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Production of α1,3-Galactosyltransferase-Knockout Cloned Pigs Expressing Human α1,2-Fucosylosyltransferase[1]

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ABSTRACT

The production of genetically engineered pigs as xenotransplant donors aims to solve the severe shortage of organs for transplantation in humans. The first barrier to successful xenotransplantation is hyperacute rejection (HAR). HAR is a rapid and massive humoral immune response directed against the pig carbohydrate Galα1,3-Gal epitope, which is synthesized by α1,3-galactosyltransferase (α1,3-GT). The Galα1,3-Gal antigen also contributes to subsequent acute vascular rejection events. Genetic modifications of donor pigs transgenic for human complement regulatory proteins or different glycosyltransferases to downregulate Galα1,3-Gal expression have been shown to significantly delay xenograft rejection. However, the complete removal of the Galα1,3-Gal antigen is the most attractive option. In this study, the 5′ end of the α1,3-GT gene was efficiently targeted with a nonisogenic DNA construct containing predominantly intron sequences and a Kozak translation initiation site to initiate translation of the neomycin resistance reporter gene. We developed two novel polymerase chain reaction screening methods to detect and confirm the targeted G418-resistant clones. This is the first study to use Southern blot analysis to demonstrate the disruption of the α1,3-GT gene in somatic HT-transgenic pig cells before they were used for nuclear transfer. Transgenic male pigs were produced that possess an α1,3-GT knockout allele and express a randomly inserted human α1,2-fucosylosyltransferase (HT) transgene. The generation of homozygous α1,3-GT knockout pigs with the HT-transgenic background is underway and will be unique. This approach intends to combine the α1,3-GT knockout genotype with a ubiquitously expressed fucosyltransferase transgene producing the universally tolerated H antigen. This approach may prove to be more effective than the null phenotype alone in overcoming HAR and delayed xenograft rejection.

assisted reproductive technology, embryo, gamete biology, gene regulation, oocyte development

INTRODUCTION

The promise of an unlimited supply of suitable pig cells, tissues, and organs for transplantation into humans is the impetus for continuing research efforts to develop a universally accepted animal source for xenotransplantation. Despite formidable obstacles and the slow rate of progress, advances in technology and genetic modification of animals can make xenotransplantation a reality [1, 2]. Discordant pig xenografts are rejected by humoral and cellular immune responses [1]. Hyperacute rejection (HAR) represents the first major immunological hurdle encountered by the xenograft and occurs within minutes to hours after transplantation [3]. HAR is an aggressive response mediated by natural antibody reactivity directed against the pig carbohydrate Galα1,3-Gal epitope followed by complement activation [4]. When HAR is averted in vascularized organs by plasmapheresis [5] or complement inhibition [6], the xenograft is still subject to delayed xenograft rejection (DXR). DXR is characterized by a strong humoral response, type II endothelial cell activation, and an acute cellular infiltrate [1]. In avascular tissues such as cartilage, the delayed rejection process also involves a strong antibody response and a cellular immune infiltrate [7]. Galα1,3-Gal antigen plays a relevant role in these delayed rejection events [7]. The Galα1,3-Gal epitope is synthesized by α1,3-galactosyltransferase (α1,3-GT), which adds a terminal galactose to the acceptor substrate N-acetyl lactosamine [8].

A number of laboratories are attempting to produce α1,3-GT knockout pigs, and two have recently reported success using primary fibroblasts on a wild-type (WT) background [9–11]. However, it is unclear whether the resulting carbohydrate phenotype will be innocuous in the transplant setting. The combination of the α1,3-GT knockout with other approaches aimed at terminally glycosylating an uncapped N-acetyl lactosamine residue may be more effective in overcoming carbohydrate-mediated rejection events. The Galα1,3-Gal epitope can be downregulated by competition between α1,3-GT and α1,2-fucosylosyltransferase (HT) for the common acceptor substrate N-acetyl lactosamine [12]. HT generates the universally tolerated H antigen [12]. Our group and others have previously shown that cells from HT-expressing mice [13–15] and pigs [16] displayed a significantly reduced expression of the Galα1,3-Gal epitope. This modification resulted in a reduction in xenogenic natural antibody reactivity and an increased resistance to human serum-mediated cytolysis. A similar effect was observed when pig cells were genetically modified with other glycosyltransferases such as α2,3-sialyltransferase [17] or acetylglucosaminyltransferase III [18]. Our study combines these approaches by producing α1,3-GT knockout pigs on an HT-transgenic background. Pigs with the α1,3-GT null phenotype with and without the HT background can be used to determine the terminal glycosylation patterns that are most beneficial for prolonging xenograft survival.

The pig α1,3-GT gene has been isolated [19] and

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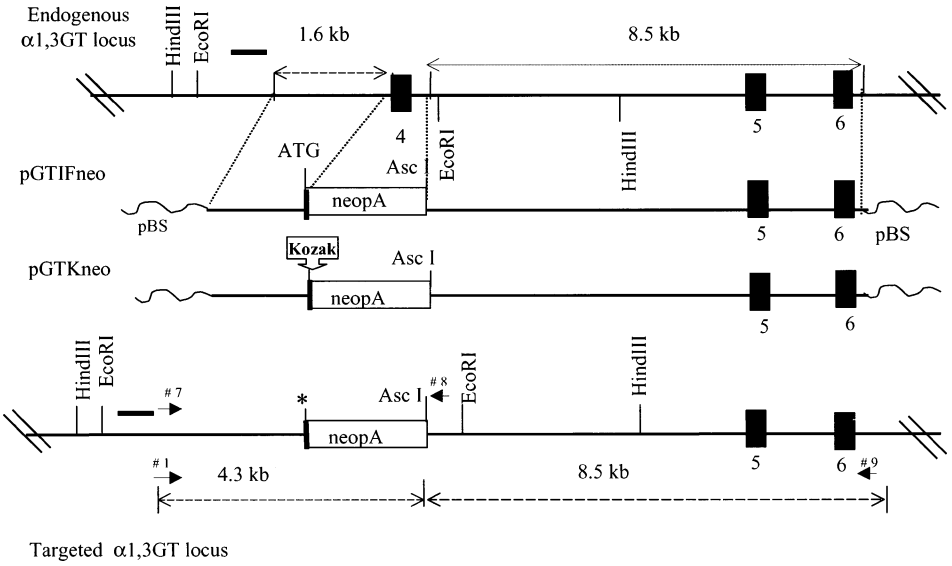

FIG. 1. Design of targeting constructs, and PCR and Southern blot screening strategies. The 5′ end of an endogenous α1,3-GT allele (exons, solid boxes; introns, thick line) showing the regions of homology used for the 5′ and 3′ arms of the targeting constructs, the position of the probe (closed bar), and the *Hin*dIII and *Eco*RI restriction enzyme sites used for Southern blot analyses. Targeting constructs pGTIFneo and pGTKneo are shown with the promoterless neo poly(A) (open box) cloned in frame with the endogenous ATG or with a Kozak consensus sequence (open arrow), respectively. A targeted allele shows the position of primers (solid arrowheads) used for PCR analyses and the unique *Asc*I site; * indicates in-frame or Kozak consensus sequence. Primers 7 and 8 amplified 3.4-kb and 2.7-kb targeted and endogenous bands, respectively. Primers 1 and 9 amplified 12.8-kb and 12.1-kb targeted and endogenous bands, respectively.

mapped to chromosome 1 region q2.10-q2.11 as a single-copy gene [20]. The number of exons and exon-intron boundries are completely conserved between the pig and the mouse, but the sizes of the introns are variable and tend to be larger in the pig [19]. Exon 4 contains the endogenous ATG translation initiation codon and encodes the transmembrane domain of the α1,3-GT enzyme [18]. The open reading frame begins within the first 10 bases of exon 4, making this gene a good candidate for gene targeting with a replacement-type construct at its 5′ end. Targeting of the 5′ end of this gene can be accomplished without the generation of fusion proteins or the use of complex internal ribosome entry site (IRES) sequences, whose functionality has positional limitations [21]. However, the difficulty of targeting the 5′ end of the α1,3-GT gene is that exons 4–8 are relatively small, ranging from 34 base pairs (bp) to 139 bp, and are separated by large introns ranging from 2 kilobases (kb) to >7 kb in length. Our 5′ targeting constructs mainly comprise intron nucleotide sequences and introduce a neomycin resistance gene to replace exon 4 at the endogenous ATG. Because intron sequences are more likely than exons to harbor spontaneous mutations, the efficiency of homologous recombination may be affected when using nonisogenic DNA [22].

Here we report the production of α1,3-GT knockout, HT-transgenic pigs. We found that promoter-trap targeting constructs consisting mainly of intron sequence and a Kozak consensus sequence to initiate translation can be used to target genes with characteristics similar to that of α1,3-GT, where the ATG start codon is located in a downstream exon and the exons are relatively small and separated by large introns. We used nonisogenic DNA in preparing the α1,3-GT gene targeting constructs and developed two efficient and accurate polymerase chain reaction (PCR) strategies to detect targeted clones. These pigs are to be used for studies aimed at overcoming the immune rejection responses usually generated by pig xenografts.

MATERIALS AND METHODS

Oocyte and Fetal Fibroblast Collection

Experiments were conducted under an animal use protocol approved by the Institutional Animal Care and Use Committee of Columbus Farming Corporation (CFC, Sherburne, NY). Oocytes from three different sources were used. One group of oocytes was flushed from the oviducts of superovulated gilts as described previously [23]. Cycling gilts were synchronized by oral administration of altrenogest (Regu-Mate; Inervet, Millsborough, DE), 18 mg/day for 5–9 days depending on the stage of the estrous cycle, and superovulation was induced by a single injection of eCG (Diosynth, Des Plaines, IL) followed by hCG (Inervet) 76 h later. Unfertilized oocytes were collected by retrograde flushing of the oviduct 45–46 h after the hCG injection. A second group of oocytes was obtained from the ovaries of gilts that received the same hormonal treatment except that ovulation was not induced with the administration of hCG. The gilts were killed 73 h after eCG treatment, their ovaries were removed, and the oocyte-cumulus complexes were collected following slicing of the 3- to 5-mm follicles. These complexes were matured in BSA-free NCSU-23 medium [24] supplemented with 10% porcine follicular fluid, 10 IU/ml eCG, 10 IU/ml hCG, 0.1 mg/ml cysteine, and 10 ng/ml epidermal growth factor. After being cultured for 22 h at 39°C under 5% CO_2 in air, the oocyte-cumulus complexes were transferred to fresh medium without hormonal supplementation for an additional 22 h. A third group of oocytes recovered from slaughterhouse pig ovaries was purchased from BoMed (Madison, WI) and shipped to our laboratory in their commercial maturation medium.

A heterozygous transgenic boar expressing the human H-transferase gene [16] was used to breed one cycling gilt (second estrus). The gilt was killed at Day 30 of pregnancy, and 12 fetuses were collected within their amniotic sacs to maintain sterility. Each fetus was processed separately. The head, limbs, and viscera were dissected away, and the remaining tissue was minced into approximately 1-mm pieces. Tissue pieces from each fetus were cultured for fibroblast outgrowth in 25-mm^2 (T-25) tissue culture flasks in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 15% fetal calf serum (FCS; Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). Genomic DNA was isolated from each fetus and analyzed by PCR for the presence of the H-transferase transgene as previously described [23]. Confluent T-25 flasks were trypsinized, and cells were transferred to T-75 flasks (passage 1), grown to 80–90% confluence, and then frozen in liquid nitrogen for future use. Approximately 2×10^6 cells/vial in culture medium containing 10% dimethyl sulfoxide (Sigma, St. Louis, MO) were frozen overnight in a −80°C freezer and subsequently stored in liquid nitrogen until use.

α1,3-GT Targeting Constructs

Two targeting constructs were generated to target the α1,3-GT gene (Fig. 1). In targeting construct pGTIFneo, a neomycin-bovine growth hormone poly(A) (neo) sequence cassette was inserted adjacent to and in frame with the endogenous ATG start codon (bases 10–12 of exon 4). The following 471 bases were deleted, which included the remaining 80 bases of exon 4 and 390 bases of intron 4 within the targeting construct. For generation of targeting construct pGTKneo, the neo cassette with a Kozak concensus sequence for the start of translation was used to replace the endogenous ATG, exon 4, and 390 bp of intron 4. An 18-kb DNA fragment from intron 3 to intron 7 was isolated from the EMBL3 porcine

TABLE 1. PCR primers used in the generation of α1,3-GT knockout pigs.

Primer[a]	Sequence
1-F1765	TCCATGAACAACTTCGATTGC
2-R14158	TCCTTCCAGACCAGCACAGTTAGC
3-F3182	AGTTTCCAACTACTACACTGACTTGC
4-R4856	ATCTGCAGTATTTTCTCCTGGGAAAAGAAAAGGAGAAGG
5-F5327	ATCGGCCGTGTATCCCTGAGCCCTTAAATACCG
6-R13831	ATGGCCGTTTCCCAGTCTTACC
7-F2725	TTGAGCCAGGCCACCTCCTCTTTATG
8-R5424	ACAATGGCAACATGGCAGGAAGGAAG
9-R13915	AAGGACGAGGCGACTGAGAAGGTCATGG
10-F2744	CTTTATGGTCATGAGAACG
11-R3055	TGGAAACAGCATCTATACC

[a] F, forward primer; R, reverse primer. Primer ID numbers represent actual positions of the primers within the 18-kb DNA fragment isolated from the porcine EMBL3 genomic library.

genomic library (BD Biosciences, Clontech, Palo Alto, CA) using an intron 3 fragment as probe for Southern blot screening. The 18-kb fragment was subcloned and sequenced in its entirety and used to design PCR primers (Table 1). DNA from a boar (different from the one used as sire for the fetuses) was used to amplify a 12.4-kb fragment from intron 3 to intron 6 by long-range PCR. Amplification was performed using the Expand Long Template PCR kit (Roche, Indianapolis, IN) with forward primer 1 and reverse primer 2 (Table 1). The cycling conditions were 94°C for 2 min, followed by 94°C for 15 sec, 60°C for 30 sec, and 68°C for 10 min for 10 cycles, 94°C for 15 sec, 60°C for 30 sec, and 68°C for 10 min + 20 sec per cycle for 20 cycles, and a final extension step of 68°C for 8 min. The PCR fragment was cloned into the pCR2.1 cloning vector (Invitrogen) and used as a template to amplify the 5′ and 3′ arms of the constructs. A 1.6-kb fragment was amplified using forward primer 3 and reverse primer 4 and used for the 5′ arm of the constructs. An 8.5-kb fragment was generated and used for the 3′ arm of the constructs using forward primer 5 and reverse primer 6 (Table 1). Except for the extension times, the PCR cycling conditions used were the same as described above. The pBluescript cloning vector (Stratagene, La Jolla, CA) was used to construct the targeting vectors. An *Asc*I restriction enzyme site was placed immediately after the neo poly(A) sequence in both pGTIFneo and pGTKneo constructs. The construct vectors were each transfected into XL10-competent cells (Stratagene), single colonies were amplified, and plasmid DNA isolated by cesium chloride gradient ultracentrifugation. Targeting constructs were linearized with *Not*I outside the region of homology and used for electroporation into primary fibroblasts.

Fibroblast Transfection Culture and Drug Selection

One transfection was carried out with targeting construct pGTIFneo using fibroblasts from a non-HT-transgenic fetus. Four transfections were carried out with construct pGTKneo using fibroblasts from both HT-transgenic and nontransgenic fetuses. Fetal fibroblasts frozen at passage 2 were thawed and cultured in a T-75 tissue culture flask for 24 h prior to electroporation in DMEM supplemented with 10% FCS, 1% penicillin and streptomycin, 5% minimal essential medium, and 2 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN) at 38°C in an atmosphere of 5% CO_2 in air and 100% humidity. Fibroblasts were harvested by trypsinization, and approximately 2×10^6 cells were electroporated with 10 μg of linearized DNA at 450 V and 350 μF using a Gene Pulser II electroporator (Bio-Rad Laboratories, Hercules, CA). Electroporated cells were cultured for 48 h before transfer into 96-well plates (2×10^3 cells/well) or 100-mm tissue culture plates (2×10^5 cells/plate) for selection in culture medium containing G418 (Geneticin, 600 μg/ml; Invitrogen). Selection was carried out for 10 days, and wells with resistant colonies were each transferred in triplicate to 96-well plates. Wells from one plate were used for DNA isolation and PCR analysis of targeted clones. Possible targets from the second plate were expanded for Southern blot analysis, and cells from the third plate were expanded and frozen as described above in small aliquots (10^5 cells/vial) for nuclear transfer (at approximately 20 cell doublings from the single cell targeting event). G418-resistant colonies from 100-mm plates were transferred by the cloning ring method to 24-well tissue culture plates for expansion and analysis.

PCR and Southern Blot Analyses of Neomycin-Resistant Colonies

DNA was isolated from G418-resistant colonies in 96-well plates by lysis in TE buffer (50 mM Tris, pH 8, 2.5 mM EDTA, 100 mM NaCl, and 0.1% SDS) for 3 h at 60°C followed by isopropanol precipitation, and pellets were washed twice in 70% ethanol and resuspended in 30 μl TE. Preliminary screening for targeted events was carried out by PCR using two sets of primers. The first pair of primers flanked the 5′ arm of the construct and the neomycin resistance gene cassette and amplified targeted and endogenous bands of 3.4 kb and 2.7 kb, respectively, in single-allele α1,3-GT knockout clones. The primers used were forward 7 and reverse 8 (Table 1). The LA *Taq* PCR kit (Takara; Panvera Corporation, Madison, WI) was used, with 2 μl of DNA from each G418-resistant colony as template. The cycling conditions were 94°C for 2 min, followed by 94°C for 15 sec, 65°C for 30 sec, and 72°C for 3 min for 10 cycles, 94°C for 15 sec, 65°C for 30 sec, and 72°C for 3 min + 20 sec per cycle for 20 cycles, and a final extension step of 72°C for 8 min.

For confirmation, long-range PCR was carried out on potential targeted clones using a second pair of primers that bind to sequences outside both arms of the targeting construct within the flanking chromosomal DNA. Both targeted and endogenous fragments of 12.8 kb and 12.1 kb were amplified, respectively. The LA *Taq* PCR kit was used, with 5 μl of DNA from each G418-resistant colony as template. Forward primer 1 and reverse primer 9 (Table 1) were used. The cycling conditions were 94°C for 2 min, followed by 94°C for 15 sec, 65°C for 30 sec, and 68°C for 10 min for 10 cycles, 94°C for 15 sec, 65°C for 30 sec, and 68°C for 10 min + 20 sec per cycle for 20 cycles, and a final extension step of 68°C for 8 min. The amplified DNA fragments were large and too close in size to be separated, but when cut with *Asc*I the 12.8-kb fragment amplified from the targeted allele produced two pieces, 8.5 kb and 4.3 kb, that were clearly distinguishable from the endogenous 12.1-kb band after electrophoresis on a 0.7% agarose gel.

Genomic DNA isolated from cells indicated as targeted by PCR and from fetuses and piglets following nuclear transfer was analyzed by Southern blotting [25] following standard procedures. For Southern blotting, DNA was digested with *Eco*RI or *Hin*dIII (New England Biolabs, Beverly, MA), and approximately 2 μg and 5 μg from cells and tissues, respectively, were separated on 0.7% agarose gels and blotted to Hybond-N+ membrane (Amersham, Pharmacia Biotech, Piscataway, NJ). A 311-bp DNA probe corresponding to intron 3 of the α1,3-GT gene just outside the 5′ end of the targeting construct was produced by PCR using forward primer 10 and reverse primer 11 (Table 1). The probe was labeled using [α-^{32}P]dCTP (Amersham) by random oligopriming (Stratagene). Membranes were subsequently hybridized overnight at 65°C with the radiolabeled probe in a hybridization solution containing Denhardt reagent, washed with increasing levels of stringency, and exposed to Kodak XAR-5 film (Sigma).

Nuclear Transfer

Oocytes were manipulated at 46–48 h after hCG for ovulated oocytes and 40–42 h after initiation of maturition for in vitro-matured oocytes. Except for minor modifications, the procedure was as previously described [23]. For enucleation, the medium was supplemented with 1% and 5% sucrose for ovulated and in vitro-matured oocytes, respectively. The reconstructed oocytes were equilibrated in Ca-free fusion medium (pH 7.1; containing 300 mM mannitol, 0.2 mM $MgSO_4$, 0.5 mM Hepes, 0.1% polyvinyl alcohol) for 5 min, and then each oocyte was placed between the two electrodes of a fusion chamber (BTX, San Diego, CA) overlaid with fusion medium. Fusion was induced with two DC pulses of 1.2 kV/cm for 60 μsec each. Two minutes after the electrical pulse, the reconstructed oocytes were transferred into Beltsville embryo culture medium (BECM) and cultured for 1 h. They were then activated by two DC pulses

TABLE 2. Results of analysis of G418-resistant colonies from five independent transfections of primary fetal fibroblasts for the generation of α1,3-GT knockout pigs.

Fibroblast cell line	Targeting construct	No. G418-resistant colonies	No. (%) PCR-positive colonies		No. (%) Southern blot-positive colonies
			Preliminary PCR	Long range PCR	
C2.1^{HT-}	pGTIFneo	217	2 (0.9)	2 (0.9)	2 (0.9)
F1.1^{HT+}	PGTKneo	299	4 (1.3)	4 (1.3)	4 (1.3)
F1.2^{HT+}	PGTKneo	656	9 (1.37)	8 (1.2)	8 (1.2)
C2.0^{HT-a}	PGTKneo	54	4 (7.4)	4 (7.4)	ND
F1.0^{HT+a}	PGTKneo	46	4 (8.7)	4 (8.7)	ND

[a] G418-resistant colonies isolated by the cloning ring method from 100-mm tissue culture plates. Southern blots were not done (ND).

as described for fusion except that the medium (pH 7.1) overlaying the fusion chamber contained 300 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM Hepes, and 0.1% polyvinyl alcohol. Nuclear transfer embryos were either transferred into recipient animals the same day or, in some cases to obtain a higher number of embryos for transfer, were cultured overnight in NCSU-23 medium before transfer. These embryos were then pooled with nuclear transfer embryos produced the following day. In this study, a total of 2918 nuclear transfer embryos were transferred to 20 recipient gilts (mean, 146 embryos/recipient; range, 97–246). Recipients were synchronized by oral administration of altrenogest (18 mg/day for 5–9 days depending on the stage of the estrous cycle) and stimulated with 500 IU hCG 72 h later. The synchrony of the recipients' estrous cycles was delayed by 24–48 h from that of the oocyte donors. Reconstructed embryos were transferred approximately 32 h after hCG injection, at which time the recipient animals had not yet ovulated.

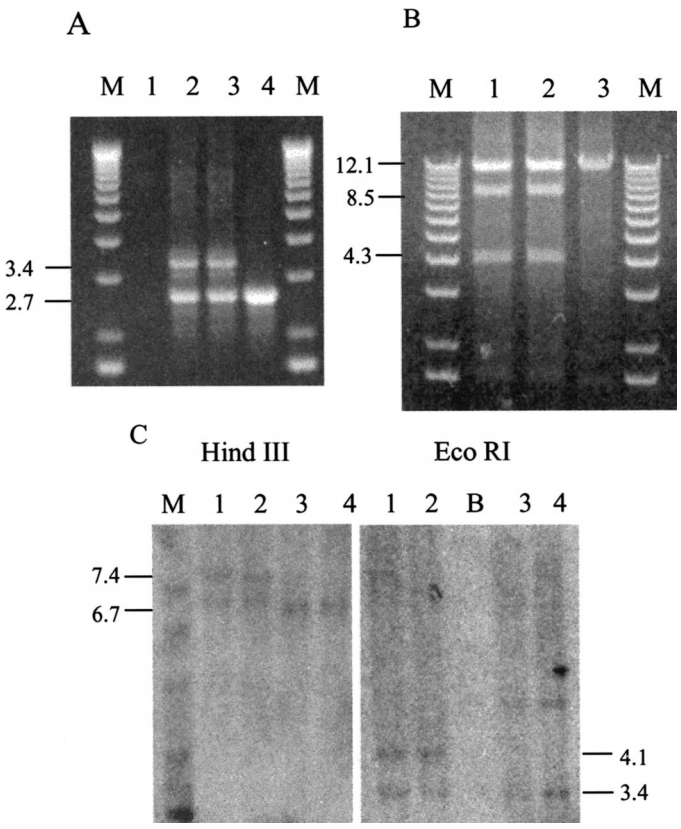

FIG. 2. PCR and Southern blot analyses of G418-resistant clones transfected with the pGTIFneo targeting construct. **A**) Preliminary PCR. Lane M: 1-kb DNA marker; lane 1: no DNA template control; lane 2: C172^{HT-}; lane 3: C173^{HT-}; lane 4: C2.1^{HT-} cell line (nontransfected control). Primers 7 and 8 amplified 3.4-kb and 2.7-kb targeted and endogenous bands, respectively. **B**) Long-range PCR. Lane M: 1-kb DNA marker; lane 1: C172^{HT-}; lane 2: C173^{HT-}; lane 3: C2.1^{HT-} cell line control. Primers 1 and 9 amplified a 12.8-kb targeted band (*Asc*I cut, 8.5-kb and 4.3-kb) and a 12.1-kb endogenous band. **C**) Southern blot analysis with *Hin*dIII- and *Eco*RI-digested DNA. First panel: *Hind* III-cut DNA. Lane M: 1-kb DNA marker; lane 1: C172^{HT-}; lane 2: C173^{HT-}; lane 3: C2.1^{HT-} nontransfected cell line control; lane 4: control pig DNA. Second panel: *Eco*RI-cut DNA. Lane 1: C172^{HT-}; lane 2: C173^{HT-}; lane B: blank; lane 3: C2.1^{HT-} nontransfected cell line control; lane 4: control pig DNA. The 7.4-kb targeted and 6.7-kb endogenous bands were produced with *Hin*dIII, and the 4.1-kb targeted and 3.4-kb endogenous bands were produced with *Eco*RI.

Flow Cytometric Analysis

Expression of the α1,3-GT gene and the HT transgene was assessed by flow cytometry of primary cultured fibroblasts. Direct fluorescence of cell-surface carbohydrate epitopes was performed with fluorescein isothiocyanate-conjugated lectins as described previously [16, 23].

RESULTS

The α1,3-GT enzyme is expressed in fetal and adult pig fibroblasts, and the Galα1,3-Gal antigen is readily detected on the cell surface; therefore, the use of a promoter-trap replacement type targeting strategy was appropriate. The targeting constructs used here are homologous to sequences at the 5′ end of the α1,3-GT gene. The ATG translation start codon located at the 5′ end of exon 4 (bases 10–12) was used as the start codon for the neomycin resistance gene in the targeting construct pGTIFneo. A second construct, pGTKneo, utilized a synthetic Kozak consensus sequence and ATG to initiate translation of the neomycin resistance gene. These replacement-type constructs contained 10.1 kb of sequence homology comprising an 8.5-kb long 3′ arm and a short 1.6-kb 5′arm. The positions of primers and the DNA probe sequence used for target analyses are indicated in Figure 1. A total of five independent electroporations were performed with primary fetal fibroblast cell lines isolated from two different fetuses (fetus F, HT$^+$; fetus C, nontransgenic; passages 2 and 3). One cell line was transfected with construct pGTIFneo, and four were transfected with pGTKneo. Table 2 gives a summary of the results obtained.

Gene Targeting with pGTIFneo

The cell line C2.1^{HT-} isolated from nontransgenic fetus C was electroporated with pGTIFneo, and 217 G418-resistant colonies (Table 2) were analyzed for successfully targeted events using primers 7 and 8. Two colonies, C17D2^{HT-} and C17D3^{HT-}, showed the 3.4-kb and 2.7-kb targeted and endogenous bands, respectively (Fig. 2A). Targeting was confirmed using primers 1 and 9 flanking the entire construct (Fig. 2B). The targeted band was cut with *Asc*I to get the 8.5- and 4.3-kb fragments indicative of a targeting event. The 12.1-kb endogenous band was uncut (Fig. 2B). Southern blot analysis of DNA from clones C17D2^{HT-} and C17D3^{HT-} and from the nontransfected C2.1^{HT-} parental cell line digested with *Hin*dIII or *Eco*RI revealed the 7.4-kb and 4.1-kb targeted bands and 6.7-kb and 3.4-kb WT bands, respectively (Fig. 2C). A 0.9% rate

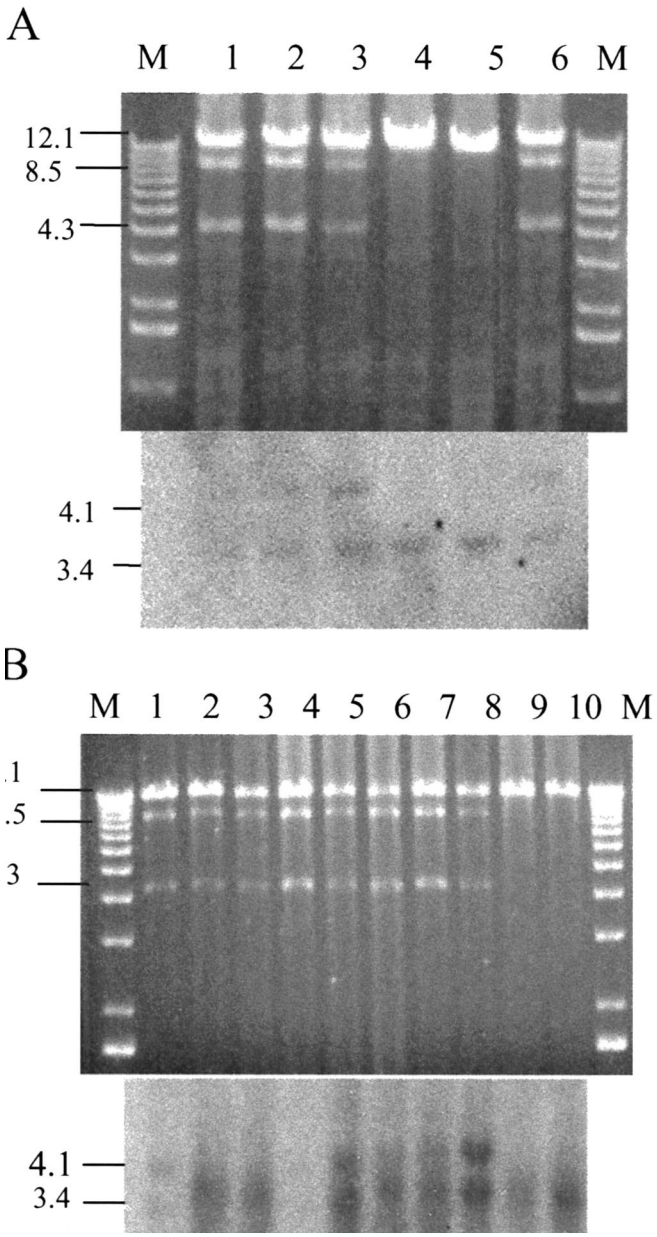

FIG. 3. PCR and Southern blot analyses of G418-resistant clones transfected with the pGTKneo targeting construct. **A**) Long-range PCR (*Asc*I-digested product, top) and Southern blot (*Eco*RI-digested DNA, bottom) analyses of G418-resistant clones of F1.1^{HT+} fibroblasts transfected with pGTkneo. Lane M: marker; lane 1: F27^{HT+}; lane 2: NF28^{HT+}; lane 3: NF29^{HT+}; lane 4: F1.3^{HT+} cell line nontransfected control; lane 5: control pig DNA; lane 6: F210^{HT+}. **B**) Long-range PCR (top) and Southern blot (bottom) analyses of G418-resistant clones of F1.2^{HT+} fibroblasts transfected with pGTkneo (*Eco*RI-digested DNA). Lane M: marker; lane 1: F13^{HT+}; lane 2: F23^{HT+}; lane 3: F54^{HT+}; lane 4: F62^{HT+}; lane 5: F612^{HT+}; lane 6: F93^{HT+}; lane 7: F71^{HT+}; lane 8: F72^{HT+}; lane 9: F129^{HT+}; lane 10: F3.1^{HT+} cell line nontransfected control. Long-range PCR primers 1 and 9 amplified 12.8-kb (*Asc*I digested, 8.5 kb and 4.3 kb) and 12.1-kb endogenous bands. For Southern blot, 4.1-kb targeted and 3.4-kb endogenous bands were produced with *Eco*RI.

of targeting was observed in all three analyses (Table 2). The C17D3^{HT-} cells were frozen in small aliquots and used as nuclear transfer donors.

Gene Targeting with pGTKneo

Results from targeting with pGTKneo are presented in Table 2 and Figure 3. PCR and Southern blotting were used to analyze a total of 1055 G418-resistant colonies from four transfections. The F1.1^{HT+} cell line was used in the first transfection, and 299 drug-resistant colonies were analyzed. Four colonies, F27^{HT+}, F28^{HT+}, F29^{HT+}, and F210^{HT+}, were detected as targeted by PCR with primers 7 and 8 (data not shown). This targeting was confirmed by long-range PCR with primers 1 and 9 flanking the entire construct, digestion of the PCR product with *Asc*I (Fig. 3A, top), and Southern blot analysis (Fig. 3A, bottom). A 1.3% rate of targeting was determined by all three methods (Table 2). Cells from targeted clones F27^{HT+}, F29^{HT+}, and F210^{HT+} were used as nuclear transfer donors.

Cell line F1.2^{HT+} was transfected with pGTKneo, and 656 drug-resistant colonies were analyzed (Table 2). PCR using primers 7 and 8 revealed that nine clones (F13^{HT+}, F23^{HT+}, F54^{HT+}, F62^{HT+}, F612^{HT+}, F93^{HT+}, F71^{HT+}, F72^{HT+}, and F129^{HT+}) were targeted (data not shown). When these clones were subjected to long-range PCR analysis using primers 1 and 9 (Fig. 3B, top) and to Southern blot analyses (Fig. 3B, bottom), eight of the nine clones were confirmed as α1,3-GT targeted mutants. Clone F129^{HT+} (Fig. 3B, lane 9) did not show a mutant banding pattern. Two additional transfections with construct pGTKneo were performed using F1.0^{HT+} and C2.0^{HT-} cell lines (Table 2). Tissue culture plates (100 mm) and cloning rings were used for selection of drug-resistant colonies instead of 96-well plates. From C2.0^{HT-} and F1.0^{HT+} transfected cells, 54 and 46 G418-resistant colonies were transferred by the cloning ring method to 24-well plates, respectively. In each case, four targeted clones were identified by PCR (Table 2), to give targeted mutation rates of 7.4% and 8.7%, respectively. Southern blot analysis was not performed on these clones.

TABLE 3. Summary of nuclear transfer results for the generation of α1,3-GT knockout male pigs.

	Donor cell lines			
	C173^{HT-}	F210^{HT+}	F27^{HT+}	F29^{HT+}
No. oocytes				
BoMed	604	483	154	454
CFC, in vitro matured	484	101	214	97
CFC, ovulated	130	94	55	52
No. embryos transferred	1218	674	423	603
No. recipients	7	4	4	5
No. pregnant on day 30	3	1[a]	1	2
No. pregnant on day 60	3	0	0	2
No. pregnancies brought to term	1	0	0	2
No. piglets at birth	4	0	0	8[b]
No. live α1,3-GT knockout^{HT-} pigs	2	0	0	0
No. live α1,3-GT knockout^{HT+} pigs	0	0	0	4

[a] Pregnancy terminated for fetal fibroblast collection.
[b] The first litter from F29^{HT+} reconstructed embryos consisted of two piglets (live births), and the second litter consisted of six piglets (two live births and four stillbirths).

Production of α1,3-GT Knockout Pigs by Nuclear Transfer

Four different targeted cell lines, C173^{HT-}, F210^{HT+}, F27^{HT+}, and F29^{HT+}, were used as donors for nuclear transfer (Table 3). The karyotype of the parent cell lines (before targeting) used in this study appeared normal, with >75% of the chromosome spreads displaying a full complement of 38 chromosomes (data not shown). A total of 2918 nuclear transfer embryos were transferred to 20 recipient gilts (mean, 146 embryos/recipient; range, 97–246). Seven preg-

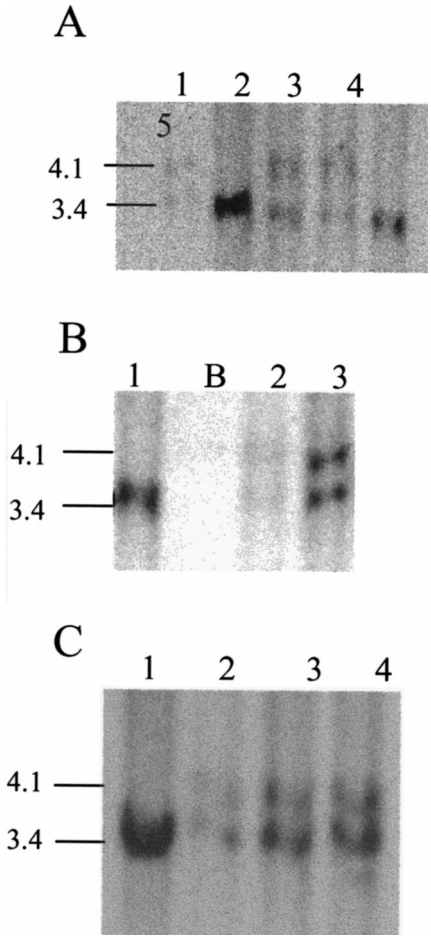

FIG. 4. Southern blot analysis of *Eco*RI-digested DNA from nuclear transfer cloned piglets and fetuses, producing 4.1-kb targeted and 3.4-kb endogenous bands. **A**) Litter 136. Lane 1: piglet 136-1^{HT-}; lane 2: piglet 136-2^{HT+}; lane 3: piglet 136-3^{HT-}; lane 4: piglet 136-4^{HT-}; lane 5: recipient, DNA control. **B**) Southern blot analysis of *Eco*RI-digested DNA from litter 141, Lane 1: recipient, DNA control; lane 2: blank; lane 3: piglet 141-1^{HT+}; lane 4: piglet 141-2^{HT+}. **C**) Southern blot analysis of *Eco*RI-digested DNA from fetuses derived from C173^{HT-} and F210^{HT+} reconstructed embryos. Lane 1: fetus 1 (reabsorbing); lane 2: fetus 2^{HT-}; lane 3: fetus 3^{HT+}; lane 4: fetus 4^{HT+}.

nancies were detected by ultrasonography at Day 30, five were detected at Day 60, and three were carried to term, resulting in eight live births (one piglet died soon after birth) and four stillbirths (Table 3). Figure 4A shows Southern blot analysis of the four piglets cloned from C173^{HT-} and a nontargeted cell line. The 4.1-kb targeted and 3.1-kb endogenous bands were detected in DNA samples isolated from piglets 1, 3, and 4 (Fig. 4A, lanes 1, 3, and 4). Figure 4B shows Southern blot analysis of the two piglets from the first litter cloned from F29^{HT+} cells. The targeted and endogenous bands were found in DNA samples from both piglets (Fig. 4B, lanes 2 and 3). DNA was isolated from each of the six piglets (four stillborn and two live) from the second litter cloned from F29^{HT+} cells and was analyzed by PCR. DNA from all six piglets showed the 3.4-kb targeted and 2.7-kb endogenous bands, using primers 7 and 8 (data not shown). The presence of a mutated allele was confirmed by long-range PCR using primers 1 and 9. A targeted band (digested with *Asc*I to give the 8.5-kb and 4.3-kb targeted bands) from the mutated allele and the 12.1-kb endogenous band were evident in all six piglet samples (data not shown). Figure 4C shows Southern blot analysis of the four fetuses harvested for fibroblasts. The 4.1-kb targeted and 3.1-kb endogenous bands were detected in DNA samples isolated from fetuses 2, 3, and 4. Fetus 1 was reabsorbing, and a targeted band was not evident in the partially degraded DNA (Fig. 4C).

To increase the number of embryos transferred per recipient, some embryos were cultured in NCSU-23 medium for 24 h and then transferred together with nuclear transfer embryos produced the following day. In some cases, nuclear transfer experiments were conducted with nontargeted cells. The cells used in the non-gene targeting experiments were from a different cell line, which produces black piglets instead of the white piglets from the targeted cell lines (see Fig. 5). The fibroblasts used in the non-gene targeting experiments also expressed the HT transgene.

Embryos reconstructed with C173^{HT-} cells were transferred to seven recipients and resulted in three established pregnancies (detected by ultrasonography at Days 30 and 60 of pregnancy). Two recipients aborted after Day 60, and one carried the pregnancy to term, producing a litter of four piglets (Table 3 and Fig. 5A, inset shows litter at birth). This litter resulted from the transfer of targeted and nontargeted nuclear transfer embryos. One of the four piglets born was clearly from the nontargeted (black) cell line. One piglet from the litter died soon after delivery from an undetermined cause, and the remaining three are healthy at 2 mo of age (Fig. 5A).

Three recipients received F210^{HT+} reconstructed embryos, and one received C173^{HT-} and F210^{HT+} reconstructed embryos. A pregnancy resulted from the recipient transferred with the combination of F210^{HT+} and C173^{HT-} reconstructed embryos (Table 3). This recipient was killed at Day 30 of pregnancy, and the fetuses were collected to establish fetal fibroblast cell lines for further experiments. Two of the fetuses developed from C173^{HT-} cells, and the remaining two were from F210^{HT+} cells, as determined by flow cytometry detecting HT expression (data not shown). One of the four transfers with F27^{HT+} reconstructed embryos resulted in a pregnancy that aborted after 30 days (Table 3). Two of the five transfers with F29^{HT+} reconstructed embryos became pregnant and produced litters of two piglets (Fig. 5B) and six piglets (four stillborn and two live; Table 3). Overall, five pregnancies were obtained with 1227 embryos produced from oocytes ovulated (331 oocytes) or in vitro matured (896 oocytes) after collection from pigs kept in our facility at CFC. In comparison, two pregnancies resulted from BoMed in vitro-matured oocytes (1695 oocytes): one aborted after Day 60 and the second produced a litter of two piglets. The first two pregnancies were allowed to proceed to 119 days (5 days past the due date of 114 days), delivery was by caesarian section, and there was no milk let down. The third pregnancy was allowed to go to term naturally, parturition occurred at 116 days, and there was milk let down; however, there were four stillborn piglets and two live piglets.

Detection of Cell Surface Expression of H and Galα1,3-Gal Carbohydrate Antigens

Nuclear transfer using α1,3-GT knockout donor cells derived from HT-transgenic or nontransgenic fetuses successfully produced piglets. The nontransgenic cell line C2.1^{HT-} exhibits a high level of expression of the Galα1,3-Gal epitope but lacks H antigen expression (Fig. 6A). The litter (no. 136) of four piglets was produced from embryos reconstructed using the nontransgenic C173^{HT-} targeted cell

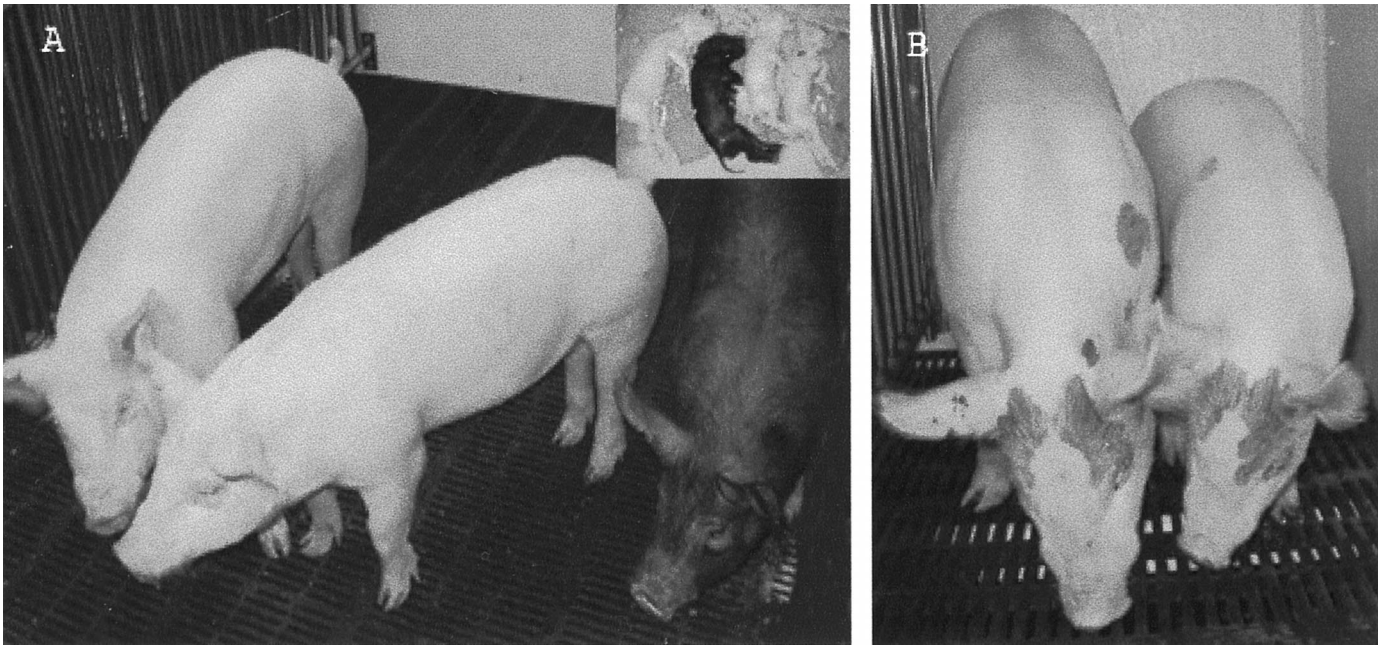

FIG. 5. Nucelar transfer live piglets. **A**) Litter 136 produced by nuclear transfer using C173^{HT-} and a nontargeted donor cell line: piglets 136-1 (α1,3-GT knockout, white), 136-2 (α1,3-GT WT, black), and 136-4 (α1,3-GT knockout, white). Inset shows the entire litter at birth: three α1,3-GT knockout (white) and one WT (black) piglets. **B**) Two α1,3-GT knockout piglets from litter 141 cloned by nuclear transfer from cell line NF29^{HT+}: piglets 141-1^{HT+} and 141-2^{HT+}.

line and from nontargeted cells that express HT (Fig. 6). Three of the piglets, 136-1, 136-3, and 136-4, did not express HT and showed a slight reduction in Galα1,3-Gal epitope expression as compared with the parental cell line (Fig. 6, A and C). However, the first litter (no. 141) of two piglets produced from the F29^{HT+} α1,3-GT targeted cell line expressed both HT and the Galα1,3-Gal epitopes as strongly as did the parental cell line F1.1^{HT+} (Fig. 6, B and D).

DISCUSSION

Flow cytometry results have indicated that the α1,3-GT gene is expressed in pig fibroblasts, which allowed us to use a promoter-trap targeting strategy. A targeting construct with a modified translation initiation site based on the Kozak consensus sequence (for review see [26]) was used to initiate translation of the neomycin resistance gene. A second construct with the neo gene inserted in frame with the endogenous ATG was also produced. The use of a Kozak consensus sequence allowed for flexibility in designing the targeting construct because 1) the selectable marker gene can be placed under the control of the endogenous promoter without it having to be cloned in frame with the coding sequence, i.e., the 5′ untranslated region of a gene can be targeted with a synthetic Kozak-ATG consensus sequence for translation initiation, 2) there was no need to generate a fusion protein with the selectable marker gene that may have reduced functionality, 3) it does not require the use of IRES sequences, and 4) it allows the arms of the targeting constructs to consist entirely of intron sequences. In targeting the 5′ end of a gene in the pig, a synthetic Kozak-ATG consensus sequence was used to initiate translation as efficiently as does the endogenous translation start site. Neomycin-resistant targeted α1,3-GT colonies were detected at similar frequencies after transfection with either pGTIFneo or pGTKneo and selection with 600 μg/ml G418.

The cell lines used here were isolated from fetuses that were not sired from the boar whose DNA was used as template for generating the targeting constructs. Therefore, there was no chance that the cells possessed an allele isogenic to the constructs. The level of isogenicity and the extent of single base changes introduced by long-range PCR were not determined in this study. Dai et al. [10] found no differences between cell lines transfected with isogenic and nonisogenic DNA in the pig. In the present study, a similar rate of gene targeting was obtained with cell lines from two different fetuses. How these factors affect the rate of homologous recombination in the pig is unknown. As in humans [27], isogenic DNA is not an absolute requirement for gene targeting in pigs.

Unlike embryonic stem cells, somatic cells progress through a finite number of population doublings before becoming senescent, which places severe constraints on their ability to proliferate sufficiently to accommodate one round of gene targeting and cloning. An estimated 30–35 cell doublings are required for one round of gene targeting [28]. We optimized the conditions to culture, select, and identify the targeted clones, allowing successful expansion of the cells that carried the targeted α1,3-GT allele.

Targeted clones were efficiently detected by a preliminary PCR assay using specific primers that flanked the short 1.6-kb 5′ arm and the neomycin resistance gene. The forward primer was homologous to sequence outside the 5′ arm of the construct, and the reverse primer was anchored to homologous sequence within the 3′ arm. Using these flanking primers, we did not get the large number of false positives detected with targeting primers in which one primer is specific to sequence outside the construct and the other is specific to neomycin resistance gene sequence (data not shown) [9]. Because both the targeted mutant and nontargeted alleles had the same specific homologous binding sites for the preliminary PCR primers, both targeted and endogenous alleles were screened in the same reaction, making it easier to identify possible targets and mixed col-

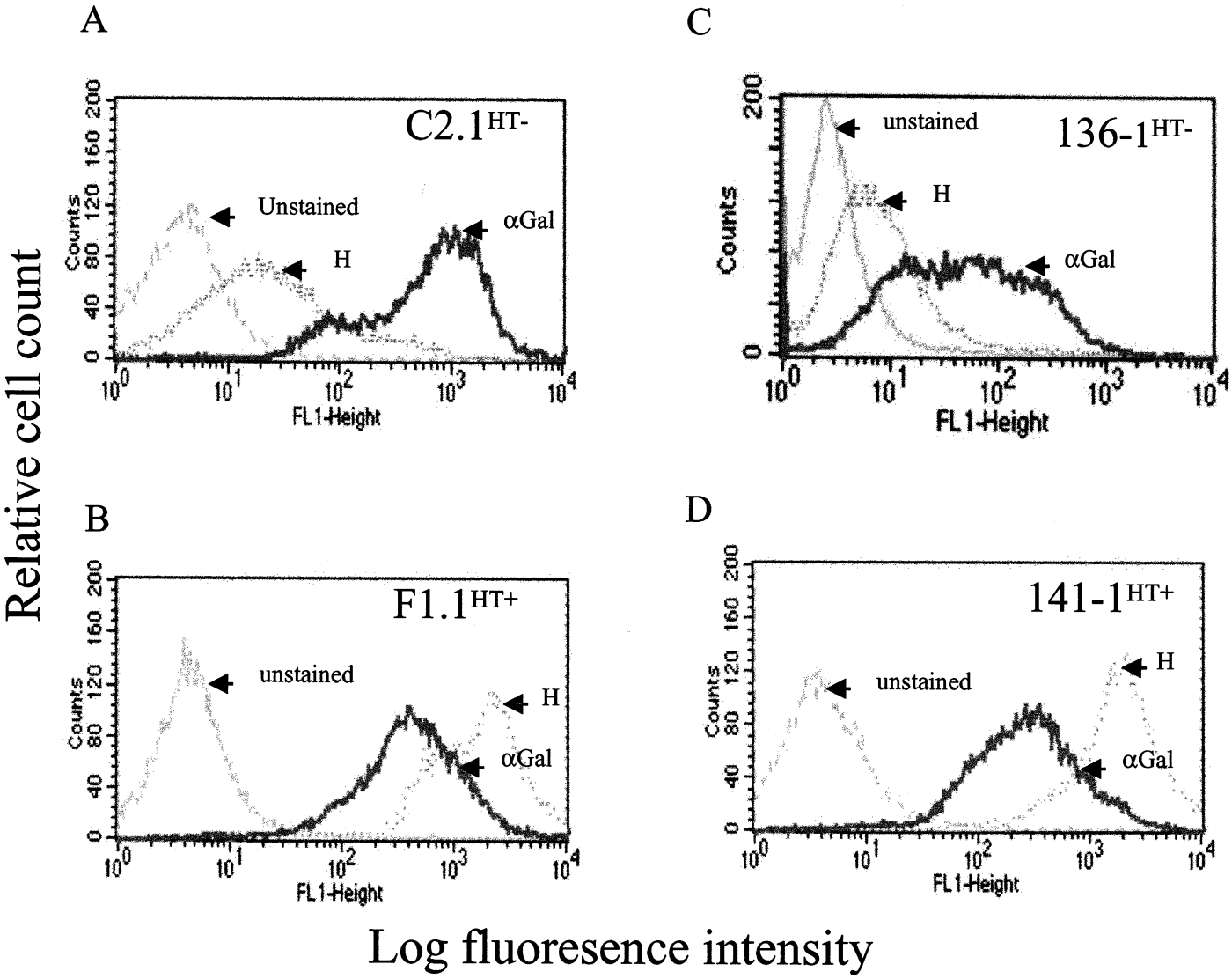

FIG. 6. Flow cytometric analysis of pig fibroblasts. The *x*-axis shows the fluorescence intensity, and the *y*-axis shows the relative cell count for unstained cells, H and Galα1,3-Gal antigen expression (arrowheads). **A**) Expression profile for the parental fibroblast cell line C2.1^{HT-} from which piglets 136-1^{HT-}, 136-3^{HT-}, and 136-4^{HT-} were derived. **B**) Expression profile for the parental fibroblast cell line F1.1^{HT+} from which piglets 141-1^{HT+} and 141-2^{HT-} were derived. **C**) Expression profile for piglet 136-1^{HT-}, which was positive for Galα1,3-Gal but not H antigen. **D**) Expression profile for piglet 141-1^{HT+}, which was positive for Galα1,3-Gal and H antigens.

onies. All but one of the clones indicated as targeted by the preliminary PCR analysis were confirmed by long-range PCR analysis. In addition, results of the long-range PCR analysis were corroborated by Southern blotting, which confirmed the presence of a mutated α1,3-GT allele. The unique *Asc*I site placed after the neo selection cassette was used to cut the targeted fragment amplified by long-range PCR using primers that flanked the whole targeting construct so that it could be distinguished from the endogenous band by agarose gel electrophoresis. Long-range PCR permitted the detection of recombination events at both ends of the construct to confirm replacement of the WT with the disrupted allele.

The efficiency of homologous recombination found in this study ranged from 0.9% to 8.7%, as determined by long-range PCR analysis, and is comparable to that obtained in the two previous reports of α1,3-GT gene knockout pigs [9, 10]. Plating of the transfected cells in 100-mm tissue culture plates and selecting clones by the cloning ring method appears to isolate a higher percentage of targeted clones. Here, each colony identified as targeted by long-range PCR was isolated from a different plate and was assumed to represent an independent targeting event. However, isolating large numbers of clones by the cloning ring method is very tedious and time-consuming. The process can be scaled up to screen larger numbers of G418-resistant clones by selection in 96-well tissue culture plates. However, we observed a drop in targeting efficiency using this method that could be related to differences in plating densities. The targeting frequencies obtained for cell lines isolated from the two fetuses were similar when plated in the 96-well plates and when plated in the 100-mm plates. Although a slightly higher percentage of targeted clones was obtained with the pGTKneo construct, there was no significant difference from the percentage obtained with the pGTIFneo construct.

To date, we have generated six healthy α1,3-GT gene knockout male piglets, four of which express the HT antigen on their cells. Except for one apparently normal piglet that died soon after birth, the remaining seven piglets de-

rived from this work are healthy (including the HT-expressing nontargeted control littermate). Pregnancies were established with embryos reconstructed from all four targeted cell lines used for cloning.

This is the first study to show that Southern blot analysis can be performed to confirm a knockout in somatic pig cells before they are used for nuclear transfer. The two targeting constructs designed and used here were effective in replacing a WT allele at frequencies comparable to those previously reported for the α1,3-GT locus. A novel preliminary PCR screening strategy was developed and was stringent enough to avoid large numbers of false positives. A unique long-range PCR screening strategy also was developed and used to confirm the presence of the mutated allele by gene targeting. The long-range PCR screening strategy was equivalent in stringency to Southern blotting, as indicated by the similarities in numbers of targeted clones detected with both assays. In targeting the 5′ end of a gene in the pig, a synthetic Kozak-ATG consensus sequence was used to initiate translation as efficiently as does the endogenous translation start site. Thus, it is not necessary to clone the selection marker gene in frame with the endogenous ATG initiation codon. A consideration with this strategy is to retain the optimum distance of the Kozak sequence 5′ to the ATG within the untranslated region. We have produced healthy α1,3-GT gene knockout HT-transgenic male piglets that can be used to generate a α1,3-GT null phenotype. In future studies, the generation of homozygous α1,3-GT knockout pigs with the HT-transgenic background will be unique and may be more effective than the null phenotype alone in prolonging xenograft survival.

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