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Lack of the *SOX9* Gene Polymorphism in Sex Reversal Dogs (78,XX; *SRY* negative)

J. Nowacka, W. Nizanski, M. Klimowicz, S. Dzimira, and M. Switonski

From the Department of Animal Genetics and Breeding, Agricultural University of Poznan, Wolynska 33, 60-637 Poznan, Poland (Nowacka and Switonski); Departments of Reproduction (Nizanski and Klimowicz) and Pathology (Dzimira), Agricultural University of Wroclaw, Grunwaldzka 49, 50-366 Wroclaw, Poland.

Address correspondence to M. Switonski at the address above, or e-mail: switonsk@jay.au.poznan.pl.

Abstract

The molecular background of the most frequent intersexuality syndrome in dogs (female-to-male sex reversal with the female karyotype and a lack of the *SRY* gene) is unknown. In this article, new cases of this syndrome are described in two unrelated American Staffordshire terrier dogs and one miniature pinscher dog subjected to cytogenetic and molecular analysis due to the presence of an enlarged clitoris. One dog was operated on and histological studies of the gonads revealed a testicular structure without signs of spermatogenesis, but the uterus wall appeared to be normal. All three dogs had female chromosome complements and lacked the Y-linked genes *SRY* and *ZFY*. Eight fragments, representing the vast majority of the coding sequence of the *SOX9* gene, and two fragments of the 5′ flanking region of this gene were analyzed. The studied fragments had identical DNA sequences when comparing the intersexual dogs with GenBank sequences (AY237827; NW139883). Thus a mutation in the coding sequence as well as the promoter region of the *SOX9* gene might be excluded as a cause of this type of intersexuality. The importance of further studies of the 5′ flanking region of this gene is discussed.

Intersexuality, a congenital defect of the reproductive system, is caused by two main mechanisms: sex chromosome abnormalities and mutations of the genes involved in sex determination. The number of sex chromosome aberrations diagnosed in the dog is not very large; however, all major sex chromosome aneuploidies were described in this species [for a review see Switonski et al. (2004b)]. On the other hand, the molecular background of the most common intersexuality syndrome in dogs, the so-called sex reversal in female dogs having normal XX chromosome complements and missing the *SRY* gene, is unknown (Switonski et al. 2004a). This syndrome, presenting a recessive mode of inheritance, has also been found in goats, horses, and pigs (Vaiman and Pailhoux 2000). Extensive studies carried out in the goat revealed that a deletion of an 11,700 bp fragment, which alters the expression of the neighbor genes (*FOXL2* and *PISRT1*) involved in mammalian sex determination, is a cause of this type of intersexuality (Pailhoux et al. 2001). Unfortunately, in the dog this chromosome region does not harbor the causative mutation (Kothapalli et al. 2003).

It is known that *SRY* controls the expression of the *SOX9* gene. Thus in intersexes missing the *SRY* gene, testicular development is possible if expression of the *SOX9* gene is maintained. Testis differentiation is clearly related with the timing of *SRY* and *SOX9* gene expression and it was suggested that an alteration of *SOX9* gene expression, in the absence of the *SRY* gene, might be responsible for testis development in the sex reversal (XX, *SRY* negative) fetus (Meyers-Wallen 2003). On the other hand, it is also known that expression of the *SOX9* gene is detected in both male and female urogenital rides prior to the time of *SRY* gene expression (Meyers-Wallen 2003; Parma et al. 1999). Therefore one can speculate that both the 5′ flanking region and the structure of the *SOX9* coding sequence are crucial while searching for a cause of testicular differentiation in sex reversal (XX, *SRY* negative) animals.

The recent study of Qin et al. (2004) indicated that *SOX9* can induce testis development in a transgenic XX mutant *Odd sex* (*Ods*) mouse, which has an extra copy of the tyrosinse minigene driven by the dopachrome tautomerase (*Dct*) promoter. This construct was incorporated about 1 Mb upstream of *SOX9* and caused the deletion of a 134 bp genome fragment. It was found that the heterozygous (*Ods*/+) mice demonstrated a complete female-to-male sex reversal syndrome due to the interaction between the *Dct* promoter and gonad-specific enhancer elements. As a result, a male pattern expression of the *SOX9* gene occurred in embryonic gonads. The deletion of a fragment of 134 nucleotides is probably required for a change in the local chromatin conformation, bringing the *Dct* promoter close enough to the *Sox9* enhancer region to cooperate with it. The latest research on XY mice confirmed that the male phenotype can

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be controlled autosomally without the *SRY* gene, but with expression of the *SOX9* gene (Qin and Bishop 2005).

In this article we report for the first time a sex reversal syndrome (XX, *SRY* negative) in American Staffordshire terrier and miniature pinscher dogs along with the results of molecular analysis of the coding and 5′ flanking sequences of the *SOX9* gene in sex reversal dogs. Moreover, a histological study of gonads and the uterine wall of the intersexual American Staffordshire terrier was performed.

Materials and Methods

Three female dogs, a 2-year-old American Staffordshire terrier (case 1), a 14-month-old American Staffordshire terrier (case 2), and a 2-year-old miniature pinscher (case 3), were subjected to cytogenetic and molecular analyses due to their ambiguous external genitalia. All three animals had an enlarged clitoris with a 3–4 cm bone (Figure 1). In two animals (cases 2 and 3), the clitoris was removed by a surgeon.

Chromosome preparations were obtained from peripheral blood lymphocyte cultures and stained with Giemsa. This type of staining facilitates easy distinguishing between one-armed autosomes and both biarmed sex chromosomes. Fifty metaphase spreads per animal were analyzed.

Genomic DNA was extracted from peripheral blood with the use of a commercial kit (Blood DNA Prep Plus, A&A Biotechnology, Gdansk, Poland). The following fragments were studied: 104 bp of the *SRY* gene (Meyers-Wallen et al. 1995) and 448 bp of the *ZFX* and *ZFY* genes (Senese et al. 1999). Eight fragments of the *SOX9* gene (GenBank AY237827) covering all three exons and two fragments of its 5′ flanking region derived from the dog contig (GenBank NW139883) were amplified by polymerase chain reaction (PCR) using a T-gradient thermocycler (Biometra, Goettingen, Germany; Table 1). The PCR conditions were as follows: 35 cycles of denaturation at 94°C for 40 s; annealing of primers at 64°C (*SRY*), 65°C (*ZFX* and *ZFY*), and 63°C to 68°C (*SOX9*) for 40 s; elongation at 72°C for 1 min; and final elongation at 72°C for 10 min. With the use of the restriction fragment length polymorphism (RFLP) technique, the products of *ZFX* and *ZFY* genes were digested by the *Hae*III restriction enzyme at 37°C for 3.5 h and the following fragments were obtained: 448 bp, 403 bp, and 45 bp for males and 403 bp and 45 bp for females. The products of PCR were separated by electrophoresis using 1.5% agarose gel with

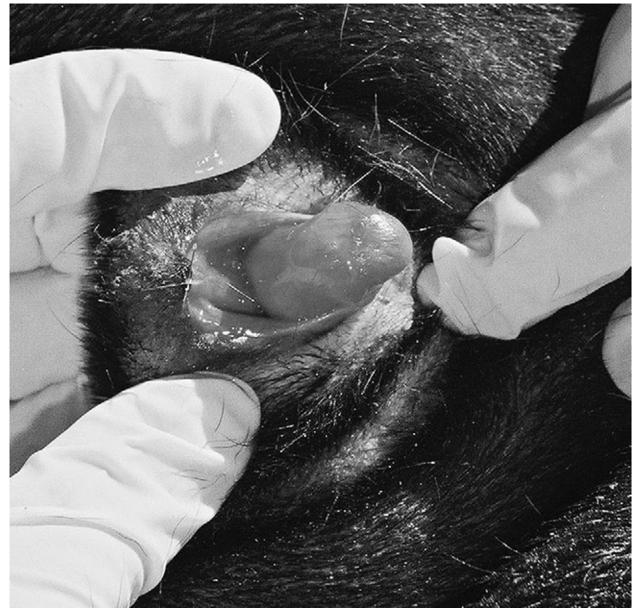

Figure 1. An enlarged clitoris of the American Staffordshire terrier (case 1).

Table 1. Primer sequences used in studies of the *SOX9* gene and its promoter with the PCR product lengths

Primers	Sequences of primes	Length of PCR product
E1	F: 5′ GCGCCCCCAGCCCCACCAT R: 5′ GCCGCCTGCGCCCACACCAT	298 bp
E1-2	F: 5′ AGATGACCGACGAGCAGGAGAAGG R: 5′CTCGCGGATGCACACGGGGAACTT	200 bp
E1-3	F: 5′ CCAAGGGCGAGCCGGACCTGAAG R: 5′CTCCAGAGCTTGCCCAGCGTCTTG	271 bp
E-2	F: 5′ AGCGGCCCTTCGTGGAG R: 5′ TGCTTGCCGGGGGAGTG	229 bp
E3-1	F: 5′ CCCCCACCACCCCGAAAACC R: 5′ CTGATGCCGTAGCTGCCCGTGTAG	256 bp
E3-2	F: 5′ CCGCCGCCCCCGCAGCAGTC R: 5′ GCGAGCGGGTGATGGGCGGGTAGG	292 bp
E3-3	F: 5′ TCCCGCACTACAGCCCGTCCTACC R: 5′ AGTGCTGGGGGCTGTGCGTCTGC	219 bp
E3-4	F: 5′ GCC GGG CAA GGC TGA CCT GAA G R; 5′ GGC GGC GCC TGC TGC TTG GAC A	291 bp
P-1	F: 5′ TGG CCG GCC GTC TCT AAG GTG AGG R; 5′ GGT GGC CGG GAA GGG CGA GAA	422 bp
P-2	F: 5′ CTC CGG GGG TGG GGG CAG AG R; 5′ CTT AGA GAC GGC CGG CCA ATC ACG	384 bp

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Table 2. Performance of three cases of the *SRY*-negative, XX sex reversal dogs

Characteristics	Case 1 (American Staffordshire terrier)	Case 2 (American Staffordshire terrier)	Case 3 (miniature pinscher)
Phenotype	Enlarged clitoris (3 cm), gonads: testes with seminiferous tubules without germ lines, small uterus	Enlarged clitoris (3.5–4 cm) with a bone	Enlarged clitoris with a bone, a very narrow vestibule of the vagina
Karyotype	78,XX	78,XX	78,XX
PCR – *SRY*	Negative	Negative	Negative
PCR-RFLP – *ZFY*	Negative	Negative	Negative
DNA (exons)			
SSCP[a]	No differences	No differences	No differences
Sequencing[b]	A/G; T/C; GCG/CGC; A/G	—	A/G; T/C; GCG/CGC; A/G
DNA (promoter)			
SSCP[a]	No differences	No differences	No differences
Sequencing	No differences	—	No differences

[a] Compared with referenced dogs (females and males).

[b] Differences in the second exon sequence (see text) were identified while comparing with GenBank (AY237827).

TBE buffer, stained with ethidium bromide, and photographed in ultraviolet light. The fragments of the coding sequence and 5′ flanking region of the *SOX9* gene were analyzed with the use of the single-strand conformation polymorphism (SSCP) technique. Electrophoresis was run at 8°C, (350 V) for 5.5 h. The 9% polyacrylamide gel was stained with 0.2% silver nitrate solution.

All the amplified fragments of this gene were sequenced (at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw) and compared with the sequence available in GenBank—AY237827 and NW139883, respectively.

The histological examination was applied to the gonads of the American Staffordshire terrier dog (case 1), which was operated on by a surgeon. The preparations were stained with hematoxylin-eosin.

Results

The intersex dogs were analyzed by cytogenetic and molecular techniques (Table 2). Cytogenetic analysis showed that all the studied dogs had a normal female chromosome complement, 78,XX (data not shown). The size and morphology of both X chromosomes were also normal. Moreover, one animal (case 1) was operated on and the testis-like gonads with the epididymis as well as a small uterus were removed. Histology of the gonads revealed the presence of seminiferous tubules without signs of spermatogenesis (Figure 2). The uterine wall appeared to be normal.

Polymerase chain reaction amplification of the Y-linked genes (*SRY* and *ZFY*) was unsuccessful for intersexual dogs, but successful for control male dogs (data not shown). For all fragments, representing the 5′ flanking and coding sequence of the *SOX9* gene, the observed SSCP patterns were concordant when comparing the studied intersexual dogs with control male and female dogs (Figure 3). To confirm this result the fragments originating from two sex reversal dogs (case 1 and 3) were sequenced. We found four positions in exon 2 at which another nucleotide was present (AY941252), compared with the GenBank sequence (AY237827): (1) G instead of A at position 582, no change in the amino acids sequence; (2) C instead of T at position 594, no change in the amino acids sequence; (3) CGC instead of GCG at position 616–618, an amino acid change: arginine → alanine; (4) G instead of A at position 656, an amino acid change: asparagine → serine. Due to the above mentioned differences, additional sequencing of exon 2 of two healthy reference dogs was performed. The obtained sequences were concordant with those for the intersexual dogs.

Analysis of the *SOX9* genomic sequence (NW139883), including 5′ and 3′ flanking regions, revealed the presence of six repetitive motives (Figure 4). There are two short tandem repeats, $(CT)_9$ and $(GC)_7$ in the first and second intron, respectively. In the 3′ region in the distance of about 1.6 kb and 4.5 kb behind the third exon, there are two motives of $(TG)_7TT(TG)_{13}$ and $(CA)_{19}$, respectively. Also in the 5′ region, which we analyzed, two repetitive motives were identified: $(CCT)_8$ and $(CA)_5GA(CA)_5$; these two repetitive motives appeared to be polymorphic.

Discussion

Intersexual development in mammals with a male sex chromosome complement (XY) and the presence of the *SRY* gene might be caused by mutations of different genes involved in the sex determination process; for instance, *SRY*, the androgen receptor (*AR*), the Mullerian inhibiting substance (*MIS*), 5-α-reductase, etc. There are also known human sex reversal cases caused by *SOX9* gene mutations, diagnosed as campomelic dysplasia syndrome. Until now, at least 27 point mutations of the *SOX9* gene have been reported and 9 of them were localized in the crucial HMG box, causing an amino acid substitution or premature stop codon (Ninomiya et al. 2000). It is also predicted that mutations in the promoter region may result in campomelic dysplasia (Kanai and Koopman 1999; Wunderle et al. 1998).

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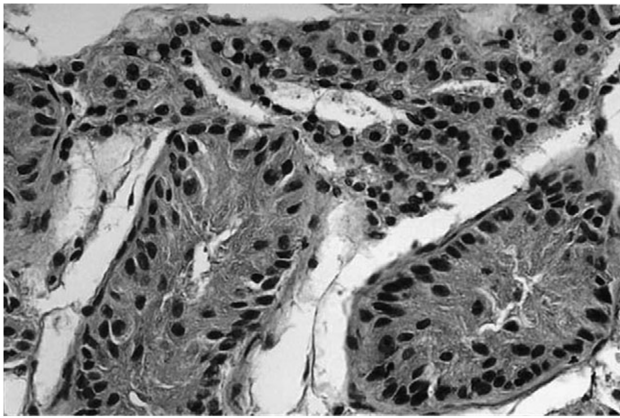

Figure 2. Seminiferous tubules without spermatocytes and the presence of Sertoli and Leydig cells in a testicle of a intersexual dog (case 1) (400×).

The molecular background of the XX sex reversed syndrome was described in goats (Pailhoux et al. 2005). The authors demonstrated that a deletion of a 11,700 bp genome fragment inhibits the expression of *FOXL2* and *PISRT1* loci. A lack of expression of *PISRT1* (noncoding RNA) upregulates the expression of the *SOX9* gene, and thus testicular development is observed. There is only one report concerning the *SOX9* gene mutation in human female-to-male sex reversal with a mosaic karyotype: 46,XX,dup(17)(q23.1q24.3)/46,XX (Huang et al. 1999). The identified small duplication included the *SOX9* gene locus, and it was suggested that an extra dose of the *SOX9* gene is sufficient for testis development in the absence of the *SRY* gene, causing female-to-male sex reversal.

A hereditary sex reversal syndrome (78,XX, *SRY* negative) has been reported quite frequently in dogs (Switonski et al. 2004a). Until now this syndrome has been diagnosed by the cytogenetic or molecular approach in 13 breeds, including the cases reported in this study. Neither aneuploidies nor structural chromosome abnormalities have been found in such animals.

At least 18 genes involved in mammalian sex determination are known and 10 of these are involved in the testis-determining pathway (Cotinot et al. 2002). Five of them (*WT1*, *DMRT1*, *GATA4*, *FOG2*, and *LHX9*) have already been excluded as a cause of dog intersexuality (XX, *SRY* negative) (Kothapalli et al. 2004; Pujar et al. 2005). The authors analyzed the segregation of microsatellite markers within the referenced American cocker spaniel dog family with 10 XX (*SRY* negative) sex reversal offspring. The marker was localized within or near the candidate gene.

Our study shows that coding and 5′ flanking sequences of the *SOX9* gene are not altered in sex reversal dogs. However, a detailed molecular analysis of this gene revealed minor differences in exon 2. In our study, also in the contig sequence (NW139883), there are four sites with different nucleotide sequences when compared with the complementary DNA (cDNA) sequence of the *SOX9* gene (AY237827). It

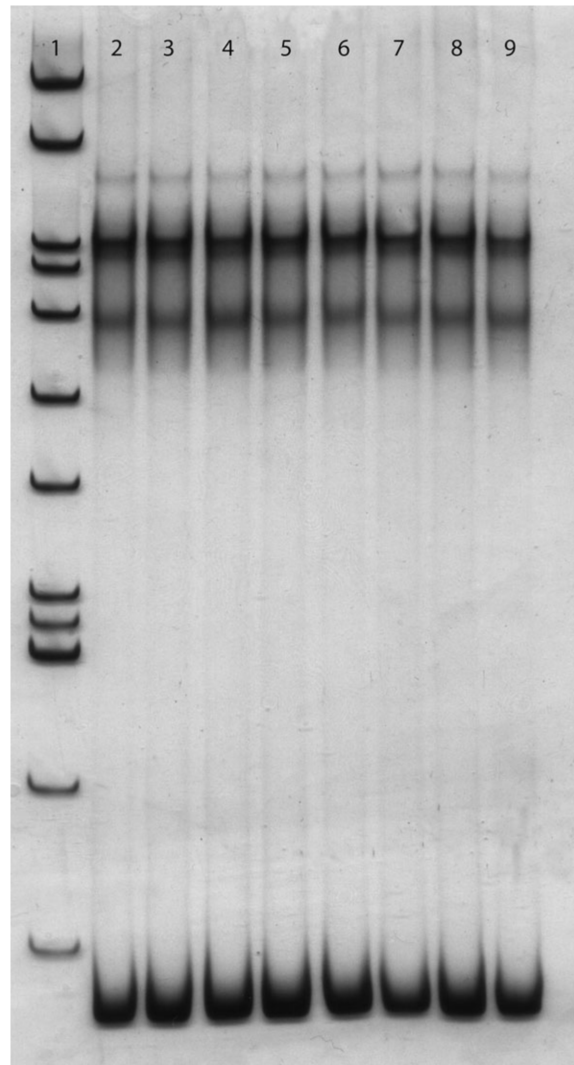

Figure 3. SSCP pattern of the E1 fragment: line 1, a length marker Gene Ruler 100 bp DNA Ladder Plus (Fermentas); lines 2–4, analyzed cases; lines 5–7, reference males; lines 8–9, reference females.

may be explained by an error in the cDNA sequence or interbreed differences. The contig sequence was derived from the boxer breed; our data concern American Staffordshire terrier and miniature pinscher breeds, while the cDNA sequence was obtained for the beagle breed. Moreover, we show that short tandem repeats are also present within the *SOX9* gene, and it might be useful in the segregation studies mentioned above.

The intersexes described in this report were subjected to cytogenetic and molecular analyses because of an enlargement of the clitoris. All of them had a female karyotype (XX) and were *SRY* negative. In only one case was histology performed that revealed the presence of inactive testes and a hypoplastic uterus. These characteristics are in agreement with previous reports demonstrating that approximately 50% of such dogs have testes and the other 50% have ovotestes,

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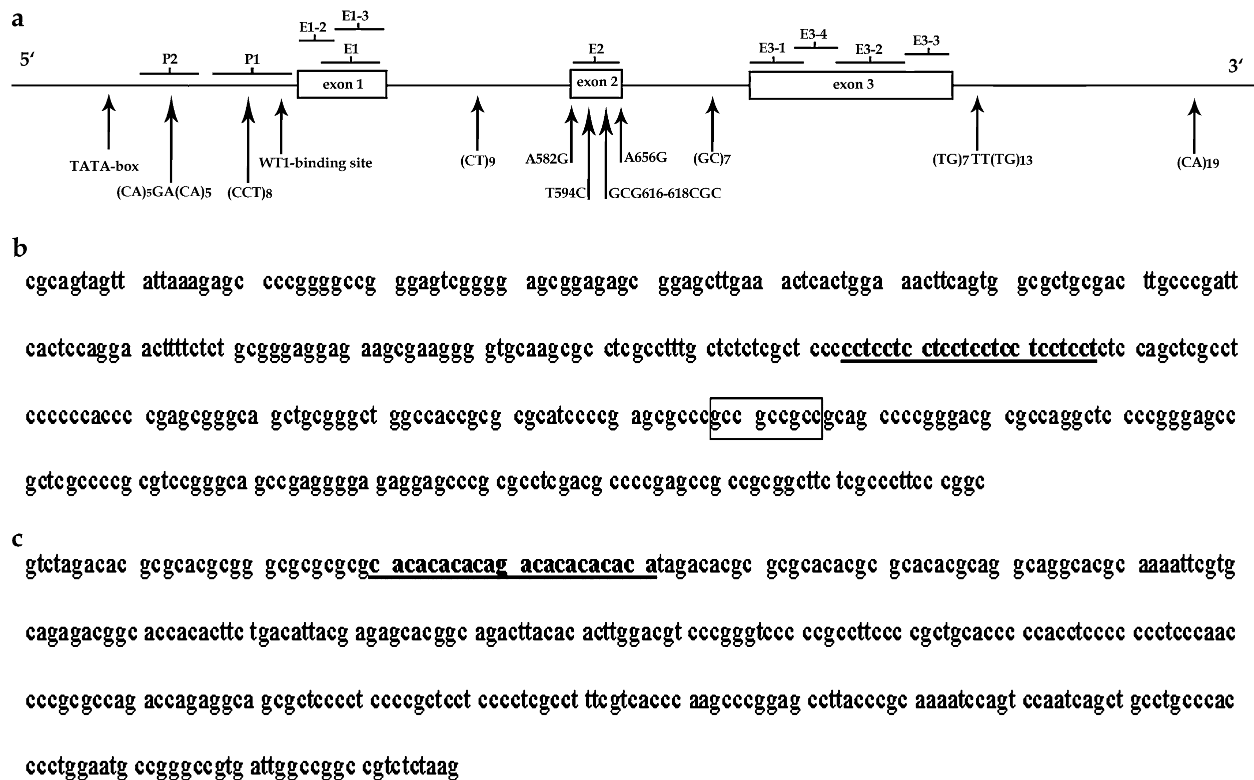

Figure 4. Molecular studies of the *SOX9* gene. **(a)** A scheme of the *SOX9* gene with indicated PCR-amplified and SSCP-analyzed fragments, repetitive sequences, TATA box, and WT1 binding site. **(b)** The sequence of P-1 fragment; underlined bold, the repetitive sequence $(CCT)_8$ (position −245 to −225 from transcription site). **(c)** The sequence of P-2 fragment; underlined bold, the repetitive sequence $(CA)_5GA(CA)_5$ (position −758, −737 from transcription site); in a frame, the WT1 binding site.

while all of them have an enlarged clitoris, usually with a bone (Meyers-Wallen et al. 1999). Taking into consideration the obtained results, two cases (cases 2 and 3) can be classified tentatively as sex reversed XX dogs, since a complete clinical examination could not be performed.

We conclude that sex reversal dogs (78,XX, *SRY* negative) have a normal coding and 5′ flanking sequence of the *SOX9* gene. To exclude a mutation of this gene as a cause of this abnormality, further studies of the 5′ flanking region or segregation analysis of short tandem repeats in a reference family should be undertaken.

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