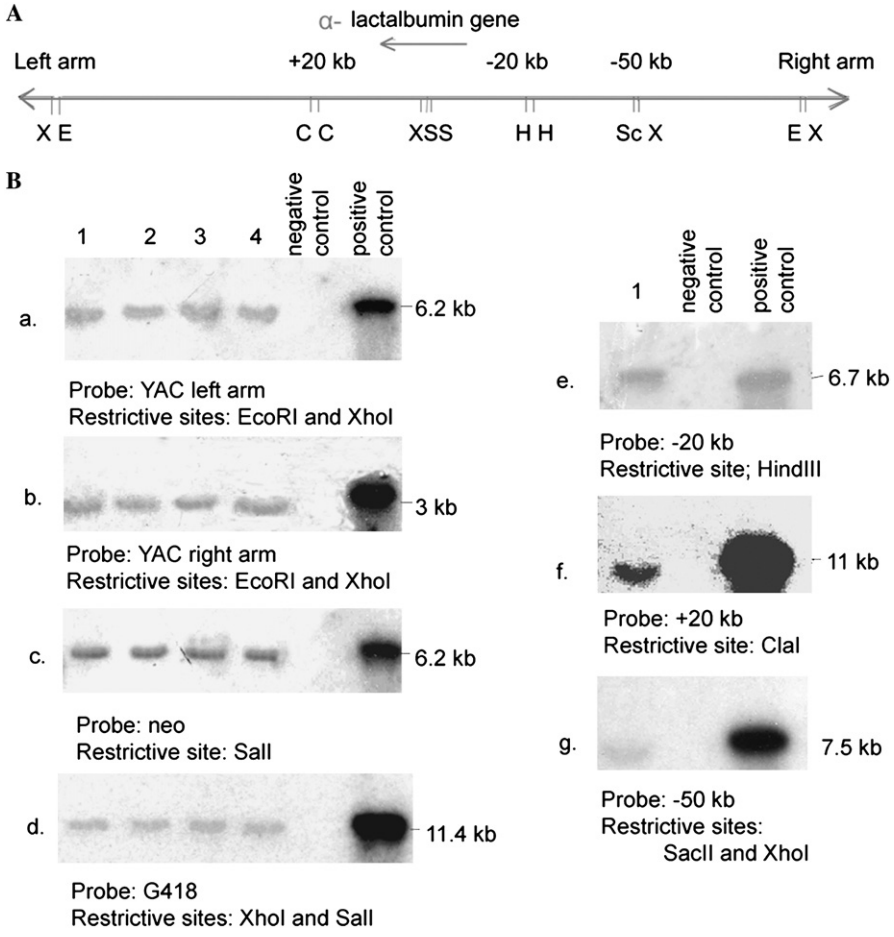

Fig. 4. Partial restriction sites of the modified YAC and Southern analysis of fusion cell clones. (A) The direction of the human α-lactalbumin gene in the hLA-YAC is indicated by an arrow. Restriction sites for the hLA(hSA)-YAC integrity analysis by Southern analysis are shown. C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sc, *Sac*II; and X, *Xho*I. (B) Each lane contained genomic DNA digested with corresponding restriction enzymes and hybridized with corresponding probes. Expected results were achieved. Lanes 1–4, cell clones; negative control, non-fusion cells; and positive control, yeasts containing the modified YAC.

Discussion

The aim of the study was to investigate the feasibility of producing YAC transgenic cell clones and for preparation to produce YAC transgenic goat by NT for mammary gland bioreactor to express human serum albumin. To ensure tissue-specific expression in the mammary gland, we selected the positive yeast colony 1 (Fig. 3) which did not contain any multiple copy integration fragment in the modified YAC and any unmodified YAC to fuse to fibroblasts. Due to only hundreds of base pairs in the 5′ targeting arm of the integration vector, this cannot direct the mammary-specific and high-level expression of the foreign gene in a head-to-tail arrangement in vectors. Meanwhile, because both the 50 kb upstream and 50 kb downstream regions of the α-lactalbumin gene are necessary for position-independent expression [18], the main analyses are on the region in our study.

Although a few groups have reported the successful production of different cell lines after fusion with spheroplasts [6,15,16,19,20], all cultured cells were immortal cell lines. It was reported that some of the mammalian cell lines could fail to fuse with spheroplast successfully [21]. Therefore, cell fusion was a bottleneck for this procedure. Fusion events can cause a loss of viability prior to cell attachment [19]. In our experiments, we found that the proliferative capacity of primary cultured cells was the most important factor to the success of fusion and the success varied among primary goat fibroblasts from different fetuses. The average efficiency of fusion was approximately 36 per 2×10^6 fibroblasts from the fetus described in Materials and methods, which was comparable to those of mouse embryonic stem (ES) cells (36, 7, or 11 per 2×10^6) [6,9] and human renal carcinoma cells (21–50 per 2×10^6) [20], but was lower than those of mouse L cells (100 per 2×10^6) [15]. Moreover, as the result of our experiment, there was a high percentage of intact YAC introduced into the cells (40–100%) [6,9,15]. In the lipofection approach, however, only about three transfectants of ES cells from 240 (approximately 1%) contained intact YAC [4]. So, far more colonies must have been picked for the analysis of intact YAC in lipofection than in fusion. In addition, as mentioned earlier, the low integration efficiency (1.5–4.1%) [11] of YAC microinjection critically increased the cost in producing YACs transgenic livestock compared to NT [22]. So, fusion appears to be a relatively simple and effective method.

Exposure time in PEG was another important factor. The optimal exposure time of different cell lines could be inconsistent [15,19,20]. Among 1, 2, and 3 min, 1 min was optimal to the fusion. It is the first time that primary cultured cells fused successfully with spheroplasts.

It was reported that transchromosomic cattle have been produced from fibroblasts fused with DT40 cells by NT to express human polyclonal antibodies [23]. We believe that this novel procedure described here will be proved as feasible and the cost of producing YACs transgenic livestock will be decreased critically compared to the other approaches. To our knowledge, this procedure used to produce YACs transgenic livestock is supposed to be an unprecedented case.

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