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Molecular analysis of 60,XX pseudohermaphrodite polled goats for the presence of SRY and ZFY genes

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The polled mutation is characterized by a recessive, incompletely penetrant, hermaphroditic effect associated with the dominant genetic factor responsible for polledness in breeds of goat. The present study describes the external morphology, anatomy of the reproductive tract, histology, chromosomal constitution and Y chromosome screening of three intersex polled goats. The animals were tested for different Y-specific sequences, including SRY and ZFY. Using Southern blot and PCR amplification, no Y-derived sequences were detected in DNA from three 60,XX pseudohermaphrodite goats. This, therefore, excludes Y chromosome translocation and XX/XY chimaerism and mosaicism. This recessive mutation leading to sex reversal might be used to map and ultimately clone the autosomal genes implicated in the sex-determining pathway.

Introduction

Since the 1950s, it has been known that sex in humans and in other mammals is determined by the presence or absence of the Y chromosome (Jost, 1947; Ford *et al.*, 1959). The concept that one or more genes on the Y chromosome induces the undifferentiated gonad to become a testis is stressed by the existence of various sex chromosomal aberrations. Certain individuals with an XX karyotype develop testes. In humans, the majority of them, known as XX males (de la Chapelle, 1987), are generated by an abnormal exchange of X- and Y-specific DNA during paternal meiosis (Seboun *et al.*, 1986; Petit *et al.*, 1987). In contrast, most XX males with genital ambiguities have been described as Y negative (Abbas *et al.*, 1990). Autosomal or X-linked mutations in the sex-determining pathway may explain this phenotype. Anomalous sexual development has been described in farm animals. In most reports, the emphasis has been descriptive and few cases of intersexuality have been reported including anatomical observations, histology of gonads and cytogenetic analysis (Hamerton *et al.*, 1969; Marcum, 1974; Hare and Singh, 1979; Hunter *et al.*, 1988; Cribiu and Chaffaux, 1990), and the presence of Y fragments has not been tested by molecular analysis.

In goats, the polled mutation is characterized by the suppression of horn formation and abnormal sexual differentiation (Soller *et al.*, 1963; Ricordeau and Lauvergne, 1967). 'Male' goats with sex anomalies include at least two distinct categories: XX individuals with testicular hypoplasia (pseudomales), and XY individuals with epididymal defects or various degrees of intersexual modifications (Basrur and Kanagawa, 1969; Soller *et al.*, 1969). Phenotypically, XX animals, all homozygous for the absence of horns (*PP*), vary from almost normal male to almost normal female showing that the expression of sex-inversion is variable (Ricordeau, 1991). Data collected by Soller and Angel (1964) on 1362 Saanen offspring of genetically defined matings showed that the polled factor has a recessive effect on sex ratio with a significant deficiency of females within homozygous *PP*. Analyses were made of the mode of inheritance of hornedness, intersexuality and infertility of male goats. There is a very strong linkage between polledness and intersexuality. The absence of horns is due to an autosomal dominant gene with complete penetrance in both sexes and probably the same gene or another closely linked gene has a recessive, incompletely penetrant, masculinizing effect on XX individuals (Ricordeau and Lauvergne, 1967; Ricordeau *et al.*, 1972).

The study of intersexuality in domestic mammals may help in understanding the mechanisms of sexual differentiation, particularly for mutations affecting non-Y downstream sex-determining genes.

In this study, we screened three polled intersex goats with ambiguous genitalia for different Y-specific sequences, including SRY (sex-determining region Y chromosome) using Southern blot and PCR analysis. Evidence suggests that SRY is the testis-determining factor (Sinclair *et al.*, 1990). Two

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cellular sources of DNA were tested (blood and gonads) to exclude a hidden mosaicism limited to the gonads.

Materials and Methods

Animals

The three unrelated goats were of alpine breed. They were singletons born from polled parents and were aged 3, 5 and 18 months.

Cytogenetic methods

Karyotyping was performed using whole blood cultures (Grouchy *et al.*, 1964) and primary gonadal cell cultures (Cribiu *et al.*, 1989). Blood samples were cultured for 3 days at 37°C and 0.5 ml of whole blood was added to 10 ml of Medium 199 (Gibco BRL, Life Technologies Inc., Grand Island, NY) supplemented with 20% fetal calf serum (Gibco), antibiotics (Gibco), L-glutamine (Gibco) and Concanavalin A (100 μg ml^{-1}; Serva, Feinbiochemica, Heidelberg/New York). Colcemid (final concentration: 0.03 μg ml^{-1}; Gibco) was added 60 min before harvesting.

Primary fibroblast cultures were initiated from gonadal fragments, disrupted and digested in a trypsin solution (2.5 g l^{-1}) and grown in an incubator as monolayer cultures in Falcon dishes (75 cm^2; Becton Dickinson Labware, Lincoln Park, NJ) containing Medium RPMI 1640 (Boehringer Mannheim Gmbh, Mannheim). G-banding was achieved using a modification of the technique of Seabright (1971). To induce R-banding, 5-bromo-2-deoxyuridine (Sigma, St Louis, MO) was added to the medium at a final concentration of 10 or 20 μg ml^{-1}. The cells were treated according to the procedure described by Hayes *et al.* (1991) and Fluorochrome-Photolysis-Giemsa (FPG) staining was performed as described by Viégas-Péquignot *et al.* (1989).

The chromosomes were arranged according to the standardized G- and R-banded caprine karyotypes (Ford *et al.*, 1980 and Di Berardino *et al.*, 1989).

Histology

Half of the gonads from all the animals were fixed in H_2O : formol (9:1, v:v) and histological sections were stained with haematoxylin safran.

DNA analysis

DNA was extracted from peripheral blood lymphocytes and from gonads of the three animals and controls using the procedure of Sambrook *et al.* (1989).

DNA blot preparation. Southern blot analysis was performed with *Eco*RI restricted genomic DNA (35 μg per well), run on 0.8% agarose gels (TBE × 1; 2–3 V cm^{-1} for 16 h). DNA transfer onto a nylon membrane (Schleicher and Schuell, Dassel) was carried out as recommended by the manufacturer, according to the method of Southern (1975).

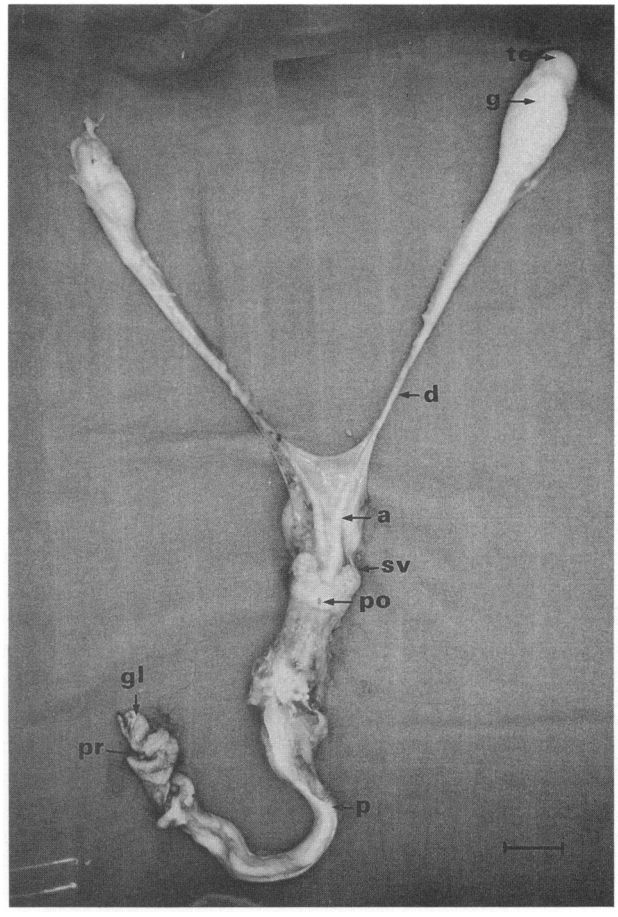

Fig. 1. Internal genital tract of the intersex 93. te: tail of epididymis; g: gonad; d: duct deferens; a: ampulla; sv: seminal vesicles; p: penis; gl: glans; pr: prepuce; po: prostate. Scale bar represents 2 cm.

Prehybridation and hybridization were performed at 40°C in a solution containing 50% formamide, 5 × Denhardt's, 6 × SSC, 0.5% SDS and 100 μg salmon sperm DNA ml^{-1} (according to Sambrook *et al.*, 1989). Probes were labelled with ^{32}P by random priming (Megaprime DNA labelling system: Amersham) and used at a final concentration of 1×10^6 c.p.m. ml^{-1} of hybridization buffer. Washings were performed three times with 2 × SSC, 0.1% SDS at 40°C for 30 min. For the ZFY probe, we used pDP 1007 a fragment of the human ZFY gene (Page *et al.*, 1987) and for SRY analysis an ovine cloned 170 bp PCR product.

PCR amplification. Approximately 1 μg of DNA was amplified using a Perkin-Elmer Cetus thermal cycler. Samples were submitted to 30 cycles of the following: denaturation 94°C for 1 min, annealing at 55°C for 1 min (SRY and BOV 97M) and at 58°C for 1 min (ZFY), and extension at 72°C for 1 min. Ten microlitres of the PCR reaction mix was subjected to electrophoresis in an ethidium bromide stained 2% agarose gel. The primers used for ZFY/ZFX amplification were those described by Aasen and Medrano (1990). The specificity of BOV 97M primers for male goat DNA was demonstrated by Appa Rao and Totey (1992). The SRY specific primers for

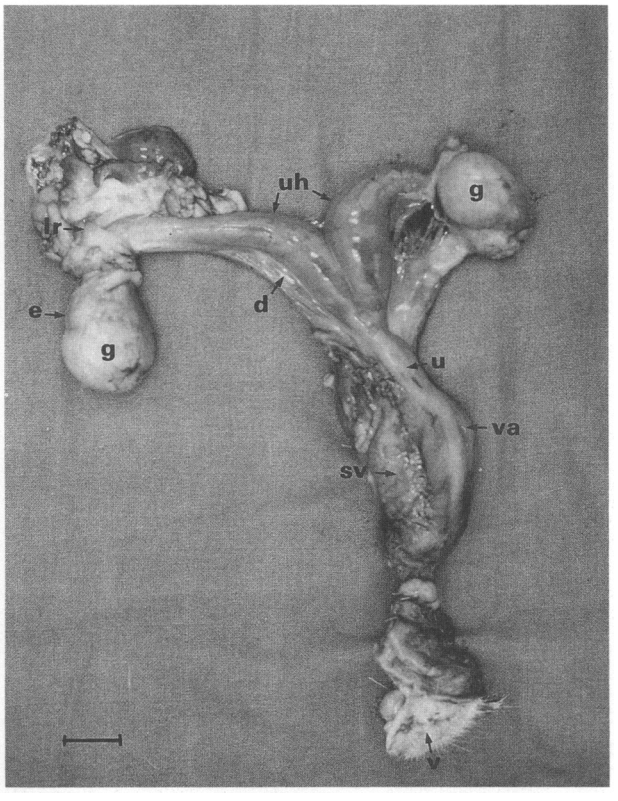

Fig. 2. Internal genital tract of the intersex 88. g: gonad; lr: inguinal ring; e: epididymis; d: duct deferens; uh: uterine horn; u: uterus; sv: seminal vesicle; v: vulva; va: vagina. Scale bar represents 3 cm.

goat DNA are M1:5′-CATTGTGTGGTCTCGTGAA-3′ and M2: 5′-TGTCTCGGTGTATAGCTAG-3′.

Results

Anatomical findings

The three animals exhibited two inguinally located udders. One of the goats had a less masculinized phenotype on the basis of general conformation and external genitalia (no. 88), whereas the other two were phenotypically male with descended testes (nos 93 and 29).

Animals 93 and 29. The anogenital distance of the two animals was greater 10.5 cm and 15 cm, respectively, than in control animals. They exhibited a penis in a ventral position and hypospadia was noted. At the back of the sheath, an empty well-developed sac (93) or rudimentary sac (29) indicated the presence of a sort of scrotum. Two gonads were present in the superficial inguinal rings region; the left gonad was smaller than the right in both animals. Post-mortem examination revealed no Müllerian derivatives. The gonads resembled a testis with a vaginal process. Well-developed epididymides, vasa deferentia and seminal vesicles were seen in both animals. Animal 93 had a prostate gland (Fig. 1).

Animal 88. The anogenital distance of this animal was short (3.6 cm). The external genitalia was represented by a 4 cm long vulva including an enlarged clitoris. At the tip of this clitoris, a vermiform appendix was present. One palpable gonad was present in the left superficial inguinal ring. After slaughter, the gonads were found to be testis-like. The right abdominal gonad was smaller than the left inguinal one and Müllerian derivatives and Wolffian structures were observed. Paired epididymides and vasa deferentia were present along the uterus which was divided into two uterine horns of different size. No oviducts or fimbriae were found in the cranial border of the uterine horn. There was no cervix and the vagina was dilated by the accumulation of secretions. Two vesicular glands were noted dorsally on both sides of the vagina (Fig. 2).

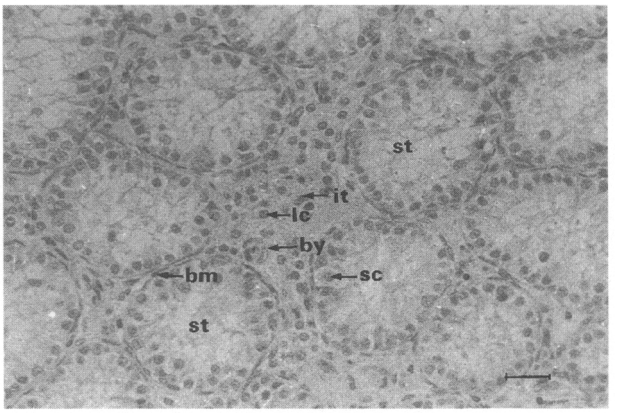

Fig. 3. Histological section of the left testis of intersex 29. st: seminiferous tubules; sc: Sertoli cell; lc: Leydig cell; it: intertubular tissue; bv: blood vessel; bm: basal membrane. Scale bar represents 40 μm.

Histology

The gonads of the three animals did not exhibit any ovarian structures and contained testicular-like structures. The tunica albuginea seemed normal.

The histological structures of the two prepubertal animals (88 and 29) were conserved. The seminiferous tubules lined by Sertoli cells and the intertubular tissue were normal; germ cells were absent (Fig. 3). The testicular structures of the pubertal intersex (93) were partially disorganized; the seminiferous tubules were narrow and lined by degenerated Sertoli cells. No germ cells were observed. Some of the tubules were surrounded by fibrosis and others by oedema. The interstitial tissue was abundant and contained Leydig cells and small cells resembling fibroblasts. Fibrosis was also observed in the rete testis.

Cytogenetic analysis

The karyotypes of the three animals obtained using blood and gonad cells were composed of only $2n = 60$,XX chromosomes with no structural anomaly (Fig. 4). No Y chromosome was observed in any of the 200 G- or R-banded metaphase spreads examined from each animal.

Molecular analysis

The presence of Y-related sequences was assayed using PCR amplification and Southern blot analysis. The six DNA samples

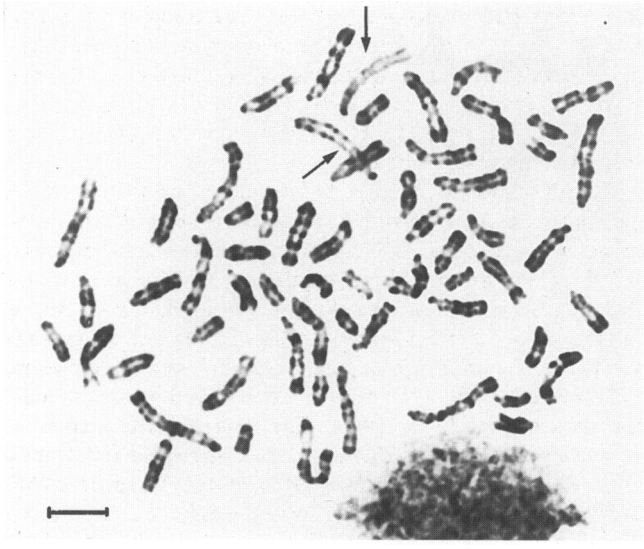

Fig. 4. R-banded metaphase spread of intersex 93. Sex chromosomes are indicated by the arrows. Scale bar represents 5 μm.

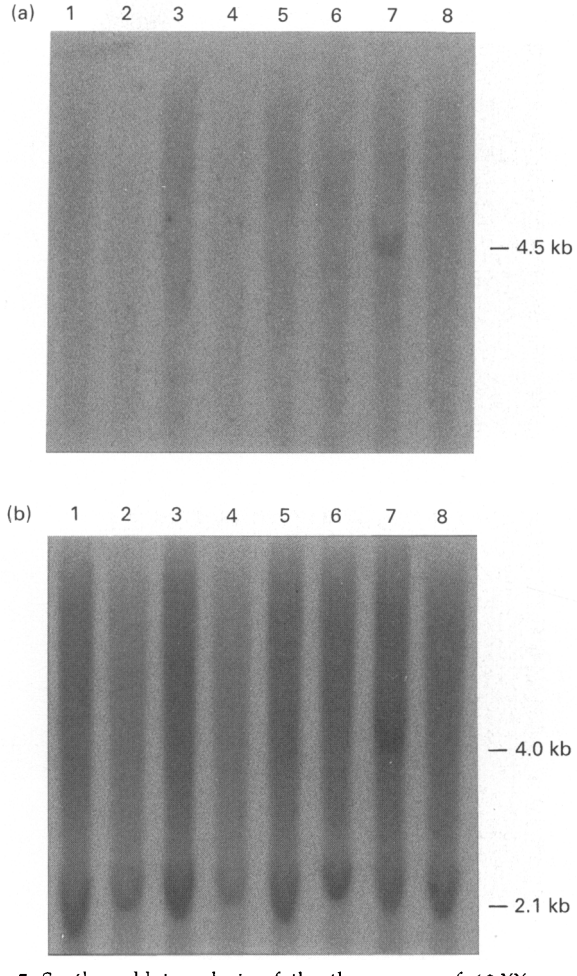

Fig. 5. Southern blot analysis of the three cases of 60,XX pseudohermaphrodite polled goats for presence of SRY and ZFY genes. DNA (35 μg) was digested by *Eco*RI and probed with the Y-specific sequences: (a) goat SRY fragment and (b) pDP 1007. Lanes 1 to 6 are intersex animals and lanes 7 and 8 are male and female animals, respectively. From blood (lanes 1, 3, 5) and from gonads (lanes 2, 4, 6), DNA of the three intersex animals shows an absence of hybridization signal. In male control (lane 7a), a specific 4.5 kb band is present with SRY probe. For ZFY, the pDP 1007 probe also recognizes the X homologue ZFY. Hence, all animals present a 2.1 kb band (b) and only male DNA has a fragment of 4.0 kb (lane 7b).

(three from blood and three from gonads) were hybridized successively with A17 probe, which recognizes the goat HMG-box region of SRY gene and with pDP 1007, a part of the human ZFY gene. A single *Eco*RI male specific band at 4.5 kb was observed in normal male goat following probing with A17. This band was absent from DNA of female goat and from DNA of the three homozygous XX polled goats (Fig. 5a). No additional band was detected after one week of autoradiography with 35 μg of blotted DNA.

With the pDP 1007 probe, a common male and female band at 2.1 kb from *Eco*RI digested DNA and a male-specific band at 4.0 kb was present only in the male control (Fig. 5b). These results indicated that SRY and ZFY genes were absent from the genome of the intersex polled goats. To exclude hidden chimaerism or mosaicism limited to the gonads, DNA was extracted from this tissue and tested for the presence of Y-related sequences by PCR amplification. Three sets of primers were used, one gave a 160 bp PCR product corresponding to Y-specific repetitive sequence (BOV 97M), the second a 169 bp fragment of the SRY gene and the third a 445 bp fragment homologous to ZFY and ZFX. The ZFY fragment remained uncut by SacI, whereas the ZFX homologue was digested to give two fragments of 272 bp and 173 bp (Fig. 6a). The results obtained from XX intersex polled goats (Fig. 6a,b,c) showed that all animals were devoid of Y material and particularly of the SRY gene.

Discussion

The external and internal genitalia of the intersexes suggest that they may be freemartins or that the phenotype resulted from the presence of Y chromosome material. The freemartinism syndrome occurs in females born co-twin to a male (Lillie, 1917) and is caused by the fusion of fetal membranes and vascular anastomosis between co-twin caprine fetuses that allows the passage of haematopoietic cells and hormones from the male to the female (Ilbery and Williams, 1967; Jost *et al.*, 1972). In this case, chromosome analysis of blood cells reveals the coexistence of XX and XY cells in the male and the female co-twins (Cribiu *et al.*, 1991).

The cytogenetic data on the three caprine intersexes in the study reported here clearly shows that they are karyotypically female without chimaerism or mosaicism XX/XY. The absence of a proportion of male cells in haematopoietic tissues confirms that they are not freemartins in which the male co-twin was dead *in utero*.

The second hypothesis that could explain the presence of testes in these XX animals is an accidental X–Y or autosomal–Y translocation including the male testis-determining gene, SRY (Sinclair *et al.*, 1990). In humans, about 75 cases of 46,XX with

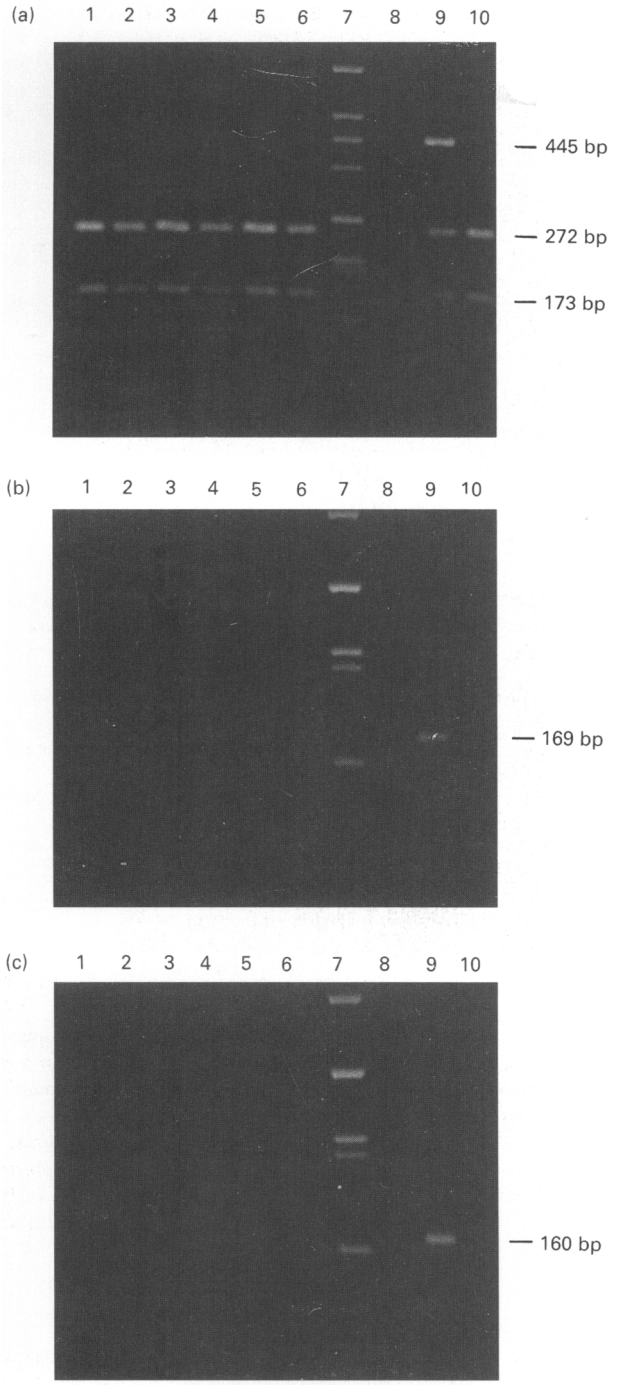

Fig. 6. PCR analysis of Y-derived sequences in DNA prepared from blood and gonadal biopsies of three cases of 60,XX pseudohermaphrodite polled goats. Amplification using oligonucleotides from (a) ZFY sequence generated a 445 bp Y-specific fragment (Y) and two X-specific bands at 272 and 173 bp (X). With SRY specific primers (b), the male PCR product contains 169 bp, whereas using BOV97M primers (c) a 160 bp Y-specific fragment was observed. Genomic DNAs used for amplification were: animal 29 (lanes 1, 2), animal 88 (lanes 3, 4), animal 93 (lanes 5, 6), normal male (lane 9), normal female (lane 10). H_2O was used as negative control (lane 8) and pBR328 BglI + HinfI digest as molecular weight marker (lane 7). For intersex animals, even-numbered lanes represent DNA extracted from blood and odd-numbered lanes those containing gonadal DNA.

testes have been described and analysed using molecular Y probes (McElreavey *et al.*, 1993). Two types of patient are clinically distinguishable according to the presence or absence of genital ambiguities. The majority of 46,XX males without ambiguities carried SRY. In contrast, most 46,XX true hermaphrodites lacked Y material.

The results of the Southern hybridization and PCR on three sporadic cases described here demonstrated that all goats lacked SRY and ZFY. They correspond to the second category of XX males with genital ambiguities described in man where SRY is absent. The present study indicates that male sexual differentiation can occur in the absence of SRY, and presumably results from a mutation of an autosomal gene. Autosomal mutations involved in sex reversal have been described in mice (Eicher and Washburn, 1986). The proposal that recessive mutations of autosomal genes can be responsible for XX sex reversal in humans has been suggested by de la Chapelle (1987) and recently a model of sex determination based on the existence of an autosomal gene, Z, the recessive allele of which confers a male phenotype, was proposed by McElreavey *et al.* (1993). The results reported here on sex-reversed polled goats support this model. However, the mode of inheritance of intersexuality in polled goats was demonstrated as recessive (Eaton and Simmons, 1939). As in humans, the phenotypic expression of XX sex-reversed animals that lack SRY is variable. The level of genitalia sex-reversal is not related to the gonadal phenotype. Indeed, the gonads of two animals (29 and 88) present the same testicular structure and yet their phenotype is very different. Conversely, animals with the same phenotype (29 and 93) possess different gonadal structures. Three hypotheses may explain this variability: (i) existence of several mutations, (ii) leakiness of the mutation and (iii) difference in genetic background of the animals. In contrast to humans, in goats, the existence of several mutations can probably be excluded because all intersex animals are carriers of the polled mutation. The lack of dissociation between intersexuality and polled trait strongly infers that the same locus is responsible for intersexuality in all polled goats. The cause of this phenotypic variability remains unknown and further investigations are required to test the validity of the two remaining hypotheses.

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