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Nucleotide sequence and embryonic expression of quail and duck *Sox9* genes

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Abstract

Sox9 is a member of the *Sry*-type HMG-box (Sox) gene family. It encodes a transcription factor and is thought to be important for sexual differentiation in chicken. In the present study we have isolated *Sox9* cDNAs from quail and duck, and examined the expression patterns of the corresponding genes in early embryonic gonads by whole-mount in situ hybridization. We developed a polymerase chain reaction-based protocol to identify the sex of quail and duck embryos before its morphological manifestation. *Sox9* expression was first detected on days 5 and 7 in the gonads of male quail and duck embryos, respectively, and was not apparent in female gonads at these stages. These expression patterns are similar to that of chicken *Sox9*. Our results thus suggest that the expression of quail and duck *Sox9* is associated with testis differentiation.

Keywords: *Sox9*; Quail; Duck; Sexing; Sex determination

1. Introduction

In mammals, the heterogametic pairing of sex chromosomes (XY) results in male development, whereas males are homogametic (ZZ) and females are heterogametic (ZW) in birds. It remains unclear whether avian sex is determined by Z chromosome gene dosage, by a master female-determining gene (or genes) on the W chromosome, or by a combination of both processes (Clinton, 1998). However W chromosome dose not seem to have a decisive role, since administration of an aromatase inhibitor to genetically female embryos before sex-determining period caused about half of treated chickens develop testes (Elbrecht and Smith, 1992). It seems likely that the in vivo exposure of estrogen at an early stage of embryonic development plays a crucial role in differentiation of an ovary in chicken. In contrast, the importance of estrogens for gonadal sex differentiation in birds is not seen in mammals. Although the systems for sex determination and differentiation differ between mammals and birds, several genes that are associated with sex determination or differentiation in mammals are expressed in similar patterns in chicken and mouse gonads, suggestive of some degree of similarity between the molecular mechanisms of sexual differentiation in these two species.

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One such gene is *Sry-type high mobility group-box containing gene 9* (*Sox9*). *Sox9* and the related gene *Sry*, which is located on the Y chromosome, are sex determination genes in mice (Koopman et al., 1991; Vidal et al., 2001). However, *Sox9* is not thought to contribute to sex determination in chicken, given that it is expressed predominantly in developing testis only after establishment of the sexually dimorphic expression pattern of *anti-Müllerian hormone* (*Amh*), a gene associated with sexual differentiation (Oréal et al., 1998; Smith et al., 1999; Takada et al., 2005). It is instead likely that *Sox9* plays a role in sexual differentiation in chicken.

Elucidation of the molecular mechanisms of sex determination and differentiation in birds will require the identification of additional genes that are essential for these processes as well as comparative analyses of gene expression patterns and mechanisms of action between birds and other vertebrates. We have now characterized the expression patterns of *Sox9* in quail and duck embryos during the early stages of gonadal differentiation before the appearance of morphological sex differences. To distinguish the sexes at these early stages, we devised an easy and accurate sexing method based on the polymerase chain reaction (PCR).

2. Methods

2.1. Animals

Fertilized Japanese quail (*Coturnix coturnix japonica*) and domestic duck (*Anas platyrhynchos*) eggs were obtained from a local supplier (Saitama Experimental Animal Supply, Saitama, Japan) and were maintained at 18 °C until transfer to an incubator at 37.8 °C. Staging of quail embryos was confirmed at dissection according to Zacchei (1961). Staging of duck embryos was compared at dissection with chicken stages (Hamburger and Hamilton, 1951). The entire urogenital ridge of each embryo was explanted for whole-mount in situ hybridization.

2.2. PCR-based sexing

A hind limb was removed from an embryo to isolate genomic DNA for PCR-based sexing as described (Clinton et al., 2001), with minor modifications. In brief, tissue was soaked in 100 μl of digestion buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1% SDS, and proteinase K (10 μg/ml)] and incubated at 50 °C for 1 h. After phenol–chloroform extraction, 80 μl sample was diluted to 400 μl with water, and 1 μl of the diluted material was subjected to PCR. The PCR protocol comprised denaturation for 4.5 min at 95 °C followed by 40 cycles of incubation at 95 °C for 30 s and 56 °C for 30 s. The reaction was performed in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphates, 0.13 μM 18S primers (Clinton et al., 2001), 0.4 μM *Wpkci* primers, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The primers qWpkciF (5′-TTGGGCATTTGAAGATT GTC-3′) and qWpkciR (5′-GTCTGAAGGGTCTGA GGGT-3′) were used for sexing of quail embryos, whereas dWpkciF (5′-CTTCTTGGGCGTTTCGTG-3′) and dWpkciR (5′-G TCTGAAGGGCCCGAGGGT-3′) were used for sexing of duck embryos. PCR products together with molecular size standards (50-bp DNA ladder; Invitrogen, Carlsbad, CA) were fractionated by electrophoresis on a 4% agarose gel.

2.3. Cloning and sequencing of quail and duck Sox9 cDNAs

Partial genomic fragments of quail and duck *Sox9* were amplified by PCR in a final volume of 25 μl containing 1× NH_4 buffer (Bioline, London, UK), 0.2 mM deoxynucleoside triphosphates, 0.4 μM primers, and 0.5 U of Biotaq DNA polymerase (Bioline). The PCR protocol comprised denaturation at 95 °C for 4.5 min followed by 40 cycles of incubation at 95 °C for 30 s and 62 °C for 30 s. The primers used were qdSox9F (5′-ATGAATCTCCTAGACCCCTT C-3′) and qdSox9R (5′-GGSACCAGSGTCCAGTCGTA-3′). The PCR products were ligated into the pT7-Adv vector (Clontech, Palo Alto, CA) and sequenced by Operon Biotechnologies (Tokyo, Japan).

Quail and duck *Sox9* cDNAs were generated by 5′ and 3′ RACE. Total RNA was purified from male quail (day 7) and duck (day 8) embryonic gonads with the use of an RNeasy Mini kit (Qiagen, Valencia, CA) and was converted to double-stranded cDNA with the use of a SMART PCR cDNA Synthesis kit (Clontech). RACE was performed in a solution containing 1× NH_4 buffer (Bioline), 0.2 mM deoxynucleoside triphosphates, 0.4 μM primers, 1.8 M betaine, and 0.5 U of Biotaq DNA polymerase. The PCR protocol comprised denaturation at 95 °C for 4.5 min followed by 40 cycles of incubation at 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 3 min. Betaine was included to facilitate the amplification of GC-rich target fragments (Henke et al., 1997). Primers used for the quail and duck 3′ RACE were 5′ PCR Primer IIA (Clontech) and uniSox9F1 (5′-CA GCCCCACCATGTCGGATGACTCCGC-3′), those for the quail 5′ RACE were 5′ PCR Primer IIA and uniSox9R1 (5′-TCCTTCTTCAGGTCCGGGTCGCC-3′), and those for the duck 5′ RACE were 5′ PCR Primer IIA and dSox9R6 (5′-TTGGCTCACCGCCTCTCGGATG-3′). RACE products were ligated into the pGEM-T easy vector (Promega, Madison, WI) for nucleotide sequencing. At least three independent clones were sequenced for each RACE reaction.

The complete coding sequences of quail and duck *Sox9* cDNAs were amplified by RT-PCR as for RACE with the exception that PCR was performed for 4.5 min at 95 °C followed by 40 cycles of incubation at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, and that the primers used for *qSox9* cDNA were qSox95′UTRF (5′-CTGGAGGCTCCATC TCTCCCTG-3′) and qSox93′UTRR (5′-TTTATTT GTCTT CACGTGGCT-3′) and those for *dSox9* cDNA were dSox95′UTRF (5′-CCCCCTCCGCCACTTTCTCG-3′) and