

Dimorphic Goat Amelogenin Alleles on Sex Chromosomes for Gender Determination of Preimplantation Embryos

Hsiao-Ling Chen, Tung-Chou Tsai, Jan-Chi Huang, De-Chi Wang, and Chuan-Mu Chen

Abstract—Amelogenin (AMEL) is a conserved gene located on the sex chromosomes of mammals. It is involved in the formation of enamel, which is the hard, white material that forms the protective outer layer of each tooth. In this study, we first cloned the coding sequences of the goat *AMELX* and *AMELY* transcripts from female and male lamb enamel tissues during tooth development. All 207 amino acids of the putative AMEL proteins from the cDNA coding regions of both sex chromosomes were the same, but the *AMEL* sequences of the 3'-untranslated regions (UTR) were different. The results showed that the nucleotide sequences of intron 5 of the goat *AMELX* and *AMELY* genes contained multiple deletions/insertions and had only 48.5% identity. Based on the dimorphic *AMEL* intron sequences, a set of sex-specific primers was successfully applied to goat gender determination. A high sensitivity for sex determination was reached with a minimal amount of template, such as a trace amount of genomic DNA from a single blastomere isolated from embryos at the blastula stage among different strains of goats.

Index Terms—Goat amelogenin gene, polymorphic DNA, embryo sexing, single blastomeres, preimplantation.

I. INTRODUCTION

Goats are important livestock for multiple purposes, such as high-quality meat, wool, and milk sources and for the production of valuable recombinant proteins in transgenic goat milk for agriculture, veterinary medicine or pharmaceutical uses [1]-[4]. Recent studies have demonstrated that goat offspring can be easily produced from *in vivo*- and *in vitro*-fertilized eggs by embryo transfer, either fresh or frozen/thawed [5]. In addition, it is desirable to control the sex ratios in the goat industry.

Amelogenin (AMEL) is the major protein found in developing tooth enamel and belongs to a family of extracellular matrix (ECM) proteins. AMEL is involved in the formation of enamel, which is the hard, white material

that forms the protective outer layer of each tooth [6]. One copy of the amelogenin gene is located on each of the sex chromosomes (X and Y). The *AMELX* gene, which is located on the X chromosome (Xp22.31- p22.1), makes almost all of the amelogenin. The copy of the amelogenin gene on the Y chromosome (Yp11.2), *AMELY*, makes very little amelogenin and is not required for enamel formation [7].

A number of approaches have been used to identify the gender of preimplantation mammalian embryos including cytological analysis of Barr bodies or sex chromosomes [8], detection of the male-specific H-Y antigen [9], quantitative comparison of X-linked enzyme activity between male and female embryos [10], hybridization with a male-specific probe [11], and determination of the differences in the developmental rates between male and female embryos [12]. These procedures are generally time-consuming or inaccurate. However, some of these problems have been improved by the use of polymerase chain reaction (PCR) [13], [14], such as amplification of the SRY gene, which is associated with the testis-determining factor [15], or relevant Y- specific repetitive sequences [16], [17].

In the present investigation, we first cloned and sequenced the goat *AMEL* gene cDNAs from the *Alpine* strain of dairy goat. The sex-specific polymorphic fragments in the goat *AMELX* and *AMELY* genes were then identified in each intron sequence. Furthermore, we extended the use of the X/Y homologous *AMEL* analysis to determine the genders of goat embryos by single- blastomere PCR amplification. The sensitivity and fidelity of the newly created PCR sexing technique also tested in different strains of dairy goat.

II. MATERIALS AND METHODS

A. cDNA Cloning of Goat Amelogenin Genes

Total RNAs were isolated from 1.0 g of goat ameloblast tissues with TRIzolTM reagent and according to the manufacturer's protocol (Invitrogen, San Diego, CA). 300µg of total RNA were used to isolate the poly(A⁺) mRNA using the FastTrack[®] MAG mRNA isolation kit (Invitrogen). An aliquot of 5 µl poly(A⁺) mRNA was used for first-strand cDNA synthesis by SuperScriptTM III reverse transcriptase (Gibco/BRL, Burlington, ON) as described [18]. A mature goat amelogenin cDNA fragment (0.7 kb) was amplified by PCR using a pair of primers, pAMEL-F (5'-CACTGAGAACGTGTGTTCAA AG-3') and pPolyT-R (5'-TTTTTTTTTTTTTTTTTTTGTGA-3'). The PCR product

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was cloned into pGEM T-Easy Vector Systems (Promega, Madison, WI) and the sequence was verified by nucleotide sequencing [19].

B. Isolation of Gender-Specific Amelogenin Gene Fragments

To identify the gender-specific sequence, the intron 5- X and intron 5-Y of goat AMEL genes were amplified by PCR. The upstream primer oligonucleotide was 5'-AGCAACAGACAAGACCAAG-3' which located on fifth exon sequence homologous to both goat AMEL-X and AMEL-Y genes. And the downstream primers were 5'-TTTACTTCAGGTCTCTTCTC-3' and 5'-GTAGAA TGATTATGGGCACAAA-3' which located on sixth exon and 3'-untranslation region homologous to goat AMEL-X and AMEL-Y genes, respectively. PCR products were generated a 1,577-bp and a 1,780-bp fragment in the female and male, respectively. These amplicons were then cloned into pCR 2.01 vector using the TOPO cloning kit (Invitrogen, San Diego). The intron 5 sequences of goat AMEL-X and AMEL-Y clones were determined using the BigDye terminator cycle sequencing system (Applied Biosystems Inc., Foster, CA), as described previously [20], [21]. The difference between intron 5-X and intron 5-Y of AMEL genes was analyzed under comprehensively comparison of nucleotide sequences through the DNASTAR nucleic acid analysis computer software (Dnastar Inc., Madison, WI, USA). The similarity alignments of AMEL amino acids sequences among different species were performed using the GCG Sequence Analysis Software (Genetic Computer Group, WI) [22].

C. Micromanipulation of Goat Embryos

Goat embryos were collected by means of surgical method, flush the oviducts at the fifth to sixth day after insemination and obtain unimplanted embryos at morula or blastula stage [23]. In order to obtain higher embryo numbers, every donor goat had been treated with endocrine so as to achieve the object of superovulation. Such a superovulation treatment comprised of administrating intramuscular of follicular stimulating hormone (FSH) to the donor goat sequentially for 4 days since eighth day of estrous cycle twice a day with 12 h interval and the dosage was decreased daily as 4-, 3-, 2- and 1-mg, respectively. As the first dosage at the third day, co-administrated with 1000 I.U. human chorionic gonadotropin (HCG) which resulted in detection of estrous after 54 hours whereupon gave two artificial inseminations (AI) with 12 h interval. The goat embryos were collected with a sterile glass capillary tube via surgical method. The collected embryo was transferred into another petri dish where it was rinsed more than ten times. Thereafter, it was placed under a phase contrast microscope at 400X amplification for sampling of single blastomere at morula or blastula stage. The single blastomere obtained was placed directly in a 0.5-mL thin wall microcentrifuge tube and frozen quickly in a refrigerator at -70°C until PCR sexing. As soon as the single blastomere had been sampled, the goat embryo was placed into a DPBS culture medium (Gibico-BRL, Gaithersburg, MD) containing 10% serum and cultured in an incubator at 39°C to cure the mechanical damage at its zone pellucida [24].

D. Single-Blastomere PCR Gender Determination

For goat embryos sexing, we developed a triple-primer set of PCR amplification system, the upstream primer (gAMEL-XY-U1: 5'-AGCAACAGACAAGACC AAGC-3') matches completely the intron 5-X and intron 5-Y of AMEL gene sequences, while the two allele-specific downstream primers (gAMEL-Y-S1: 5'-TGCCA TATAGATAGACAAGC-3' and gAMEL-X-S2: 5'-ACC CACCATAAAAAGCTATTG-3'), allows unambiguous identification of both Y and X chromosome signals in a single reaction, respectively. The PCR products derived from a female embryo (XX) has a length of 246-bp, while those products derived from a male embryo (XY) have lengths of 246-bp and 206-bp, thereby determining the sex type of the goat embryo. The biopsy sample was spin down for a few seconds and denatured by heating the tube on a PCR apparatus (MJ Research Inc., Waltham, MA) at 97°C for 5 min then chilling on ice. A reaction mixture consisting of 20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 10 mM MgSO₄, 0.1% Triton-X 100, 0.1 mg/ml BSA, 0.4 μM of each primer, 0.2 mM of dNTP and 2.5 units of Taq polymerase (Perkin-Elmer Cetus, Foster) was added to each sample in a 0.5 ml tube (final volume, 50 μl). A total of 50 cycles of polymerization was carried out by two-stage procedure. The first stage was aimed at increasing the binding efficiency of the primers on the single-cell template, the time of incubation for denaturation, annealing and extension were set at 10, 10 and 15 sec at 94, 54 and 72°C, respectively. The following 30 cycles was focused on the amplification of gene fragment and the specificity of PCR products by means of increasing the annealing temperature to 55°C. The final extension time was increased to 1 min at 70°C and then cooling down to room temperature (Chen *et al.*, 1999). PCR products were analyzed on 2% high-resolution Syner gel (Diversified Biotech, Boston, MA) containing ethidium bromide.

E. Statistical Analysis

All of the data were performed by using the student t- test statistical analysis. A difference between two means was considered statistically significant based on **P* < 0.05.

III. RESULTS AND DISCUSSION

A. Molecular Cloning of Amelogenin mRNA Transcripts from Male and Female Goats

The full-length 624-bp goat amelogenin (gAMEL) mRNAs each encoded an identical 16-aa secretory signal peptide followed by a 191-aa mature polypeptide, as shown in Fig. 1. In this study, RT-PCR was applied to identify and clone the goat AMEL cDNA fragments from enamel tissue of both male and female lambs during tooth development. A major 752-bp gAMEL cDNA product was amplified from the AMELX gene (GenBank accession number: AF215889) and was present in male and female samples, whereas a minor 746-bp gAMEL cDNA product were amplified from the AMELY gene (GenBank accession number: AF215890) and was only present in male samples. The sequences of the 5'-UTR and exons were identical between the goat AMELX and AMELY mRNA transcripts, but high nucleotide diversity

was found in their 3'-UTRs (Fig. 1).

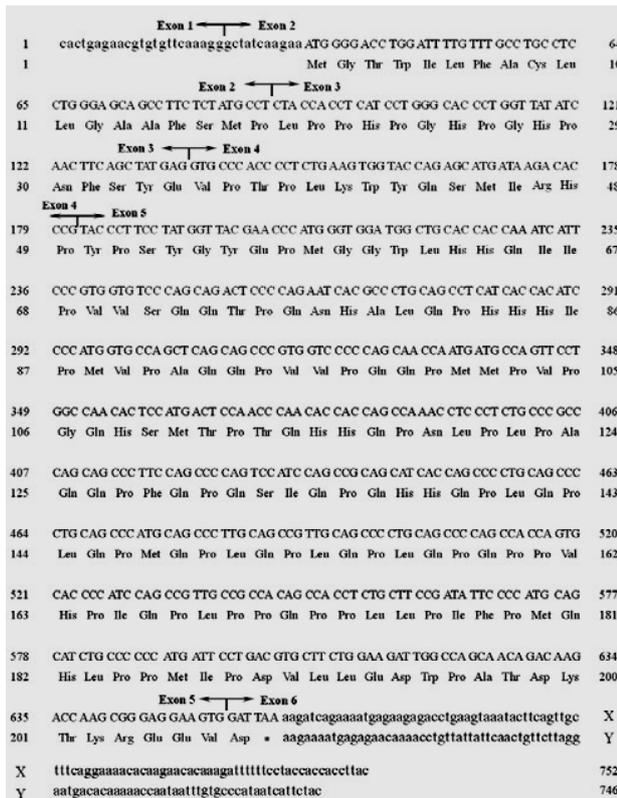


Fig. 1. The complete coding sequence of the goat AMELX and AMELY genes cloned from developing ameloblast tissues of female and male alpine dairy goats. The numbering of the amelogenin cDNA and translated protein sequences begins at the translational start codon. The number of the first nucleotide in each row is provided at the left. "T" bars with arrows indicate exon junctions and the exon numbers that border the sites. An asterisk (*) marks the translational stop codon. The different sequences of the 3'-UTRs present in AMELX and AMELY are marked with X and Y, respectively.

B. Structure Analysis of Amelogenin Protein among Different Species

The putative 207-amino acid sequence of the goat AMEL protein from the coding sequence was deposited in NCBI database (GenBank accession number: AAG43997). The alignment of the goat AMEL peptide sequence with sequences from different species, including bovine, pig, human, and mouse, was constructed using the GCG Sequence Analysis System. The 16-aa secretory signal peptide and biomineral matrix domain located in the N-terminus of the goat AMEL mature protein are highly conserved among different mammalian species (Fig. 2). More diversity was found in the C-terminus of the AMEL protein in this study. It has also been reported that an alignment of the variable region of the exon 6-derived amino acid sequence of 17 other species shows more deletions and variations (Sire *et al.*, 2005). The secondary structure of AMEL has been a challenging problem for structural biology because it has a rigid structure that arises from contiguous β -turns, which impart a β -spiral structure (Renugopalakrishnan, 2002). In this study, we found that goat AMEL contains a 24-residue tandem repeat sequence of β -spiral structure, QHH-QPL-QPL-QPM-QPL-QPL-QPQ (Fig. 2). This β -spiral turns may offer an ideal structure for the passage of calcium ions through amelogenin.

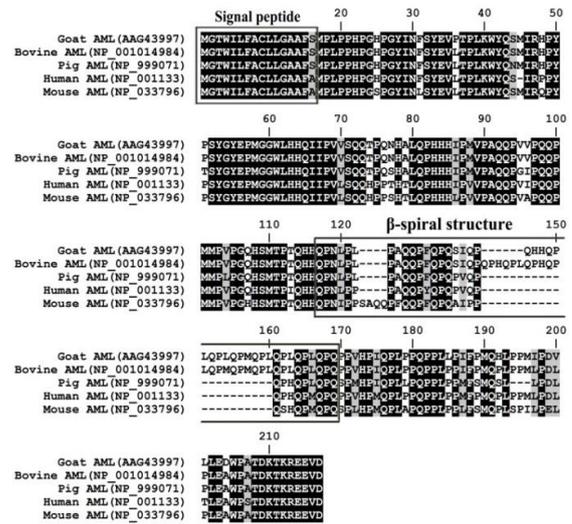


Fig. 2. Alignment of the goat, bovine, pig, human and mouse amelogenin amino acid sequences deduced from genomic DNA (Human) or cDNA (goat, bovine, pig and mouse). Amino acids are represented by the one-letter code. Identical sequences are marked with a black background, and similar sequences are marked with a gray background. The dashes represent gaps in the amino acid sequence. The signal secretion peptide is encoded by amino acids 1-16, as shown in the N-terminus. A special β -spiral structure present in the highly variable region of AMEL is marked with an open box.

C. Identification of Sex-Specific Sequences between AMEL-X and AMEL-Y Genes

To identify the gender-specific sequences, the sequences of intron 5 of the goat AMEL alleles on the X and Y chromosomes were amplified by PCR. The PCR products generated were 1,577-bp and 1,780-bp in females and males, respectively. The similarity between the newly cloned intact intron 5 sequences of goat AMELX and AMELY was only 48.5%. These results are consistent with the previous report that the sequence similarity of intron 5 is less than 30% between different species, whereas high degrees of similarity (>80%) in the coding region are found among humans, swine, and bovine [25].

D. A Multiplex PCR Sexing Using Amelogenin Gene Polymorphisms

For PCR-based goat embryo sexing, a set of three primers was developed that spanned a 58-bp multiple deletion region in AMELY. The upstream primer (gAMEL-XY-U1) matched the exon 5 sequence that is identical between both goat AMELX and AMELY. The two downstream primers (gAMEL-Y-S1 and gAMEL-X-S2, as shown in Materials and Methods) matched sequences in intron 5-Y and intron 5-X that are identical between goat AMELX and AMELY, respectively. The three primers enabled the unambiguous identification of both Y and X chromosome signals in a single reaction. The PCR product derived from a female embryo (XX) had a length of 264-bp, while the products derived from a male embryo (XY) had lengths of 264-bp and 206-bp. Therefore, the number of bands generated indicated the sex of the goat embryo.

E. A Multiplex PCR Sexing Using Amelogenin Gene Polymorphisms and Comparison with Chromosomal Karyotyping

The sensitivity of the AMEL-based sexing assay was

evaluated on a dilution panel of genomic DNA from male and female goats to simulate the DNA quantities that could be isolated from embryo biopsies. The *AMELX* and *AMELY* PCR products were detected with at least 5 pg of genomic DNA, suggesting that this assay is suitable for sex diagnosis with as little as single cell. The PCR protocol and the three primers were successfully applied to sex determination in single blastomeres taken from 6- day-old embryos (Fig. 3 (A)). Sample Nos. 3 and 4 showed a single 264-bp fragment and were interpreted as female. Sample Nos. 1 and 2 showed both the 264- and 206-bp amplification products and were interpreted as male (Fig. 3 (C)). The results were fully confirmed by chromosomal karyotyping (Fig. 3 (B)) and live births. Although the primer sequences were based on the cloned *AMEL* gene of *Alpine* dairy goats, they worked equally well with embryonic blastocysts isolated from the *Saanen*, *Nubian*, and *Taiwan* goat strains (data not shown). Therefore, this system may be useful for various popular dairy goats.

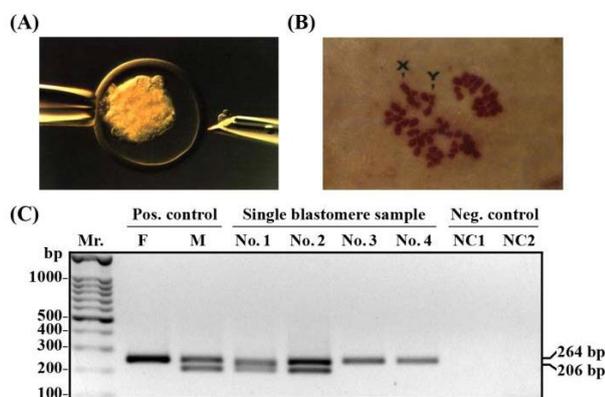


Fig. 3. PCR-based sexing by triplex primers of the goat *AMEL* gene in single blastomeres. (A) Image represented the goat single blastomere biopsy. (B) Chromosomal karyotyping of goat blastomere to confirm the sexing results. (C) PCR products from a single biopsied goat blastomere in an embryo-sexing experiment. The gender-neutral (264 bp) and the male-specific PCR products (206 bp) are indicated. Mr.: molecular size marker. A PCR reaction without an embryo blastomere was used as a negative control (NC). The amplified products were applied individually to the wells of a 2.0% high-resolution Synergel.

IV. CONCLUSION

The performance trait of milk production is the most important genetic variable in the dairy industry. There is increasing demand in the marketplace for determination of the sex of an unborn bovine or goat fetus [24]. Owners want to know whether male and female goat contracts have been filled or they need to continue producing embryos from donor goats. The sex of the fetus can change the value of the pregnancy. Another economic benefit of embryo sexing is that it gives owners the ability to control the genetic background [26]. In addition to improved genetic control, gender preselection provides additional advantages for management and facility usage.

The *AMEL* gene-based tests can be used for sex diagnosis of small amounts of genomic DNA obtained from different sources, including blood DNA and embryo biopsies. It also can be extensively used in several other applications including forensics, archaeozoology, and meat production and processing. Moreover, the sequences obtained of the

AMELX and *AMELY* alleles showed unanticipated highly polymorphisms, especially in the β -spiral structure, which would be interesting to study further from an evolutionary perspective.

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