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Evidence for activation of *Amh* gene expression by steroidogenic factor 1

Shuji Takada [a,*], Tomoaki Wada [a], Ruri Kaneda [a,b], Young Lim Choi [a], Yoshihiro Yamashita [a], Hiroyuki Mano [a,b]

[a] *Division of Functional Genomics, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan*
[b] *CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan*

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Abstract

The *anti-Müllerian hormone* gene (*Amh*) is responsible for regression in males of the Müllerian ducts. The molecular mechanism of regulation of chicken *Amh* expression is poorly understood. To investigate the regulation of chicken *Amh* expression, we have cloned *Amh* cDNAs from quail and duck as well as the promoter regions of the gene from chicken, quail, and duck. The expression patterns of *Amh* during embryonic development in these three species were found to be similar, suggesting that the regulatory mechanisms of *Amh* expression are conserved. The sequence of the proximal promoter of *Amh* contains a putative binding site for steroidogenic factor 1 (SF1), the protein product of which can up-regulate *Amh* in mammals. We showed here that SF1 is able to activate the chicken *Amh* promoter and binds to its putative SF1 binding site. These results suggest that SF1 plays a role in regulation of *Amh* expression in avian species.

Keywords: *Anti-Müllerian hormone*; Steroidogenic factor 1; Quail; Duck; Chicken; Sex determination; Gonadogenesis

1. Introduction

In birds, the heterogametic pairing of sex chromosomes (ZW) results in female development, whereas males are the heterogametic sex (XY) in mammals. It remains unclear whether avian sex is determined by a master female-determining gene (or genes) on the W chromosome, by Z chromosome gene dosage, or by a combination of both mechanisms (Clinton, 1998). Although the systems for sex determination and differentiation differ between mammals and birds, several genes that are associated with sex differentiation in mammals are expressed in similar patterns in mouse and chicken gonads during development, suggesting that the molecular mechanisms of sexual differentiation are similar to some extent in the two species.

One such gene is that for *anti-Müllerian hormone* (*Amh*), also known as *Müllerian inhibiting substance*, which is expressed from early stages of sexual differentiation, predominantly in pre-Sertoli cells of male embryonic gonads, in mice and chickens (Münsterberg and Lovell-Badge, 1991; Oréal et al., 1998). The product of this gene, AMH, is a member of the transforming growth factor-ß (TGF-ß) superfamily of secreted signaling molecules and induces regression of the Müllerian ducts, the anlagen of the female reproductive tract including the uterus, oviducts, upper vagina, and fallopian tubes (Josso et al., 2001). However, there is a difference between mouse and chicken in the expression patterns of *Amh* that are evident before the appearance of structural differences between the sexes. At this stage, *Amh* is expressed at similar levels in male and female gonads of chicken (Oréal et al., 1998) but is not expressed in mice (Münsterberg and Lovell-Badge, 1991).

The sex and temporal specificities of its expression during embryonic development indicate that *Amh* is a highly regulated gene. The molecular mechanisms of *Amh* expression have been extensively analyzed in mammals, but they are not well characterized in chicken. Analysis of the mammalian *Amh* promoter has revealed that the transcription factors SOX9 and steroidogenic factor 1 (SF1, also known as AD4BP) play central roles in male-specific up-regulation of mouse *Amh*. Targeted mutagenesis of the SOX binding site at nucleotide position −142 in the proximal promoter of mouse *Amh* prevented the initiation of gene transcription (Arango et al., 1999), indicating that SOX9 is essential for the induction of *Amh* expression. In vitro transfection experiments with

* Corresponding author. Tel.: +81 285 58 7449; fax: +81 285 44 7322.
E-mail address: stakada@jichi.ac.jp (S. Takada).

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a minimal *Amh* promoter (180 bp) revealed that mutation of the SF1 binding site at position −90 resulted in a marked decrease in transcriptional activity (Shen et al., 1994). Introduction of the same mutation into transgenic mice led to only a slight decrease in *Amh* expression (Arango et al., 1999). This discrepancy may be explained by the existence of another functional SF1 binding site at position −218 (Watanabe et al., 2000).

Characterization of the chicken *Amh* promoter (Oréal et al., 1998) revealed little sequence similarity to that of mouse *Amh*, although two putative SOX binding sites and a putative SF1 binding site were detected. Whether these predicted binding sites are functional has remained unknown, but analysis of the expression of chicken *Sox9* and *Sf1* suggests that the products of these genes are not essential for male-specific up-regulation of chicken *Amh*. *Sox9* is expressed predominantly in developing male gonads during sexual differentiation in the chicken; however, in contrast to the mouse, up-regulation of *Amh* precedes that of *Sox9* (Oréal et al., 2002; Smith et al., 1999a; Takada et al., 2005). Chicken *Sf1* is expressed at similar levels in male and female gonads before sexual differentiation, as is chicken *Amh*; however, after the onset of sexual differentiation, *Sf1* is expressed at a higher level in female gonads than in male gonads, suggesting that SF1 is not responsible for the male-specific up-regulation of *Amh*.

Interaction of the *Amh* promoter with its regulatory factors has not been demonstrated in the chicken. To investigate the molecular mechanism of regulation of *Amh* expression in chicken, we have compared the promoter sequences of three avian species: chicken, quail, and duck.

2. Results

2.1. Cloning and expression patterns of quail and duck Amh

To clone the promoter regions of quail and duck *Amh*, we first attempted to clone quail and duck *Amh* cDNAs by 5′ and 3′ RACE. Primers for RACE were designed on the basis of the sequences of partial genomic fragments of quail and duck *Amh* amplified by PCR with the primers cAmh-4 and TAMHF3, which, in turn, had previously been designed on the basis of the chicken *Amh* cDNA sequence (Western et al., 1999) and used to clone *Amh* cDNA from the red-eared slider turtle, *Trachemys scripta* (Takada et al., 2004). These primers, which are located in the 3′ terminal region of the open reading frame of chicken *Amh*, yielded 391-bp products from the quail and duck genomes (data not shown). Comparison of the DNA sequences of these products with that of chicken *Amh* cDNA (GenBank accession no. X89248) revealed a 27-bp conserved sequence with no mismatches. Sense and antisense oligonucleotides corresponding to this 27-bp sequence were synthesized and used for 3′ and 5′ RACE, respectively. Given that sex differentiation occurs before day 5 in quail embryos and day 7 in duck embryos (Takada et al., 2006), we used RNA purified from the gonads of male quail and duck embryos on days 7 and 8, respectively, as a template for RACE. The nucleotide sequences of 5′ and 3′ RACE products were determined and assembled to yield the corresponding cDNA sequences.

To verify the authenticity of the sequences determined by 5′ and 3′ RACE for each species, we attempted to amplify cDNAs containing the entire coding regions of quail and duck *Amh* by RT-PCR with primers that map to the corresponding 5′ and 3′ untranslated regions. A 2.0-kb cDNA was amplified from RNA prepared from quail or duck. Nucleotide sequencing of each of the amplified fragments confirmed that the 5′ and 3′ RACE sequences were linked in tandem for both quail and duck, demonstrating that the assembled sequences correspond to single transcripts.

The putative proteins encoded by the quail and duck cDNAs comprise 644 and 670 amino acids, respectively. A search for protein motifs with CD-Search (Marchler-Bauer and Bryant, 2004) revealed that both deduced amino acid sequences contain the 99-residue TGF-ß motif at their COOH-termini, with 97, 94, and 94 residues of this motif being identical in the quail protein and chicken AMH, in the duck protein and chicken AMH, and in the quail and duck proteins, respectively, (Fig. 1). The overall sequence identities of the three avian proteins are 94.0, 76.5, and 75.3% for quail and chicken, duck and chicken, and quail and duck, respectively, despite the previous finding that the amino acid sequence of AMH is poorly conserved among vertebrates (Carré-Eusèbe et al., 1996; Neeper et al., 1996; Western et al., 1999). Given that a BLASTP search of the non-redundant GenBank database with the deduced amino acid sequences of the quail and duck proteins as queries yielded *Gallus gallus* (chicken) AMH followed by *Macropus eugenii* (wallaby) AMH and *Alligator mississippiensis* (American alligator) AMH as the most similar sequences, we conclude that the isolated quail and duck cDNAs are derived from the corresponding *Amh* genes. The nucleotide sequences of these quail and duck cDNAs have been deposited in GenBank under the accession numbers AY904049 and AY904047, respectively.

We next examined the spatiotemporal expression patterns of quail and duck *Amh* during the early stages of gonadal differentiation with the use of whole-mount in situ hybridization. Gonad–mesonephros complexes were isolated from quail embryos on days 4, 5, 6, and 7 (Zacchei stages 17 to 18, 20 to 21, 22, and 24, respectively), (Zacchei, 1961) and from duck embryos on days 6, 7, 8, and 9. Duck embryos were staged by comparison with chicken (Hamburger and Hamilton, 1951). The morphological stages of duck are essentially the same as those of chicken, although development is slightly delayed in duck embryos (days 6, 7, 8, and 9 for duck embryos correspond to Hamburger and Hamilton stages 25 to 26, 28, 29 to 30, and 31 to 32, respectively). *Amh* mRNA was not detected in quail gonads on day 4 (Fig. 2A,F) or in duck gonads on day 6 (Fig. 2J,O). *Amh* expression was observed in male and female gonads both of quail on days 5, 6, and 7 (Fig. 2B–D,G–I) and of duck on days 7, 8, and 9 (Fig. 2K–M,P–R), with expression levels being higher in male than in female. Sense control probes yielded no specific labeling (Fig. 2E,N). The earliest

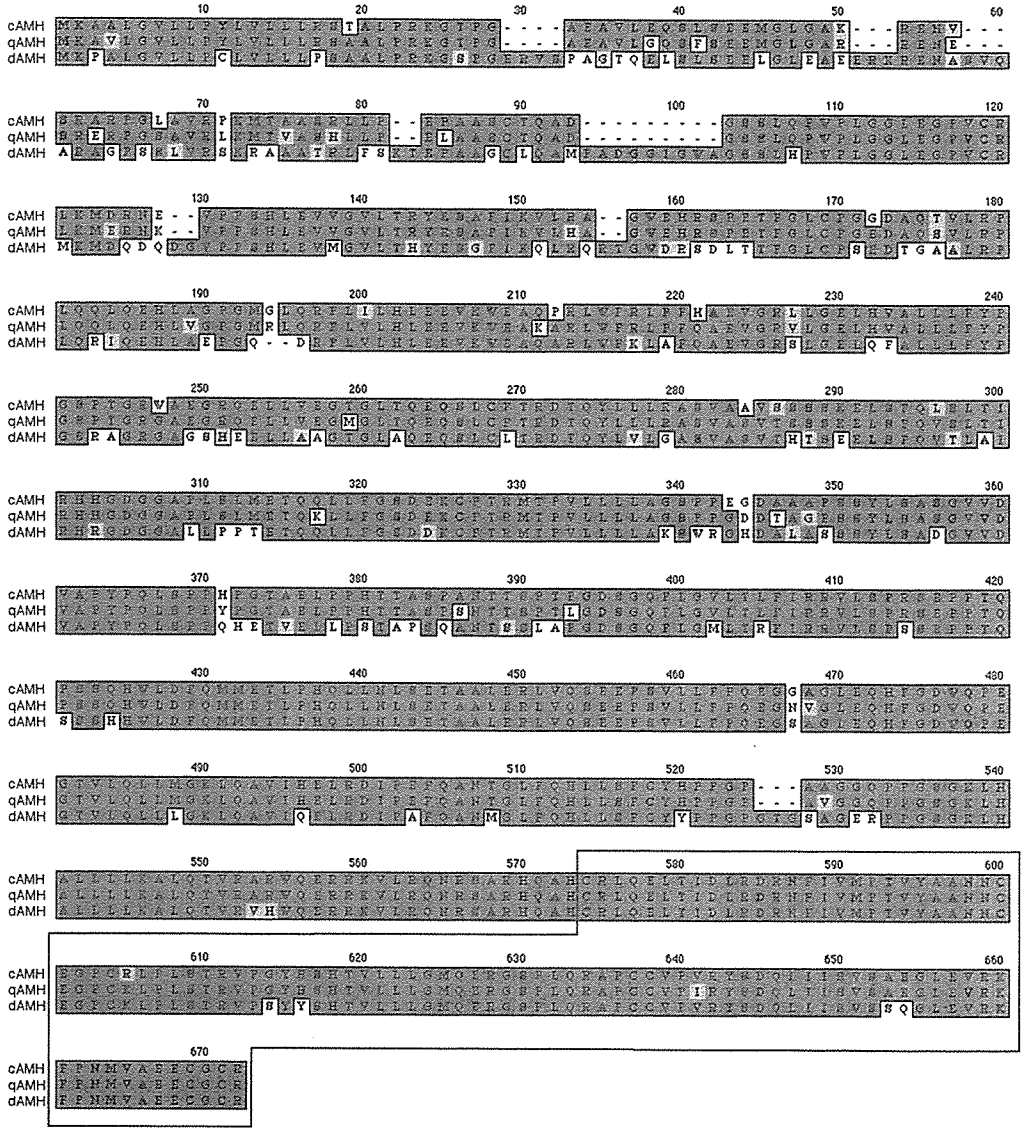

Fig. 1. Alignment of the deduced amino acid sequences of chicken (c), quail (q), and duck (d) AMH. Dark and light gray shading indicate identical and similar amino acids, respectively. The boxed region corresponds to the TGF- ß motif.

detectable stages for male-specific up-regulation of *Amh* were thus similar for chicken (stages 28–30) (Loffler et al., 2003; Morais da Silva et al., 1996; Oréal et al., 1998; Smith et al., 1999a), quail (day 5, corresponding to Zacchei stages 20 to 21 and Hamburger and Hamilton stages 27–29) (Zacchei, 1961), and duck (day 7, corresponding to Hamburger and Hamilton stage 28). These similar expression patterns suggest that regulation of *Amh* expression is conserved among these three avian species. The expression patterns of quail and duck *Amh* are also similar to those of quail and duck *Sox9* (Takada et al., 2006). The earliest detectable stages examined so far for male-specific up-regulation of *Amh* and *Sox9* is same in quail and duck (quail at day 5and duck at day 7), however, there was a difference in expression patterns between *Amh* and *Sox9* in female gonads of quail and duck; *Amh* is expressed at low levels but *Sox9* is not.

2.2. Cloning and nucleotide sequence analysis of chicken, quail, and duck Amh promoters

To isolate the promoters of chicken, quail, and duck *Amh*, we used PCR with primers based on the sequences of *Amh* and of a gene located upstream of *Amh* in the avian genome. BLAT analysis (February 2004 assembly, http://genome.ucsc.edu/cgi-bin/hgBlat, International Chicken Genome Sequencing Consortium, 2004) with the chicken *Amh* sequence as a query identified *Sap62* (also known as *Sf3a2*) as being located upstream of and adjacent to *Amh*. Primers were thus designed on the basis of open reading frame sequences conserved between chicken (NM_001004397) and mouse (NM_013651) *Sap62* and among chicken (X89248), quail, and duck *Amh*. These primers yielded 5328- and 4651-bp products from the chicken and quail genomes, respectively,

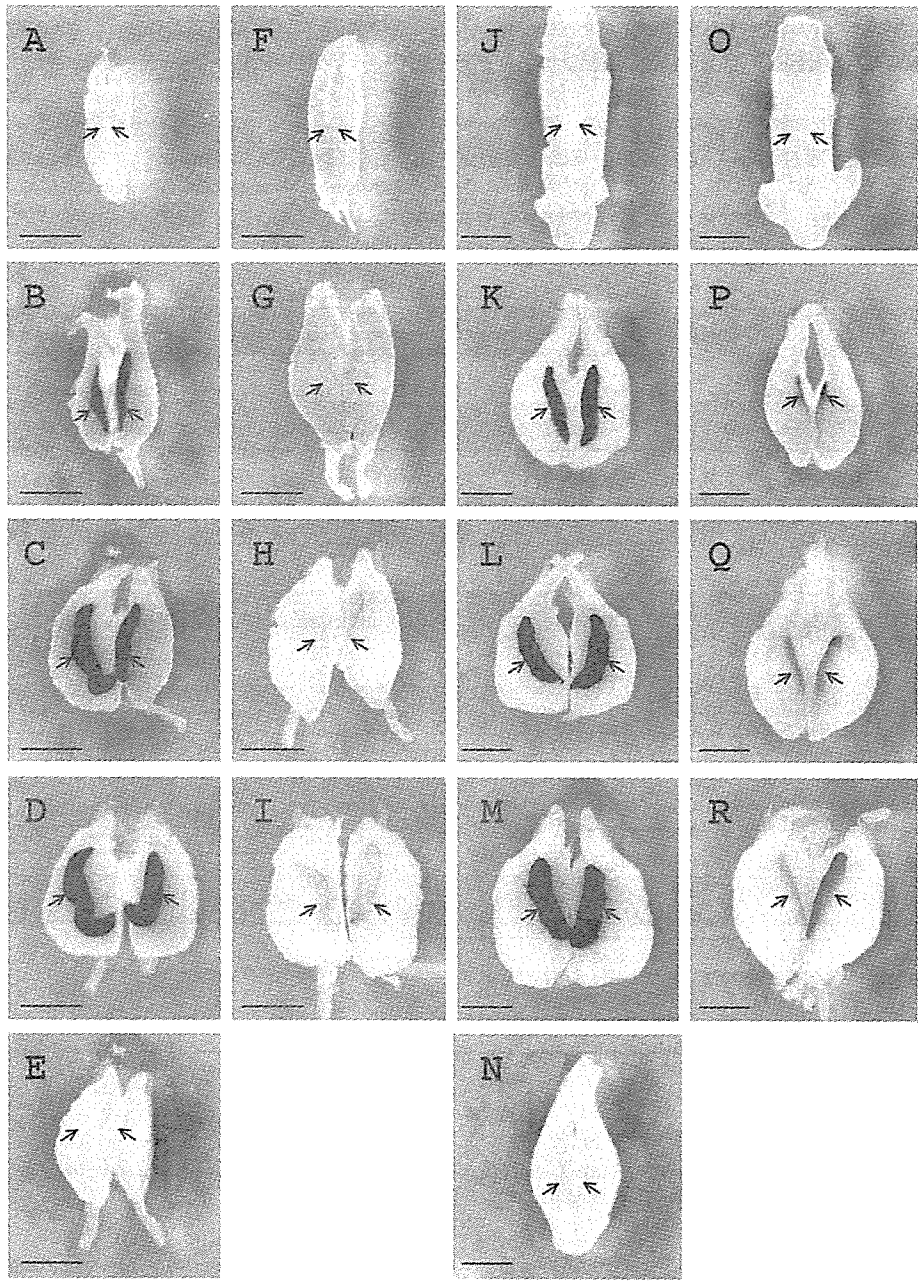

Fig. 2. Whole-mount in situ hybridization analysis of *Amh* expression in the embryonic gonad–mesonephros of quail and duck. Male (A–E) and female (F–I) quail embryos were analyzed on day 4 (A, F) day 5 (B, G), day 6 (C, E, H), and day 7 (D, I). Male (J–N) and female (O–R) duck embryos were analyzed on day 6 (J, O) day 7 (K, P, N), day 8 (L, Q), and day 9 (M, R). All embryos were subjected to hybridization with an antisense probe, with the exception of those in (E) and (N), for which a sense probe was used as a control. Arrows indicate the positions of the gonads. Scale bars, 1 mm.

but no product was obtained from the duck genome. The chicken and quail products were sequenced and the resulting sequences were used to design a primer for amplification of the duck *Amh* promoter. A 5793-bp PCR product was thus obtained from the duck genome. Sequence analysis revealed that the orientations of *Sap62* and *Amh* are the same in all three avian species. The nucleotide sequences of these genomic fragments (excluding the primer sequences) have been deposited in GenBank under the accession numbers DQ269189 for chicken, DQ269190 for quail, and DQ269191 for duck.

The similarity in the expression patterns of *Amh* among chicken, quail, and duck embryos suggested that the regulatory sequences responsible for the control of *Amh* expression are also conserved among these species. To identify regulatory elements that might mediate up- or down-regulation of *Amh* expression, we first compared the promoter sequences among the three species. The sequences obtained by PCR from chicken and quail spanned from exon 2 of *Sap62* to exon 1 of *Amh*, whereas that obtained from duck spanned from exon 3 of *Sap62* to exon 1 of *Amh*. To compare corresponding

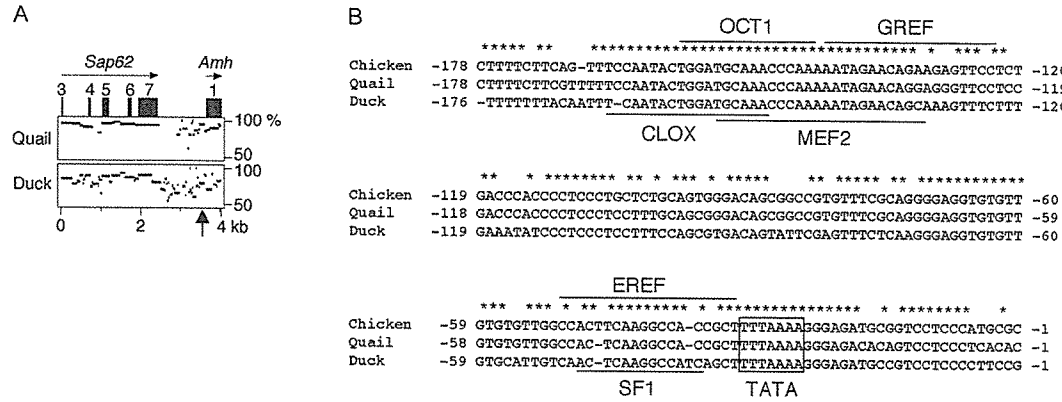

Fig. 3. Comparison of the nucleotide sequences of the 5′ flanking regions of chicken, quail, and duck *Amh*. (A) MultiPipMaker analysis of the 4016-, 3387-, and 5745-nucleotide sequences of the chicken, quail, and duck *Amh* promoter regions. Regions of significant similarity are indicated by horizontal lines. A region of marked similarity within the proximal promoter of *Amh* is indicated by the vertical arrow. The exons of chicken *Sap62* and *Amh* are indicated by black boxes with exon numbers. Horizontal arrows show the transcriptional orientation of *Sap62* and *Amh*. The *y*-axis represents percentage nucleotide identity; the *x*-axis denotes distance in kilobases. (B) Nucleotide sequence alignment of the conserved region within the *Amh* proximal promoter. Asterisks indicate identical nucleotides. Potential transcription factor binding sites are indicated by horizontal lines. The boxed region is the TATA box.

genomic regions of the three species, we extracted nucleotide sequences corresponding to the duck fragment (entire sequence of DQ269191) from the chicken (nucleotides 1083–5098 of DQ269189) and quail (nucleotides 1039–4420 of DQ269190) fragments. Comparison of these nucleotide sequences with MultiPipMaker revealed the existence of a highly conserved region corresponding to nucleotides −178 to −1 of the chicken sequence (the major transcription start site of chicken *Amh* was designated as position +1) (Fig. 3A). We next searched for binding elements within this conserved region with the use of FrameWorker software. This analysis identified several candidate binding factors: octamer-binding protein 1 (OCT1), CLOX and CLOX homology factors (CLOX), glucocorticoid responsive and related element (GREF), myocyte-specific enhancer-binding factor 2 (MEF2), EGR/nerve growth factor-induced protein C and related factors (EREF), and SF1 (Fig. 3B). Among these factors, only the gene for SF1 has been shown to be coexpressed with *Amh* in the developing chicken gonad (Oréal et al., 2002), further implicating SF1 as a regulatory factor for control of chicken *Amh* expression.

2.3. Regulation of chicken Amh expression by SF1

To localize regulatory sequences within the 5′ flanking region of chicken *Amh* experimentally, we tried to perform transient transfection assays with nested deletion constructs of the *Amh* promoter (nucleotides −2217 to +45, corresponding to the genomic region spanning intron 5 of *Sap62* and the entire 5′ untranslated region of *Amh*) fused to a luciferase reporter gene and primary culture cell prepared from chicken embryonic gonads. (data not shown). However, this experiment could not work well because real-time RT-PCR analysis of *Amh* expression showed that sexual dimorphism of *Amh* expression was lost during 2 days of cell culture. Given that neither a chicken Sertoli cell line nor culture conditions that maintain *Amh* expression in primary chicken Sertoli cells that maintains *Amh* expression have been established at this time, it will be difficult to identify regulatory elements essential for sexual dimorphism in *Amh* expression by transient transfection assays with nested deletion constructs of the *Amh* promoter region. We therefore focused our analysis on the factors implicated in such regulation by sequence analysis.

Among the factors identified by comparison of the chicken, quail, and duck *Amh* promoter sequences (Fig. 3B), only the gene for SF1 is known to be coexpressed with chicken *Amh*. We therefore, examined the effect of SF1 on the expression of chicken *Amh* by co-transfection of BOSC23 cells with an expression vector for SF1 (pcDSf1B2) and the *Amh* promoter

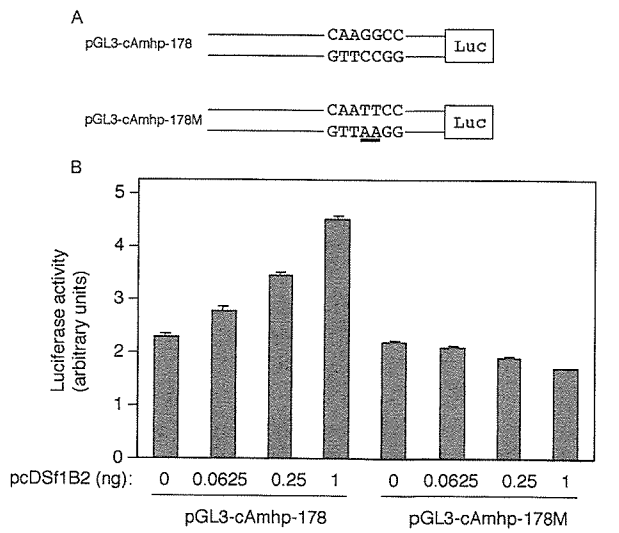

Fig. 4. Trans-activation of the chicken *Amh* promoter by SF1. (A) Schematic representation of the *Amh* promoter constructs used for transfection. The sequences of the putative SF1 binding site and of a mutated version are shown. Mutated bases are underlined. (B) Co-transfection of BOSC23 cells with the *Amh* promoter-reporter constructs and various amounts of an SF1 expression vector. Luciferase activity of the transfected cells was determined as described in (C).

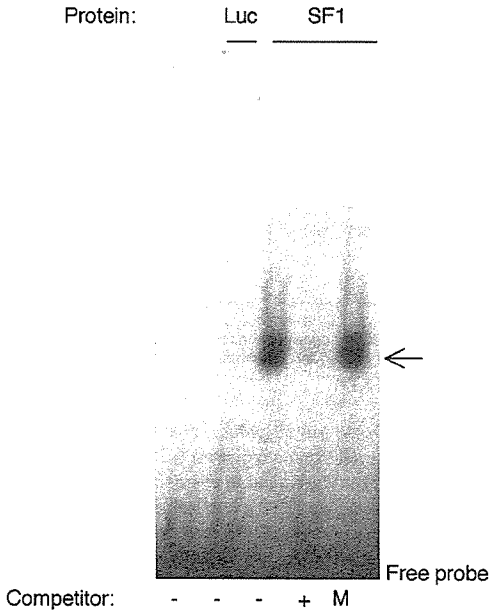

Fig. 5. Binding of SF1 to the chicken *Amh* promoter. EMSA analysis was performed with a ^{32}P-labeled fragment of the *Amh* promoter containing the putative SF1 binding site and with in vitro-translated SF1 or luciferase (control). Competitors included the unlabeled probe (+) and a corresponding oligonucleotide containing a mutated SF1 binding site (M). The lanes without competitors are indicated with '−'. The arrow indicates the SF1–probe complex.

constructs pGL3-cAmhp-178 and pGL3-cAmhp-178M, the latter of which contains a substitution of TT for GG in the putative SF1 binding site (Fig. 4A). The luciferase reporter activity derived from pGL3-cAmhp-178 was increased twofold by transfection with pcDSf1B2 in a manner dependent on the amount of the latter vector, whereas no such effect was observed in cells transfected with pGL3-cAmhp-178M (Fig. 4B). These results thus suggested that SF1 is able to activate expression of chicken *Amh*.

2.4. Binding of SF1 to the chicken Amh promoter

To examine directly whether SF1 binds to its putative binding site in the chicken *Amh* promoter, we performed EMSA analysis with a 96-bp fragment of the promoter as a probe and with in vitro-translated SF1 and luciferase (negative control). SF1 bound to the ^{32}P-labeled probe whereas luciferase did not (Fig. 5). The binding of SF1 to the labeled probe was not detected in the presence of an excess amount of unlabeled probe but was unaffected by the presence of an excess of a corresponding oligonucleotide containing a GG to TT substitution in the putative SF1 binding site. These data thus indicated that SF1 binds directly and specifically to the putative SF1 binding site in the chicken *Amh* promoter.

3. Discussion

We have cloned quail and duck *Amh* cDNAs and examined the expression patterns of the corresponding genes during embryonic development. The expression patterns of the quail and duck genes were similar to that of chicken *Amh*, suggesting that the molecular mechanisms of *Amh* gene regulation are conserved among avian species. We compared the *Amh* promoter sequences of the three species and identified putative binding sites for several transcription factors. Further examination revealed that one of these sites, that for SF1, mediated activation of *Amh* expression in transfected cells and that SF1 binds directly to this site.

Although *Sf1* and *Amh* are expressed in the same cells during early embryonic development in the chicken (Oréal et al., 2002), the expression patterns of these genes are not identical after the onset of sexual differentiation. *Amh* is expressed at similar levels in male and female gonads at stage 25; however, after stage 28, its expression level in male gonads is increased whereas that in female gonads remains similar to that apparent at stage 25 (Oréal et al., 1998; Smith et al., 1999a). *Sf1* is also expressed at similar levels in male and female gonads at stage 25, but these expression patterns persist until stage 30 and *Sf1* expression is then up-regulated only in the female, with expression in the male remaining at a level similar to that apparent at stage 30 (Oréal et al., 2002; Smith et al., 1999a,b). It is thus possible that SF1 regulates *Amh* expression only before the onset of sexual differentiation, when *Amh* and *Sf1* are each expressed at similar levels in male and female gonads.

It is also possible that the relatively low level of *Sf1* expression in male gonads is sufficient to activate chicken *Amh* in cooperation with an unidentified factor that is expressed at a higher level in male gonads than in female gonads. A similar model has been proposed for the male-specific up-regulation of *Amh* expression by SF1 and SOX9 in mammals (Arango et al., 1999; Shen et al., 1994). In mouse and human, whereas SF1 can up-regulates the *Amh* gene expression only twofold, SF1 together with SOX9 can up-regulates the *Amh* gene expression fivefold (De Santa Barbara et al., 1998; Schepers et al., 2003). However, SOX9 is not a candidate for such a factor in chicken because up-regulation of *Amh* precedes that of *Sox9* in this species (Oréal et al., 2002; Smith et al., 1999a; Takada et al., 2005). It would be rather possible that SOX9 plays a role in maintenance and/or amplification of *Amh* expression level directly or indirectly in male gonads once transcription is initiated. If SOX9 has such a function, it would be reasonable to postulate that male-specific up-regulation of *Amh* precedes that of *Sox9* in quail and duck gonads as well. This issue needs to be clarified in the future.

Another member of the SOX protein family may thus substitute for SOX9 in this role in chicken, given that SOX proteins are functionally redundant (Chaboissier et al., 2004; Collignon et al., 1996; Downes and Koopman, 2001; Kanai-Azuma et al., 2002; Pennisi et al., 2000; Schepers et al., 2003; Smits et al., 2001; Stolt et al., 2003). However, with the exception of *Sox9*, the chicken *Sox* genes known to be expressed in the gonads to date are not expressed in a male-specific manner (Oréal et al., 1998; Smith et al., 1999a; Takada et al., 2005).

Oréal et al. (1998) detected two putative SOX binding sites in the chicken *Amh* promoter by sequence analysis. If these

putative SOX binding sites are functional, they would likely be conserved in quail and duck, given the similarity in expression patterns of *Amh* among these three species. In quail, two SOX binding sites are present in the *Amh* promoter at positions corresponding to those of chicken, but neither site is present in the duck *Amh* promoter (data not shown), suggesting that these SOX binding sites are not functional. This finding may thus indicate that the mechanism of *Amh* regulation in chicken differs from that in mouse.

To determine whether a conserved SOX binding site might be present in the *Amh* promoters of chicken, quail, and duck, we searched the entire intergenic sequence between *Sap62* and *Amh* for such a site with the FrameWorker program but failed to detect one (data not shown). However, we are still not able to exclude the possibility that chicken *Amh* expression is regulated by a SOX protein. Further, extensive cloning of chicken *Sox* genes and analysis of the chicken *Amh* promoter will be necessary to clarify this issue.

Watanabe et al. (2000) described that there are two SF1 binding sites in mammalian *Amh* promoter, one locates proximal (proximal SF1 site) and the other more distal (distal SF1 site), and both of them are conserved in various species of mammals and essential for full promoter activity in Sertoli cells. If there would be a distal SF1 binding site in addition to the SF1 binding site, which we analyzed, in avian species like mammals, it is reasonable to postulate that such sequence is conserved among chicken, quail and duck *Amh* promoter. However, we could not find such a site searched by FrameWorker program using the entire intergenic sequence between *Sap62* and *Amh* (data not shown). It might be possible that the numbers of SF1 binding site, which is required for full *Amh* regulation are different between mammals and avians. If so, this may imply that protein complexes for male-specific up-regulation of *Amh* are also different between them.

Given that chicken *Amh* is expressed in a sexually dimorphic manner at the early stages of sexual differentiation, its expression might be regulated by the product of the testis-determining gene or by a target of this product. Further analysis of the chicken *Amh* promoter may also lead to the identification of such a factor. Such studies would be facilitated by the establishment of a Sertoli cell line or of primary culture conditions for Sertoli cells that support the normal pattern of *Amh* expression.

In conclusion, we showed the first evidence that SF1 can up-regulate *Amh* gene expression in chicken, like in mammals. However, *Amh* is expressed higher levels in male than in female gonads (Oréal et al., 1998; Smith et al., 1999a) and *Sf1* is expressed at high levels in female gonads, but at the relatively low level in male gonads as well, after onset of sexual differentiation (Oréal et al., 2002; Smith et al., 1999a,b). It can be possible that low level expression of *Sf1* is sufficient for *Amh* up-regulation. If it is so, there should be an unidentified factor that is expressed at a higher level in male than in female gonad and up-regulates *Amh* gene expression together with SF1. In mammals, one of critical factors with such activity is SOX9, however in chicken it is not likely because male specific up-regulation of *Amh* precedes that of *Sox9* (Oréal et al., 2002; Smith et al., 1999a; Takada et al., 2005). In addition, our comparative sequence analysis failed to identify conserved binding site for SOX factor. The nucleotide sequences of entire intergenic sequence between *Sap62* and *Amh* in chicken, quail and duck would be useful to identify such a factor.

4. Experimental procedures

4.1. Animals

Fertilized Japanese domestic duck (*Anas platyrhynchos*), quail (*Coturnix coturnix japonica*), and chicken (*Gallus gallus domestica*) eggs were obtained from a local supplier (Saitama Experimental Animal Supply, Saitama, Japan) and maintained at 18 °C until their transfer to an incubator at 37.8 °C. Staging of chicken and quail embryos was confirmed at dissection as described by Hamburger and Hamilton (1951) and Zacchei (1961), respectively. Staging of duck embryos was compared at dissection with chicken stages (Hamburger and Hamilton, 1951). The urogenital ridge of each embryo was explanted for whole-mount in situ hybridization, and the hind limb was used for extraction of genomic DNA and polymerase chain reaction (PCR)-based sexing as described (Clinton et al., 2001; Takada et al., 2006).

4.2. Cloning and sequencing of quail and duck Amh cDNAs

Partial genomic fragments of quail and duck *Amh* were amplified by PCR with the primers cAmh-4 (Western et al., 1999) and TAMHF3 (Takada et al., 2004). The PCR products were ligated into the pT-Adv vector (Clontech, Palo Alto, CA) and sequenced by Operon Biotechnologies (Tokyo, Japan).

Quail and duck *Amh* cDNAs were generated by 5′ and 3′ rapid amplification of cDNA ends (RACE) (Frohman et al., 1988). 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). Primers for 3′ RACE were 5′ PCR Primer IIA (Clontech) and uniAmhF1 (5′-GGCTGCAG-GAGCTGACCATCGACCTGC-3′), and those for 5′ RACE were 5′ PCR Primer IIA and uniAmhR3 (5′-GCAGGTCGATGGTCAGCTCCTGCAGCC-3′). The complete coding sequences of quail and duck *Amh* cDNAs were also generated by reverse transcription (RT) and PCR with the primers qAmh5′UTRF (5′-CTGGCGGCTCTGAGTGCGTTGG-3′) and qAmh3′UTRR (5′-AAGGGGCTGCAGGTGGGAACC-3′) for quail *Amh* and dAmh5′ORFF1 (5′-AGTGGTATCAACGCAGAGTACG-3′) and dAmh3′UTRR (5′-CTCCAGGGCTATGGGCAGGAGCCT-3′) for duck *Amh*. RT-PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI). At least three independent clones were sequenced for each RT-PCR product.

4.3. Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Xu and Wilkinson, 1998) with maleic acid buffer. Digoxigenin-labeled RNA probes were synthesized by in vitro transcription of DNA fragments obtained by digestion of 5′ RACE clones of quail and duck *Amh* with ApaI-NotI and SmaI-HincII, respectively. The probes correspond to nucleotides 1208–1714 of quail *Amh* cDNA (GenBank accession no. AY904049) and nucleotides 1180–1753 of duck Amh cDNA (AY904047).

4.4. Cloning and sequence analysis of chicken, quail, and duck Amh promoters

The promoters of chicken and quail *Amh* were obtained by PCR amplification of genomic DNA with primers based on chicken, quail, and duck *Amh* as well as chicken and mouse *Sap62* sequences: uniSap62F2 (5′-CTGACGCTCCATAA-CAATGAGG-3′) and uniAmhR7 (5′-ACCTTCCTCCAGGTGCAGGA-3′). The promoter of duck *Amh* was obtained by PCR with genomic DNA and the primers

dSap62F12 (5′-GCACCAGAAAAAGTCAAAGTGG-3′) and dAmhR9 (5′-TGAGAACACCCATGACTTCCAGGTGG-3′). At least three independent clones were sequenced for each PCR product.

Chicken, quail, and duck *Amh* promoter sequences were compared with MultiPipMaker software (http://pipmaker.bx.psu.edu/pipmaker) (Schwartz et al., 2000) after examination for the existence of repeat units with RepeatMasker (http://repeatmasker.org). Searches for binding motifs were performed with FrameWorker (Genomatix, Munich, Germany).

4.5. Cell culture and transfection assay

The open reading frame of *Sf1* was amplified by PCR with the primers cSf1F (5′-ATGGACTATTCGTATGATGAGG-3′) and cSf1RXhoI (5′-CTCGAGTCAAGTCCGCTTGGCGTGCAGC-3′) from cDNA prepared from the gonads of female chicken embryos at day 7 and was cloned into pGEM-T Easy. An Sf1 expression vector (pcDSf1B2) was then constructed by subcloning the open reading frame into pcDNA3.1 (Invitrogen, Carlsbad, CA).

Genomic fragment for co-transfection experiment was amplified by PCR using chicken *Sap62-Amh* genomic clone as template and the primers cAmh-178F (5′-CTCGAGCTTTTCTTCAGTTTCCAA-3′) and GL3AmhR (5′-GGCGCCGGGCCTTTCTTTATGTTTTTGGCGTCTTCCATCCTCC-CTGTTCTGCT-3′). The amplified fragment, which contains 178 bp of the promoter, the transcription start site, the full-length 5′ untranslated region and the initiation codon of *Amh*, was cloned into pGL3-basic (Promega) at XhoI/KasI site (pGL3-cAmh-178). A plasmid containing a mutated version of the proximal promoter of chicken *Amh* (pcAmhp-98/1M) was constructed by cloning into pGEM-T Easy of a PCR product obtained from pGL3-cAmhp-178 with the primers cAmhp-98FPstI (5′-CTGCAGTGGGACAGCGGCCG-3′) and cAmh-1RMut (5′-GCGCATGGGAGGACCGCATCTCCCTTT-TAAAAGCGGTGGAATTGAAGTG-3′); pGL3-cAmhp-178M was then constructed by substituting the 93-bp PstI-HinPI fragment of pGL3-cAmhp-178 with that of pcAmhp-98/1M. All constructs were verified by nucleotide sequencing.

Co-transfection experiments were performed in triplicate by transfection of BOSC23 cells, which are derived from human kidney (Pear et al., 1993), with 5 μg of pGL3-cAmhp-178 or pGL3-cAmhp-178M, 0.05 μg of pGL4-hRL-tk as a transfection control, and pcDSf1B2 (0, 0.0625, 0.25, or 1 ng; the total amount of DNA was maintained constant by the addition of pcDNA3.1 as appropriate). Transfection was performed with the use of Lipofectamine 2000 (Invitrogen). Luciferase assays were performed with a Dual-Luciferase Reporter Assay System (Promega).

4.6. Electrophoretic mobility-shift assay (EMSA)

For preparation of a probe, a DNA fragment amplified by PCR from pGL3-cAmhp-178 with the primers cAmh-98FPstI and cAmh-1R (5′-GCGCATGG-GAGGACCGCATC-3′) was cloned into pGEM-T Easy. The probe and a mutated competitor (insert of pcAmhp-98/1M) were excised from the respective pGEM-T Easy-based plasmids with PstI and EcoRI and purified by electrophoresis. Probe DNA was labeled with [γ-^{32}P]ATP (Amersham Bioscience, Piscataway, NJ) with the use of T4 polynucleotide kinase (Takara-Bio, Shiga, Japan). SF1 and luciferase were produced by in vitro transcription and translation with pcDSf1B2 and Luciferase T7 control DNA (Promega), respectively, and a TNT Coupled Reticulocyte Lysate System (Promega). Binding reactions were performed in a final volume of 15 μl of a solution containing 10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 25 mM $MgCl_2$, 0.5 μg of poly(dI-dC), 10% glycerol, and 0.7 mM phenylmethylsulfonyl fluoride. For the competition experiment, non-labeled probe or mutated competitor was added to the binding reaction at an ~100-fold excess. Reaction mixtures containing 2.5 μl of in vitro-synthesized SF1 or luciferase were incubated for 20 min on ice before addition of the ^{32}P-labeled probe. After incubation of binding reaction mixtures for 20 min at room temperature, DNA–protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel with 0.5×Tris–borate–EDTA buffer at 4 °C.

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

Shuji Takada [a,*], Jun Ota [a,b], Norio Kansaku [c], Hideji Yamashita [d], Tokukazu Izumi [e], Madoka Ishikawa [a], Tomoaki Wada [a], Ruri Kaneda [a], Young Lim Choi [a], Koji Koinuma [a], Shin-ichiro Fujiwara [a], Hirotaka Aoki [a], Hiroyuki Kisanuki [a], Yoshihiro Yamashita [a], Hiroyuki Mano [a,b]

[a] *Division of Functional Genomics, Jichi Medical School, 3311-1 Yakushiji, Kawachigun, Tochigi 329-0498, Japan*
[b] *CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan*
[c] *Laboratory of Animal Genetics and Breeding, Azabu University, Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan*
[d] *Laboratory of Genome Exploration and Informatics, Department of Bioscience, Kyushu Tokai University, Kawayo, Aso-gun, Kumamoto 869-1404, Japan*
[e] *Laboratory of Animal Reproduction, Department of Bioproduction Science, Faculty of Bioresources and Environmental Science, Ishikawa Prefectural University, Nonoichi-machi, Ishikawa 921-8836, Japan*

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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.

* Corresponding author. Fax: +81 285 44 7322.
E-mail address: stakada@jichi.ac.jp (S. Takada).

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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

dSox93′UTRR (5′-ATGGCTTTTAGGGTCTG GTGAG-3′). RT-PCR products were ligated into pGEM-T easy.

2.4. Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Xu and Wilkinson, 1998) with the maleic acid buffer method. Digoxigenin-labeled RNA probes were synthesized by in vitro transcription of cDNAs obtained by *Bam*HI digestion of 3′ RACE clones of *qSox9* and *dSox9*. The probes corresponded to nucleotides 391–568 of *qSox9* cDNA (Accession No. AY904048) and nucleotides 194–371 of *dSox9* cDNA (Accession No. AY904046).

3. Results and discussion

3.1. Sexing of quail and duck embryos

To distinguish between female and male early embryos of quail or duck before the appearance of morphological sexual differences, we developed an assay based on the absence (males) or presence (females) of *Wpkci* (also known as *Asw*), which is located on the W chromosome (Hori et al., 2000; O'Neill et al., 2000). This gene was detected by PCR performed in a single tube with *Wpkci*-specific primers and, as a positive control, primers specific for the gene for 18S rRNA. Female quails are expected to generate two distinct PCR products of 101 and 256 bp for quail *Wpkci* (*qWpkci*) and the 18S rRNA gene, respectively, whereas males are expected to generate only the latter product (Fig. 1A). Female ducks are expected to yield two bands of 104 and 256 bp for duck *Wpkci* (*dWpkci*) and the 18S rRNA gene, respectively, whereas males should again generate only the latter band (Fig. 1B). The accuracy of this PCR-based sexing method was confirmed by the amplification of genomic DNA from adult birds of each species. In all cases, the results of PCR-based sexing were identical to those obtained by morphological examination (data not shown).

3.2. Cloning and sequence analysis of quail and duck Sox9 genes

To isolate *Sox9* cDNAs for quail and duck, we used the 5′ and 3′ rapid amplification of cDNA end (RACE) techniques (Frohman et al., 1988) with RNA purified from the gonads of male quail or duck embryos on days 7 and 8, respectively, given that *Sox9* would be expected to be expressed in the developing testis if it functions in male sexual differentiation. To design the 3′ RACE primer, we amplified partial genomic fragments of quail and duck *Sox9* by PCR with a primer set based on conserved cDNA sequences for the 5′ untranslated region (UTR) and the high mobility group (HMG)-box region of *Sox9* of chicken (*Gallus gallus*, U12533), American alligator (*Alligator mississippiensis*, AF106572), red-eared slider turtle (*Trachemys scripta*) (Spotila et al., 1998), mouse (*Mus musculus*, AF421878), and rainbow trout (*Oncorhynchus mykiss*, AB006448). These primers yielded 266- and 269-bp products from the quail and duck genomes, respectively (data not shown), and these products were then subjected to nucleotide sequencing.

The 3′ RACE reaction was then performed with gonadal cDNA from quail or duck males and a primer based on the sequence of the conserved PCR products. The nucleotide sequences of the resulting amplicons were determined and used to design the primers for 5′ RACE. The nucleotide sequences thus obtained by 5′ and 3′ RACE were assembled.

To confirm that the sequences determined by 5′ and 3′ RACE were derived from the same transcripts, we attempted to amplify cDNAs containing the entire coding regions by reverse transcription (RT) and PCR with primers localized in the 5′ and 3′ UTRs. A 1.6-kb cDNA was amplified from quail RNA and a 1.5-kb cDNA from duck RNA. Nucleotide sequencing of these amplified fragments confirmed that the 5′ and 3′ RACE sequences were linked in tandem in quail and in duck, demonstrating that the assembled sequences correspond to single transcripts.

The deduced amino acid sequences of the proteins encoded by the quail and duck cDNAs revealed that the 79-residue *Sry*-type HMG-boxes of the two proteins were identical to each other and differed by only one amino acid from that of chicken Sox9 (Fig. 2). The overall sequence identities of the three proteins were 94.3, 85.5, and 94.3% between quail and chicken, duck and chicken, and quail and duck, respectively. Given that the NH_2- and COOH-termini of the deduced amino acid sequences of the quail and duck proteins were highly conserved compared with

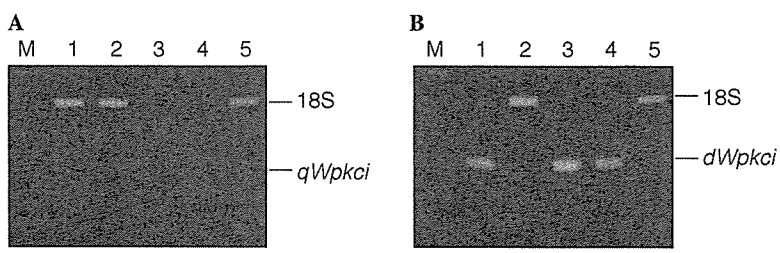

Fig. 1. PCR-based sexing of quail and duck embryos. Sexing of five embryos each of quail (A) and duck (B) was performed. Quail embryos in lanes 1, 2, and 5 are males; those in lanes 3 and 4 are females. Duck embryos in lanes 2 and 5 are males; those in lanes 1, 3, and 4 are females. The positions of PCR products corresponding to the 18S rRNA gene and *Wpkci* are indicated. Lanes M contain DNA size markers (50-bp ladder).

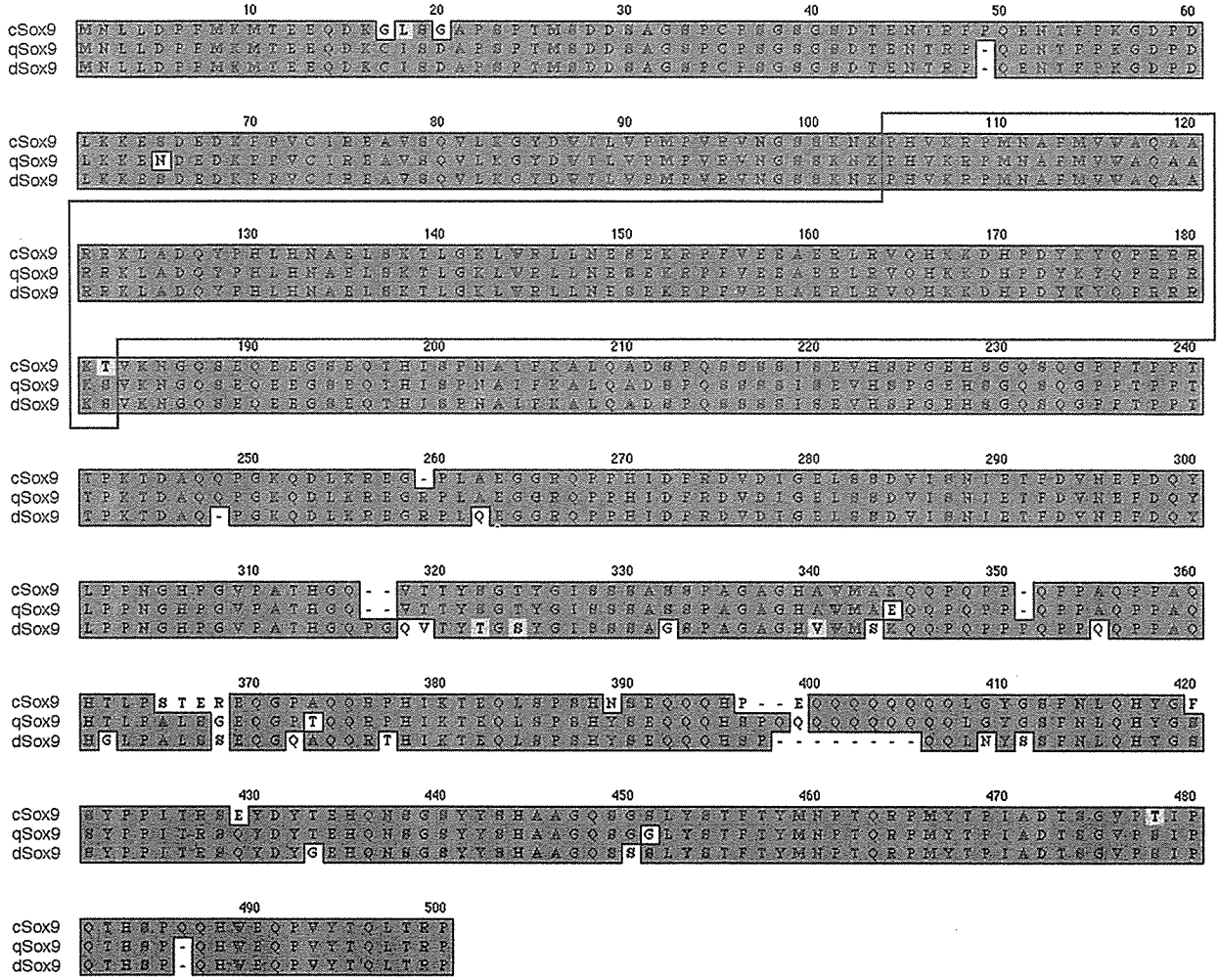

Fig. 2. Alignment of the deduced amino acid sequences of chicken (c), quail (q), and duck (d) Sox9. Dark and light gray shadings indicate identical and similar amino acids, respectively. The boxed region corresponds to the *Sry*-type HMG-box.

those of chicken Sox9 and that BLASTP searches of GenBank nonredundant database with the deduced amino acid sequences of the quail and duck proteins as queries yielded chicken and alligator Sox9 as the most similar sequences, we conclude that the isolated quail and duck cDNAs are derived from the corresponding *Sox9* genes (*qSox9* and *dSox9*, respectively). The nucleotide sequences of these cDNAs have been deposited in GenBank under the Accession Nos. AY904048 for *qSox9* and AY904046 for *dSox9*.

3.3. Expression of Sox9 in developing gonads of quail and duck

We examined the temporal and spatial expression patterns of quail and duck *Sox9* during the early stages of gonadal differentiation by whole-mount in situ hybridization with gonad–mesonephros complexes isolated from quail embryos on days 4, 5, 6, and 7 (Zacchei stages 17–18, 20–21, 22, and 24, respectively) (Zacchei, 1961) and from duck embryos on days 6, 7, 8, and 9. Duck embryos were staged by comparison with chicken (Hamburger and Hamilton, 1951); the morphological stages of duck are similar to those of chicken, although development is slightly delayed in duck (days 6, 7, 8, and 9 for duck embryos correspond to Hamburger and Hamilton stages 25–26, 28, 29–30, and 31–32, respectively).

Sox9 mRNA was not detected in quail gonads on day 4 (Figs. 3A and E) or in duck gonads on day 6 (Figs. 3J and N). *Sox9* was expressed at higher levels in male gonads than in female gonads of quail on days 5, 6, and 7 (Figs. 3B–D and F–H) as well as of duck on days 7, 8, and 9 (Figs. 3K–M and O–Q). Sense control probes yielded no specific labeling (Figs. 3I and R). The earliest detectable stages for the male-specific expression of *Sox9* were thus similar for chicken (stages 28–30) (Loffler et al., 2003; Morais da Silva et al., 1996; Oréal et al., 1998; Smith et al., 1999), quail (day 5 corresponds to Zacchei stages 20–21 and Hamburger and Hamilton stages 27–29) (Zacchei, 1961), and duck (day 7 corresponds to Hamburger and Hamilton stage 28). The expression patterns of *Sox9* in the gonads of these three avian species are also similar to that in mouse (Kent et al., 1996; Loffler et al., 2003; Morais da Silva et al., 1996; Oréal et al., 1998; Smith et al., 1999).

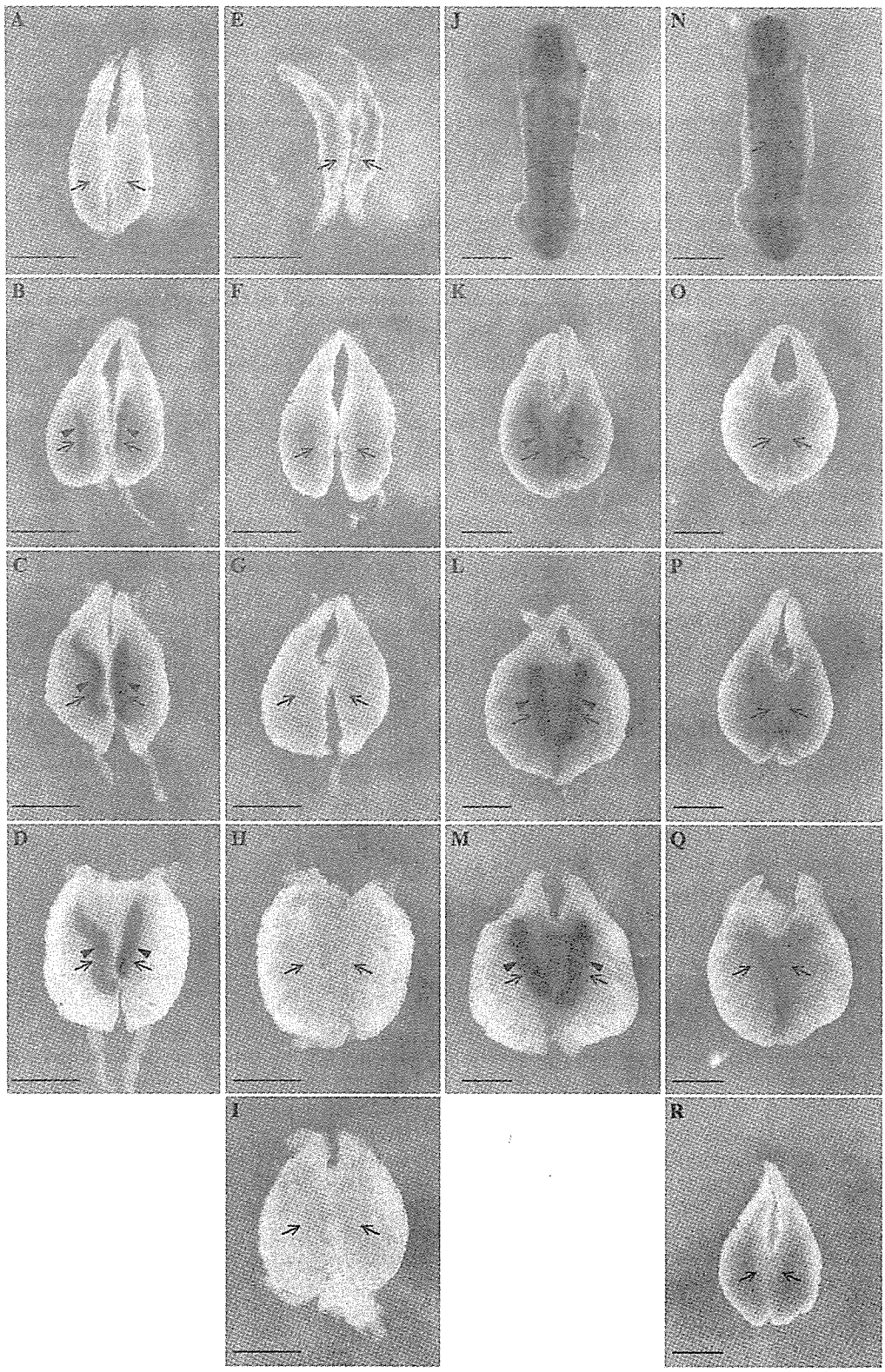

Fig. 3. Whole-mount in situ hybridization analysis of *qSox9* and *dSox9* expression in the embryonic gonad–mesonephros of quail and duck, respectively. Male (A–D and I) and female (E–H) quail embryos were analyzed on day 4 (A and E), day 5 (B and F), day 6 (C, G, and I), and day 7 (D and H). Male (J–L and M) and female (N–R) duck embryos were analyzed on day 6 (J and N), day 7 (K, O, and R), day 8 (L and P), and day 9 (M and Q). All embryos were subjected to hybridization with an antisense probe, with the exception of that in (I and R), for which a sense probe was used as a control. Arrows indicate the position of the gonad. Arrowheads indicate the region of staining. Scale bar, 1 mm.

The similarities of *Sox9* expression patterns between birds and mammals suggest that Sox9 has conserved functions in the differentiation of the embryonic gonads toward testes or ovaries. It has been postulated that Sox9 has more than one target in gonads. One of them in mammals is *Amh*, however previous studies suggested it is not a target in birds (Oréal et al., 1998; Smith et al., 1999; Takada et al., 2005). Another target in mammals identified so far is KIAA0800, which is preferentially expressed in testis while the function of which is not known (Zhao et al., 2002). Although it is not known whether KIAA0800 is expressed in avian embryonic testes or not, it can be possible that this gene and/or other genes which play a role in gonadal differentiation are regulated by Sox9 in avian and mammalian gonads. It is important to identify the targets of Sox9 in birds to understand how different hormones are produced in gonads between males and females.

In summary, the developmental expression of *qSox9* and *dSox9* is associated with testis differentiation. In quail embryos, a difference in gonadal expression of *Sox9* between the sexes was first apparent on day 5 (stages 20–21), indicating that sexual differentiation occurs at or before day 5 of incubation. In duck embryos, sexually dimorphic expression of *Sox9* was first apparent on day 7, showing that testis differentiation begins at the molecular level no later than day 7. Finally, given the similarity in the temporal and spatial expression patterns of *Sox9* during gonadogenesis in chicken, quail, and duck, comparative analysis of the *Sox9* promoters among these species may provide insight into the molecular events of sex determination or differentiation in birds.

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Mouse microRNA profiles determined with a new and sensitive cloning method

Shuji Takada[1], Eugene Berezikov[2], Yoshihiro Yamashita[1], Mariana Lagos-Quintana[3], Wigard P. Kloosterman[2], Munehiro Enomoto[1], Hisashi Hatanaka[1], Shin-ichiro Fujiwara[1], Hideki Watanabe[1], Manabu Soda[1], Young Lim Choi[1], Ronald H. A. Plasterk[2], Edwin Cuppen[2] and Hiroyuki Mano[1,4,*]

[1]Division of Functional Genomics, Jichi Medical University, 3311-1 Yakushiji, Shimotsukeshi, Tochigi 329-0498, Japan, [2]Hubrecht Laboratory, Uppsalalaan 8, Utrecht, The Netherlands, [3]Laboratory of RNA Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA and [4]CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan

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ABSTRACT

MicroRNAs (miRNAs) are noncoding RNA molecules of 21 to 24 nt that regulate the expression of target genes in a post-transcriptional manner. Although evidence indicates that miRNAs play essential roles in embryogenesis, cell differentiation and pathogenesis of human diseases, extensive miRNA profiling in cells or tissues has been hampered by the lack of sensitive cloning methods. Here we describe a highly efficient profiling method, termed miRNA amplification profiling (mRAP), as well as its application both to mouse embryos at various developmental stages and to adult mouse organs. A total of 77 436 Small-RNA species was sequenced, with 11 776 of these sequences found to match previously described miRNAs. With the use of a newly developed computational prediction algorithm, we further identified 229 independent candidates for previously unknown miRNAs. The expression of some of these candidate miRNAs was confirmed by northern blot analysis and whole-mount *in situ* hybridization. Our data thus indicate that the total number of miRNAs in vertebrates is larger than previously appreciated and that the expression of these molecules is tightly controlled in a tissue- and developmental stage-specific manner.

INTRODUCTION

MicroRNAs (miRNAs) are short noncoding RNA molecules that inhibit gene expression through incomplete base pairing with the 3′-untranslated region (3′-UTR) of target mRNAs (1,2). The miRNA system is conserved from worms to mammals and contributes to the regulation of a wide variety of cellular functions. In *Caenorhabditis elegans*, for instance, larval development is regulated by a set of miRNAs that include members of the *lin-4* and *let-7* families (3,4), and the function of *Dicer1*, which encodes an enzyme essential for miRNA biogenesis, is indispensable for mouse embryonic development (5). Furthermore, the miRNA miR-181 has been implicated in the differentiation of mouse B lymphocytes (6).

Evidence indicates that miRNAs also play a role in the pathogenesis of human disorders including cancer. The expression profiles of miRNAs are thus effective for classification of human cancers (7,8). Human *let-7* miRNAs target transcripts of the proto-oncogene RAS and are down-regulated in a large proportion of lung cancer specimens (9). Localization of miRNA genes to the fragile sites of human chromosomes indicates that many more miRNAs may be linked to carcinogenesis (10).

Although the recent public miRNA registry (miRBase release 7.1 at http://microRNA.sanger.ac.uk) contains 326 entries for human miRNAs, a large number of additional human miRNAs are thought to exist (11,12). Given the relation of miRNAs to cell growth and differentiation and to human disease, it is important to compare the expression profiles of miRNAs (both known and unidentified previously) among normal tissues and clinical specimens. Such studies have been hampered, however, by the lack of sensitive cloning methods for miRNAs. Current standard procedures for miRNA isolation require several 100 μg of total RNA as a starting material (13), an amount that is difficult to obtain from small tissues or clinical specimens. To overcome such limitations, we have developed a highly sensitive cloning method for miRNAs, which we have termed miRNA amplification profiling (mRAP).

*To whom correspondence should be addressed. Tel: +81 285 58 7449; Fax: +81 285 44 7322; Email: hmano@jichi.ac.jp

MATERIALS AND METHODS

mRAP

A Small-RNA fraction was directly isolated from cells with the use of a mirVana miRNA Isolation Kit (Ambion). In our experience, the yield of Small-RNA with this kit was about 40–50% of that for total RNA obtained by conventional methods from the same number of cells. A portion of this Small-RNA fraction together with size markers (19, 24 and 33 nt) was subjected to electrophoresis on a 15% polyacrylamide gel under denaturing conditions. The region of the gel containing RNA of 19–24 nt was excised, and the RNA molecules were recovered, dephosphorylated by incubation for 30 min at 50°C with calf intestinal alkaline phosphatase (New England Biolabs) and ligated to the 3′ adaptor [5′-(Pu)uuAACCGCGAATTCCAG(idT)-3′], where lowercase letters indicate RNA, uppercase letters indicate DNA, Pu denotes 5′-phosphorylated uridine, and idT represents 3′-inverted deoxythymidine (Dharmacon). The ligated RNA was subjected to reverse transcription with PowerScript reverse transcriptase (Clontech) and the RT primer (5′-GACTAGCTGGAATTCGCGGTTAAA-3′) in the presence of the 5′ adaptor (5′-GACCACGCGTATCGGGCACCACGTATGCTATCGATCGTGAGATGGG-3′). The products were amplified by PCR for 32 cycles of incubation at 95°C for 30 s and 65°C for 30 s with AmpliTaq Gold DNA polymerase (Applied Biosystems), the 5′ PCR primer (5′-GCGTATCGGGCACCACGTATGC-3′), and the 3′ PCR primer (5′-GACTAGCTTGGTGCCGAATTCGCGGTTAAA-3′). The resulting amplicons were fractionated by electrophoresis, and those from 90 to 95 bp were eluted, digested with BanI endonuclease (New England Biolabs), and subjected to concatamerization with the use of a Ligation High Kit (Toyobo, Osaka, Japan). Products from 500 to 2000 bp were isolated by electrophoresis and cloned into the pGEM-Teasy vector (Promega). A more detailed description of the mRAP protocol is provided as Supplementary Data on the NAR web site.

Prediction of novel miRNAs

Base calling and quality trimming of sequence chromatograms were performed with phred software (14). After masking of vector and adaptor sequences and removal of redundancy, inserts of ⩾18 bp were mapped to genomes (ncbi35 assembly for human, ncbim34 assembly for mouse) with the use of the megablast program in the NCBI software suite (ftp://ftp.ncbi.nlm.nih.gov/blast). For every genomic locus that matched an insert, repeat annotations were retrieved from the Ensembl database (http://www.ensembl.org) and repetitive regions were discarded. Genomic regions containing inserts with 100 nt flanking sequences were retrieved from Ensembl, and a sliding window of 100 nt was used to calculate RNA secondary structures with RNAfold software from the Vienna RNA Secondary Structure Package (15).

To detect homologous hairpins in other genomes, we performed a BLAST search with mature regions of each RNA sequence against human, mouse, rat, dog, cow, opossum, chicken, zebrafish and fugu genomes. Hits of ⩾20 nt with an identity of ⩾70% were extracted from the genomes together with flanking sequences of a size similar to that observed for the original hairpins. Extracted sequences were checked for hairpin structures with the use of RNAfold, and positive hairpins were aligned with the original hairpin with CLUSTAL W (16). For remaining hairpins, randfold (17) values were calculated for every sequence in an alignment by mononucleotide shuffling and 1000 iterations. A cutoff of 0.01 was used for randfold, and only regions that contained a hairpin below this cutoff for at least one species in an alignment were considered as candidates of miRNA genes. Berezikov *et al.* (18) describe the computational method for prediction of miRNAs in more detail.

Northern blot analysis

Small-RNA fractions (0.1 to 0.5 μg) were subjected to electrophoresis on a 15% polyacrylamide gel under denaturing conditions, and the separated molecules were transferred electrophoretically to a Hybond-N nylon membrane (Amersham Biosciences). The membrane was incubated with ^{32}P-labeled locked nucleic acid (LNA) corresponding to mature miRNA sequences in ULTRAhyb-Oligo solution (Ambion), and signals were detected with a BAS-1500 image analyzer (Fuji Photo Film).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described (19). LNA-modified oligonucleotides were synthesized by Thermo Electron (Ulm, Germany), and digoxigenin labeling was performed with a DIG Oligonucleotide 3′ End Labeling Kit (Roche Diagnostics, Penzberg, Germany).

RESULTS

Development of mRAP

To isolate miRNAs from small quantities of RNA, we first tried to amplify the miRNA fraction by incorporating simple PCR steps into the conventional miRNA cloning procedures (13). However, all such trials resulted in an amplification of non-specific products from degraded RNAs and adaptor-primer concatamers without miRNA-derived cDNAs (as shown in Figure 1A). To circumvent this limitation, we invented the mRAP procedure by utilizing (1) the SMART method (Clontech) for an efficient cDNA amplification and (2) a long, sophisticated 5′ adaptor. All nucleotide sequences of the 5′ adaptor originally invented by Lagos-Quintana (13), SMART IIA oligonucleotide (Clontech), and a BanI site (for a uni-directional concatamerization of PCR products) were incorporated into our initial 5′ adaptor sequence, which was subsequently optimized by addition/removal of nucleotides to reduce non-specific PCR products. In addition, the length of the 5′ adaptor (46 bases) was determined so that the miRNA-derived products can be easily separated from the two major byproducts (see Figure 1A).

In the mRAP procedure, isolated Small-RNA molecules are first ligated at their 3′ end to a 3′ adaptor and then reverse-transcribed with the use of a primer (RT primer) complementary to the 3′ adaptor (Figure 1A). Because of the fact that certain reverse transcriptases possess terminal deoxynucleotidyl transferase activity a few nucleotides

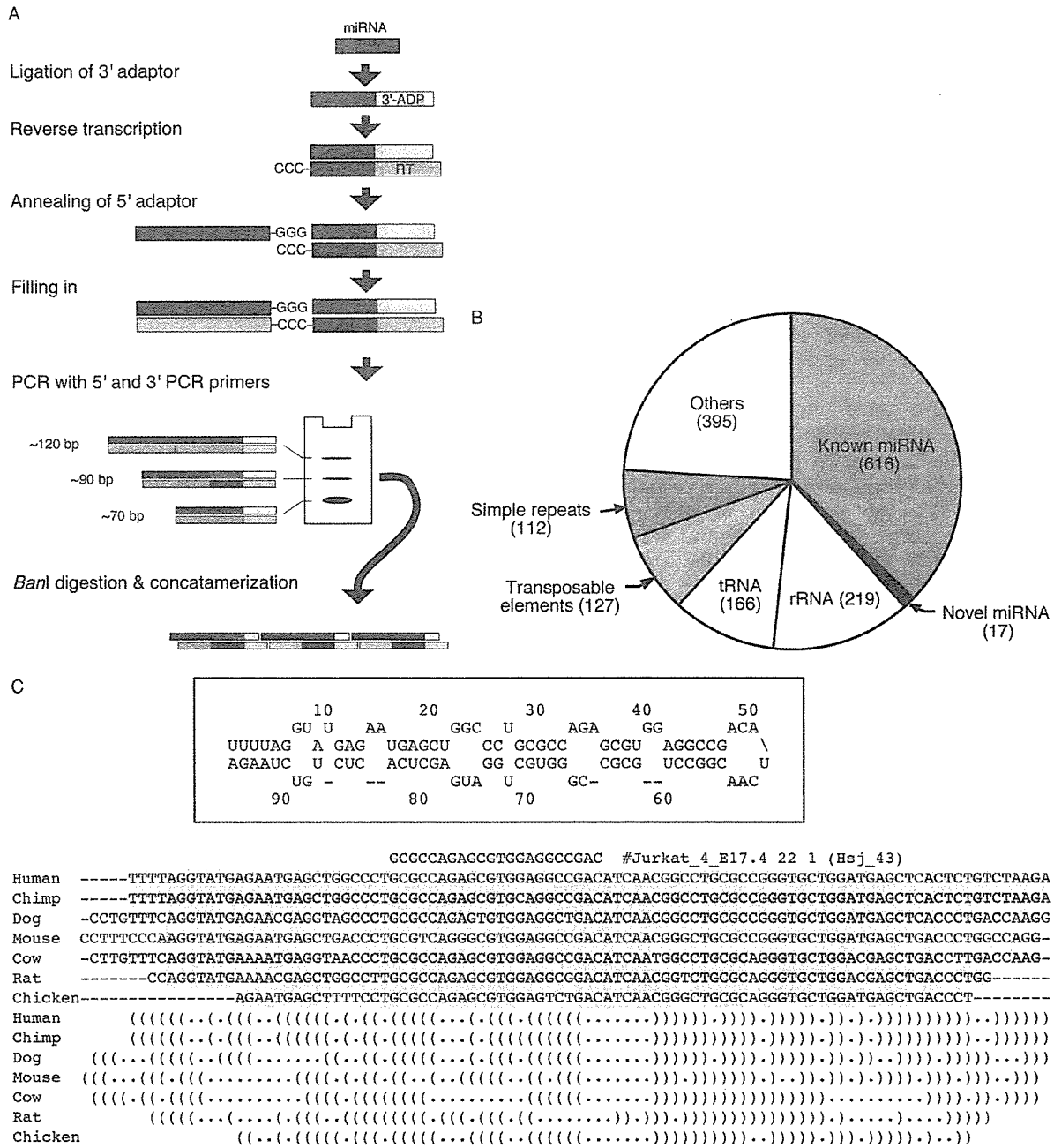

Figure 1. The mRAP protocol and its application to Jurkat cells. (**A**) Isolated Small-RNA molecules are ligated to the 3′ adaptor (3′-ADP) and subjected to reverse transcription with the RT primer. After annealing of the 5′ adaptor (5′-ADP) to the poly(C) overhang at the 3′ end of the synthesized cDNAs, the latter are subjected to PCR with the 5′ and 3′ PCR primers. After an extensive cloning/sequencing of the PCR products, we noticed that, of the three major sizes of amplicon generated, only the middle one includes cDNAs derived from miRNAs. The large product of ~120 bp is composed of two 5′ adaptors and one 3′ adaptor without miRNA sequences. The small product of ~70 bp is, on the other hand, composed of only one 5′ adaptor and one 3′ adaptor. The product of ~90 bp are thus isolated, digested with BanI, and self-ligated to yield concatamers. (**B**) Among 1652 mRAP clones of Jurkat cells that matched the human genome sequence, 616 clones corresponded to known miRNAs, 17 are candidates for novel miRNAs and 219 corresponded to rRNAs, 166 to tRNAs, 127 to transposable elements, 112 to simple repeats and 395 to other genomic sequences that do not fold into a hairpin or otherwise fail the miRNA prediction pipeline. (**C**) Alignment of the nucleotide sequence (red) of one predicted novel miRNA (Hsj_43) with genomic sequences of human, chimpanzee, dog, mouse, cow, rat and chicken. Nucleotides conserved between human and other species are shaded in gray. Possible base pairing schemes for the respective Hsj_43 precursors are shown below the aligned sequences and, for the human sequence, in the upper inset.

(mostly deoxycytidine) are added to the 3′ end of each cDNA strand (20). After the annealing of a 5′ adaptor to the poly(C) overhang of the cDNAs, PCR is performed with 5′ and 3′ PCR primers to exponentially amplify the cDNAs. The isolated cDNAs are digested with the BanI restriction endonuclease and self-ligated to generate concatamers, which are then inserted into a plasmid vector for nucleotide sequencing.

As a test case, we first applied mRAP to 5 μg of a Small-RNA fraction isolated from the human T cell line Jurkat. The procedure readily generated >1 × 10^4 colony-forming units of the concatamer library. A total of 958 clones was randomly chosen from the library and subjected to nucleotide sequencing. Each plasmid insert consisted of multiple short cDNAs (average of 2.59 cDNAs per insert), and the dataset contained a total of 2392 such cDNAs of ⩾18 bp. The 1652 cDNA sequences that passed quality assessment were subjected to computational screening for previously unidentified miRNAs with an algorithm developed in-house. In brief, after filtering of repeat, rRNA, tRNA and small nucleolar (snoRNA) sequences, the remaining sequences predicted to fold into stable stem–loop structures were selected and checked for overlap with known miRNA genes (18).

As shown in Figure 1B, the Jurkat dataset contained 616 clones of known miRNAs (corresponding to 60 independent miRNAs) and 17 clones of newly predicted miRNAs (corresponding to 15 independent miRNAs) (see Supplementary Tables S1 and S2). The proportion of miRNA clones among our Jurkat cDNA sequences (38.3%) was slightly smaller than that (46.9%) obtained by the conventional method by Lagos-Quintana *et al.* (13).

One such candidate for the novel miRNA sequences ('Hsj_43' according to our tentative nomenclature system) is shown aligned with vertebrate genomes in Figure 1C. The precursor of this miRNA is presumed to comprise 95 nt in human, and its nucleotide sequence is conserved among various vertebrates and can fold into an incompletely complementary hairpin structure (Figure 1C).

To determine whether mRAP is able to efficiently isolate miRNAs from a small number of cells, we prepared a Small-RNA fraction (7 μg, 700 and 70 ng, respectively) from 1 × 10^6, 1 × 10^5 and 1 × 10^4 Jurkat cells. We found that mRAP readily generated >1 × 10^4 colony-forming units of concatamer libraries from all three samples (data not shown). Nucleotide sequencing of randomly chosen clones revealed that the most abundant hsa-miR-142-3p occupies 36.6% (26 reads out of 71 total miRNA reads), 26.1% (24 out of 92) and 20.0% (17 out of 85) of total miRNA clones isolated from the 1 × 10^6, 1 × 10^5 and 1 × 10^4 cells, respectively (data not shown). Similarly, another abundant miRNA, hsa-miR-143, could be found in 11.3% (8 reads), 9.8% (9 reads) and 15.3% (13 reads) of miRNAs from the 1 × 10^6, 1 × 10^5 and 1 × 10^4 cells, respectively. The proportion of isolated rRNAs was also constant among the samples, indicating the high fidelity of mRAP even when performed with a small number of cells. These data confirmed that mRAP is highly sensitive for characterization of miRNA profiles, needing <0.1% of the initial RNA quantity required for current methods (13).

miRNA profiling of mouse embryos

We next applied mRAP to obtain miRNA profiles of mouse. We first isolated mouse embryos at 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5 and 17.5 days postcoitum (dpc) and subjected them to miRNA profiling. A total of 25 944 small cDNAs was sequenced for all embryos (average of 2359 clones per embryo); 3362 of these clones corresponded to 150 known miRNAs (miRBase release 7.1), and 198 of them corresponded to 75 novel miRNAs (see Supplementary Tables S3 and S4). These data indicated that many miRNAs are expressed from an early stage of embryogenesis (at 6.5 dpc, for instance, 9.63% of Small-RNA species corresponded to miRNAs). Furthermore, novel candidate miRNAs were detected throughout embryogenesis; the proportion of novel miRNAs among all known and unknown miRNAs was 6.54 ± 3.67% (mean ± SD) for the developmental stages examined.

The expression profiles of miRNAs at each developmental stage of the mouse embryo are summarized in Figure 2A and Supplementary Table S3. Whereas some miRNAs, (such as mmu-mir-124a) are expressed throughout embryonic development, many others are expressed only at specific stages. Expression of mmu-mir-206, e.g. was almost undetectable up to 13.5 dpc but was increased markedly at 14.5 dpc and thereafter. Expression of mmu-mir-148a was largely restricted to 10.5 dpc, at which time it constituted 16.07% of all miRNAs. Similarly, 24 cDNA clones (7.55% of all miRNA species) derived from the mouse embryo at 7.5 dpc corresponded to mmu-mir-23b, whereas only 0 to 3 such clones were identified at other stages of development.

We performed Northern blot analysis to confirm the miRNA profiles identified by mRAP screening. As shown in Figure 2B, northern analysis revealed that the expression of mmu-mir-206 increased progressively with time of embryonic development, whereas that of mmu-mir-124a remained relatively stable (with a slight increase apparent at 13.5 to 15.5 dpc). Direct comparison revealed that the temporal profiles of mmu-mir-206 expression determined by northern blot analysis and by mRAP were similar, with a slight difference in detection sensitivity (Figure 2C).

Northern analysis also detected the putative novel miRNA Mmj_157 at an appropriate size and with preferential expression in mid to late stages of embryogenesis (Figure 2B). We examined the localization of putative miRNAs in whole-mount preparations of mouse embryos at 10.5 dpc by *in situ* hybridization with LNA-modified DNA as a probe. Some of the novel miRNAs were found to be expressed in a tissue-specific manner. Both Mmj_163 and Mmj_157 putative miRNAs were detected specifically in the central nervous system, with the former being preferentially expressed in the telencephalon and the latter in the myelencephalon (Figure 2D). Despite its abundance in the central nervous system of embryos, we were not able to detect Mmj_157 in adult brain (Figure 3A and Supplementary Table S5), indicating that expression of this putative miRNA is both spatially and temporally restricted. In adult mice, a substantial amount of Mmj_157 was apparent only in the placenta, in which it constituted 12.85% of all miRNA clones.

miRNA profiling of adult mouse organs

We next determined the miRNA profiles for 21 organs of the adult mouse with the mRAP procedure. A total of 51 492 clones derived from Small-RNAs (average of 2452 clones per organ) was sequenced and found to include 8141 clones of known miRNAs and 287 clones of novel candidate miRNAs. The distribution of abundant miRNAs in each organ is shown schematically in Figure 3A, with the complete