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Sex Differentiation and mRNA Expression of P450c17, P450arom and AMH in Gonads of the Chicken

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ABSTRACT The present study was conducted to reveal effects of in ovo injection of nonsteroidal aromatase inhibitor (Fadrozole) or estradiol at day 3 of incubation on mRNA levels of P45017αhydroxylase (P450c17), P450 aromatase (P450arom) and anti-Müllerian hormone (AMH) in the chicken gonads. The mRNA levels in the gonads at days 4–8 of incubation were assessed by in situ hybridization analysis using digoxigenin labeling method. The in situ hybridization data were analyzed by relative expression of specific hybridizable signals of each mRNA corrected by the non-specific background by employing an image analyzer. P450c17 mRNA expression increased rapidly at day 6 of incubation in the male but decreased thereafter. In contrast to the transient expression in the male, the expression was gradually increased in the female. P450arom mRNA was not expressed in the male but was detectable in the female as early as day 6 and increased subsequently with days of incubation. AMH mRNA was expressed as early as day 5 of incubation followed by a sharp increase on day 6, which was maintained in the male thereafter. In contrast, the female showed very little expression. The injection of Fadrozole caused no effect on P450c17 mRNA expression, while it suppressed P450arom mRNA expression but increased AMH mRNA expression in the female. In contrast, the injection of estradiol induced P450arom mRNA expression significantly but suppressed AMH mRNA expression in the male. These results indicate that expression of P450arom and AMH is sexually dimorphic and is reciprocally regulated during early ontogenic life in chicken gonads. *Mol. Reprod. Dev. 55:20–30, 2000.* © 2000 Wiley-Liss, Inc.

Key Words: chicken embryo; sex differentiation; gonads; P450arom; AMH; mRNA

INTRODUCTION

Differentiation of the indifferent gonad into a testis is induced by the product of the sex-determining region of the Y chromosome termed SRY/Sry in mammals (Gubbay et al., 1990; Sinclair et al., 1990). SRY/Sry is considered to act as a switch to initiate the transcription of a cascade of other genes which contribute to testicular development. In birds, however, Sry or its equivalent gene has not been found (Griffiths, 1991; Coriat et al., 1993). Although the sex is genetically determined in birds, it remains sensitive to hormonal influences while it is at the indifferent stage. For example, injection of estrogen in ovo induces genetically male gonads to develop as ovaries in the chick embryo although the female organization is transitory since it regresses after hatching and testicular differentiation progresses (Scheib, 1983). Since estrogen is necessary to induce feminization in normal females, and cytochrome P450aromatase (P450arom) is a key enzyme that converts testosterone to estrogen, expression of the P450arom gene is one of the essential steps of ovarian differentiation. Indeed, P450arom mRNA is expressed mainly in the female gonad, which results in the aromatization of testosterone to estradiol (E2), whereas in the male gonad the absence of P450arom results in the accumulation of testosterone (Woods and Podczaski, 1974; Woods and Erton, 1978; Yoshida et al., 1996).

On the other hand, testosterone induces masculinization in the genetic male. The masculinizing effect of testosterone treatment in ovo is only temporary in the genetic female, probably due to endogenous ovarian aromatase produced by the ovary. Anti-Müllerian hormone (AMH) is a member of the transforming growth factor B (TGF-B) family and is responsible for regression of the Müllerian ducts in males during embryonic development (Vigier et al., 1987). AMH also represses P450arom biosynthesis indicating an indirect masculinizing effect of AMH (Vigier et al., 1989). In mammals, Sertoli cells of the testis produce AMH from early fetal

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life, while AMH production by granulosa cells of ovaries begins only after birth (Munsterberg, 1991). In birds, AMH is synthesized in the gonads of both sexes at the same stage during development (di Clemente et al., 1992) although the AMH mRNA expression is very low in females (Carre-Eusebe et al., 1996; Clinton, 1998, Oreal et al., 1998).

In mammals, AMH gene expression occurs after the expression of Sox9 and Sry genes. It has been suggested that SRY/Sry induces AMH expression (Haqq et al., 1994). In females, AMH is not expressed because Sox9 and Sry are not expressed. In the chicken, the mechanism of induction of the AMH gene remains unknown although recent reports demonstrated the male-specific expression of Sox9 gene at an early stage of development (Kent et al., 1996; Morais da Silva et al., 1996; Clinton, 1998). This may suggest some involvement of Sox9 in AMH expression in the male chicken as is observed in mammals (Neeper et al., 1996). However, recently, Oreal et al. (1998) made simultaneous analyses of Sox9 and AMH mRNA expression by in situ hybridization in chicken embryos and found that the early sexually dimorphic expression of AMH precedes testicular Sox9 expression which challenges the primacy of Sox9 as the common ancestral sex-determining gene in vertebrates.

Elbrecht and Smith (1992) demonstrated that treatment of chicken embryos with a nonsteroidal aromatase inhibitor (AI) at the indifferent stages causes a genetic female to develop into a male phenotype. Abinawanto et al. (1996) measured mRNA levels of gonadal P45017αhydroxylase (P450c17) and P450arom in the chicken embryo, and reported that AI treatment reduces P450arom mRNA levels in sex-reversed female embryos. Since supplemental treatment of estrogen restored P450arom mRNA expression, estrogen or an estrogen-induced substance might be involved in P450arom gene expression. The present study aimed first to reveal a more detailed profile and timing of mRNA expression of P450c17, P450arom and AMH of the gonads in normal males and females during sexual differentiation, by quantification of the mRNA using in situ hybridization analysis. In addition, inhibition of P450arom mRNA expression and induction of AMH mRNA expression after AI treatment and induction of P450arom mRNA expression and inhibition of AMH mRNA expression after estrogen treatment in individual embryos are reported. Consequently, the attempt is made to reveal molecular mechanisms of sexual differentiation in avian gonads.

MATERIALS AND METHODS

Aromatase Inhibitor (AI) Treatments

Fertile eggs from White Leghorn hens were incubated at 37.5°C in a commercial incubator using standard conditions. On day 3 of incubation the eggs were treated with a single injection of AI (Fadrozole, Ciba-Geigy Co., Summit, NJ) at a dose of 0.1 mg in 0.1 ml of 0.9% NaCl solution (saline) into the air sac of the eggs as described previously (Abinawanto et al., 1996). For control embryos 0.1 ml saline was injected. Shell holes were sealed with paraffin and eggs were replaced in the incubator. Embryos and blood were collected individually on days 4, 5, 6, 7, and 8 of incubation. Each embryo was used for in situ hybridization analysis of P450c17 mRNA, P450 arom mRNA, and AMH mRNA.

E2 Treatments

Fertilized eggs from White Leghorn hens were incubated as above. On day 3 of incubation the eggs were treated with a single injection of E2, at a dose of 0.1 mg in 0.1 ml of propanediol (PD) solution into the air sac of the egg while control embryos received 0.1 ml PD only. Shell holes were sealed with paraffin and eggs were replaced in the incubator. Embryos and blood were collected individually on days 6 and 8 of incubation.

Determination of Genotype of Chicken Embryos

Genotypic sex was determined according to the method of Uryu et al. (1989). Red blood cells were treated to expose the cellular DNA, and the samples were applied to Hybond$^+$ (Amersham, Inc., UK). A 0.7-kb fragment of W-chromosome-specific DNA (Kodama et al., 1987) was labeled with [α-^{32}P]dCTP (3000 Ci/mmol, ICN Bio-chemicals Inc., Irvine, CA) using a random-primed labeling kit (Boehringer Mannheim, Mannheim, Germany). The radioactive probe was separated from unincorporated [α-^{32}P]dCTP by gel filtration on Sephadex G-50 column and used for the hybridization with a specific activity of 1×10^8 cpm/μl as previously reported (Kato et al., 1995). The accuracy of the technique for determining the sex of embryos was verified using Southern blotting in which males showed no hybridizable signals.

Northern Hybridization

Fertilized eggs from White Leghorn hens were incubated as above and the ovaries or testes were collected from developing embryos at day 10 of incubation. After homogenization of ovaries and testes total RNA was extracted according to the method described by Chomczynski (1993). RNA (20 μg) was fractionated in denaturing agarose gel (1.2%) by electrophoresis and transferred to Hybond-N$^+$ membrane. Chicken AMH cDNA probe (Carre-Eusebe et al., 1996) was ^{32}P-labeled by the random-priming method and hybridization was performed as described (Kato et al., 1995). The membranes were washed at in 1× SSC/0.1% SDS for 15 min at 42°C, and 0.1× SSC/0.1% SDS for 15 min at 60°C twice. The membranes were exposed either to X-ray film (Amersham, Inc., UK) or to the Image Plate and analyzed by BAS 2000 Bio-Imaging Analyzer (Fuji Photo Film Co., Ltd., Japan).

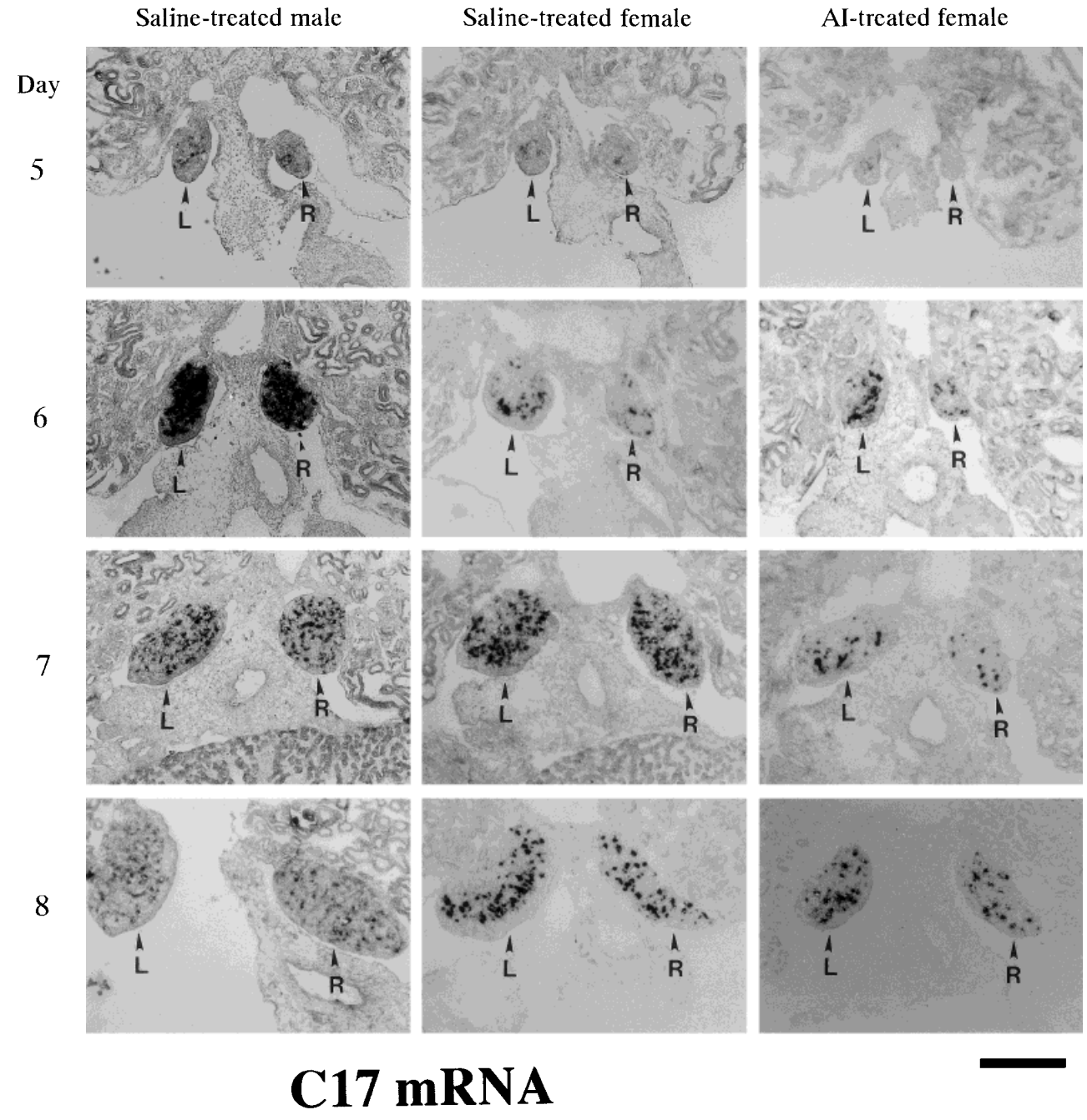

Fig. 1. In situ hybridization of P450c17 mRNA in gonads of saline-treated male and female embryos and AI-treated female embryos on days 5 to 8 of incubation. Transverse sections of the left (L) and right (R) gonads are shown in each photograph. Top and bottom of each photograph indicate dorsal and ventral regions of the tissue, respectively. Scale = 300 μm.

In Situ Hybridization

In situ hybridization with P450c17, P450arom and AMH digoxigenin (DIG)-labeled RNA probes were essentially the same as those described in our previous reports (Yoshida et al., 1996). Briefly, sense and antisense RNA probes were transcribed in vitro from linearized pSPT18, pBluescript SKII(+), and pBluescript KSII(+) plasmid containing chicken P450c17cDNA (Ono et al., 1988), quail P450arom cDNA (Harada et al., 1992) and chicken AMH cDNA (Carre-Eusebe et al., 1996), respectively. Transcription was performed using the RNA DIG-labeling kit (Boehringer Mannheim, Mannheim, Germany). The probes were reduced with base (60 mM Na_2CO_3 and 40 mM $NaHCO_3$) to an average size of 150 nucleotides according to the method described by Cox et al. (1984). The embryos were fixed in 4% paraformaldehyde containing PBS, processed into OCT compound (Miles, Inc., Elkhart, IN), frozen,

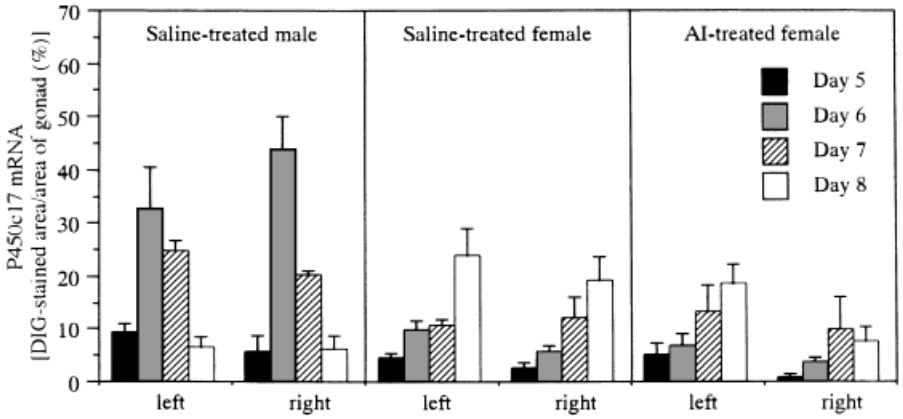

Fig. 2. Changes in P450c17mRNA levels in the section of gonads of embryos treated with saline or AI on days 5 to 8 of incubation. Each datum point is mean ± SEM (n = 3).

and 10 μm sections were cut. The sections were treated with proteinase K (1 mg/ml PBS) at 37°C for 5 min, fixed with 4% paraformaldehyde containing PBS, and hybridized with sense or antisense DIG-labeled cRNA probes overnight at 45°C. On the following day, sections were washed with 50% formamide containing 2× SSC at 45°C and treated with RNase A. DIG-RNA was detected with anti-DIG antibody coupled to alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) and reacted with Nitro blue tetrazolium salt, 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Mannheim, Germany), and levamisole (Sigma, St. Louis, MO).

Statistics

All statistical analyses were done with Statview (Abacus Concepts Inc.) on Macintosh. The size of DIG-stained area was compared using factorial ANOVA and Fisher protected least significant difference test if appropriate ($P < 0.05$; ANOVA).

RESULTS

Figure 1 demonstrates a typical example of in situ hybridization analysis for P450c17 mRNA in the gonads of the saline-injected males and females, and in the AI-treated females of chicken embryos at days 5 to 8 of incubation. Specific signals were first detected in the gonad at day 4 (data not shown) in all the groups. Specific mRNA signals were localized in the medullary cords of both left and right gonads. There was no apparent difference in the distribution pattern of signals among the gonads of any group. There was no specific P450c17 mRNA signal in the gonads of any group when the sense RNA probe was used for in situ hybridization (data not shown).

The relative expression of specific hybridizable signals of P450c17 mRNA to the non-specific background level is shown in Figure 2. Very little expression was observed in the section of the gonad of the control males at day 5 of incubation, but the signal increased abruptly and reached maximum levels at day 6 of incubation. By day 8, however, expression decreased to low levels. The levels at day 6 of incubation were significantly different from those at either day 5 or 8 of incubation ($P < 0.01$). On the other hand, the relative mRNA expression gradually increased between days 5 and 8 of incubation in both left and right gonads of the control females. The levels at day 8 of incubation are significantly different from those at day 5 of incubation. In AI-treated females, the expression of the left gonad showed a similar increase between days 5 and 8 of incubation to that observed in the control female.

Figure 3 illustrates an example of in situ hybridization analysis for P450arom mRNA in the gonads of the control males and females and the AI-treated females of chicken embryos at days 5 to 8 of incubation. No specific P450arom mRNA was detected in any regions of the control male gonad at any days of incubation. In contrast, specific mRNA of P450arom was first detected in gonads in the control females at day 6 of incubation and more intense signals were observed at later days of incubation. Specific mRNA signals were observed in the medullary cords of the gonad, but were not found in the germinal epithelium or the cortical cords of the gonads. In the AI-treated females, weak signals were found in both left and right gonads.

The relative expression of specific hybridizable signals of P450arom mRNA to the non-specific background level is shown in Figure 4. As in the control male gonads at any period examined, the expression of P450arom mRNA in both left and right female gonads was below the limits of detection at day 5 of incubation. The expression markedly increased from that of day 6 of incubation and reached the highest levels at day 8 of incubation in the control female gonad. The increased levels at days 7 and 8 were significantly higher when compared to those at days 5 and 6 of incubation. In the AI-treated females the expression levels of P450arom at day 7 of incubation were significantly higher than those at any other day, but were significantly lower

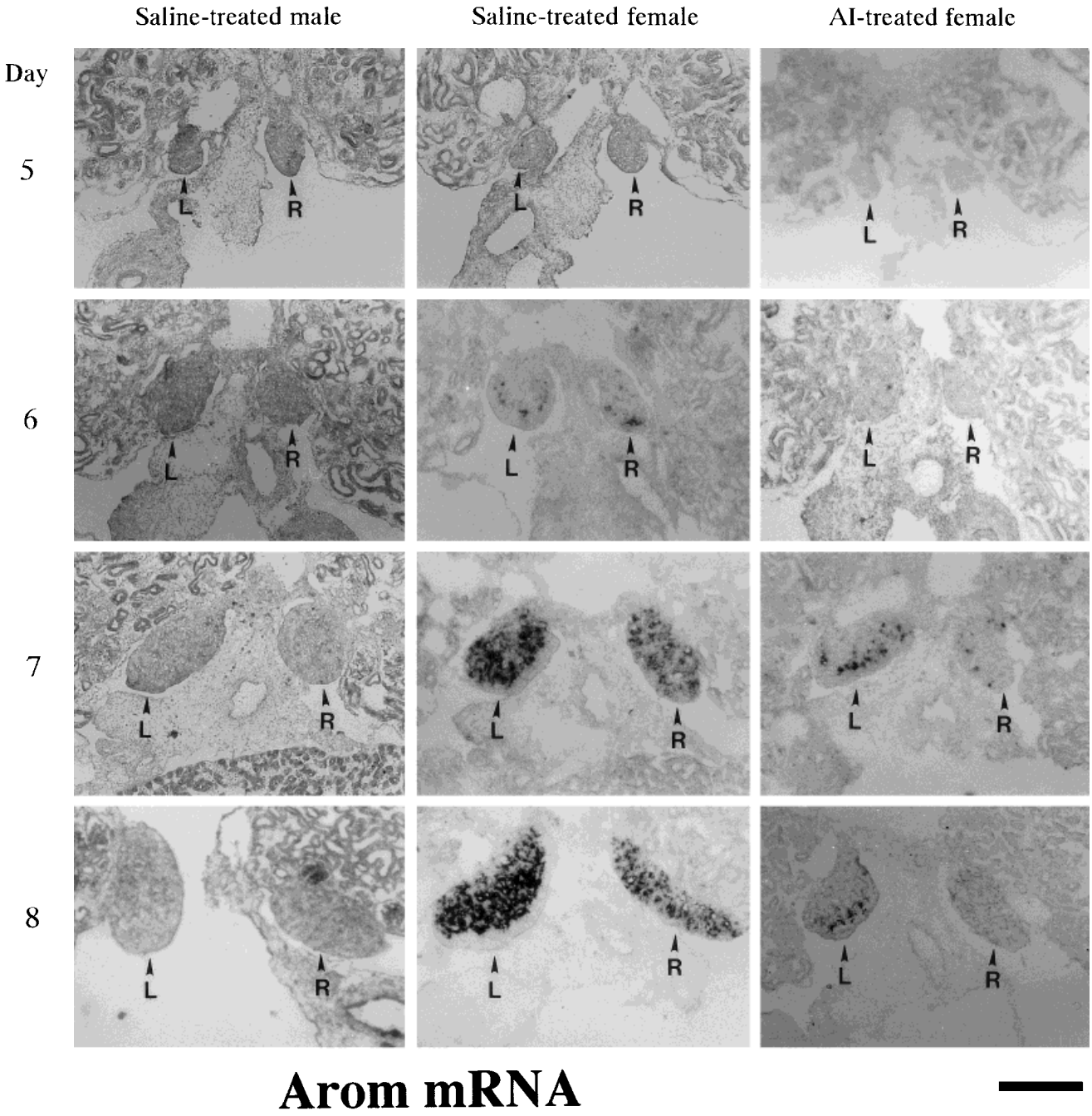

Fig. 3. In situ hybridization of P450arom mRNA in gonads of chicken embryos on days 5 to 8 of incubation. Remainder of legend is the same as Fig. 1.

when compared to those at day 7 in the left gonad of the control female ($P < 0.05$).

Prior to using the chicken AMH probe for in situ analysis, specificity of the cDNA probe was assessed using Northern blot analysis. A representative autoradiogram of a Northern blot of RNA extracted from female and male gonads of chicken at day 10 of incubation is shown in Figure 5. A distinct species of 2.8-kb AMH transcript was shown in the testicular RNA samples (Fig. 5A), whereas no bands were detected in the ovary by the conventional autoradiography using X-ray film exposed for 20 hr (Fig. 5B). However, a distinct species of 2.8-kb transcript of AMH was detected in both right and left ovaries by using the more sensitive BAS 2000 Bio-Imaging Analyzer (Fig. 5C). No AMH mRNA was detected in the liver of the chicken by either methods.

Figure 6 shows an example of in situ hybridization analysis for AMH mRNA in the gonads of the control males and females and the AI-treated females between

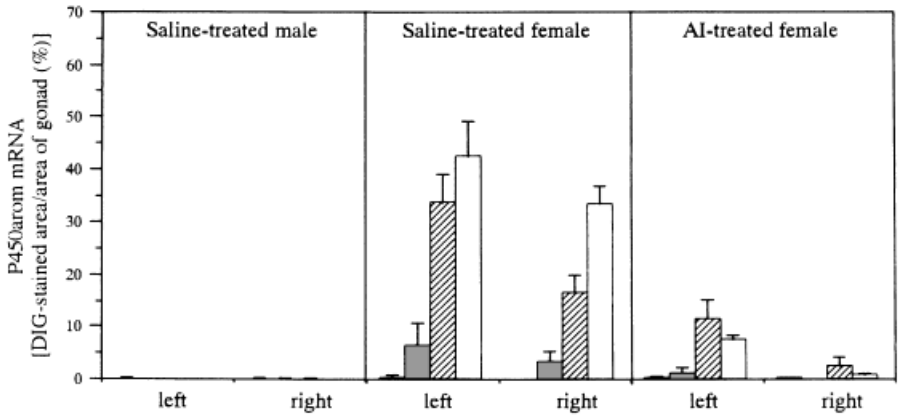

Fig. 4. Changes in P450arom mRNA levels in the section of gonads of embryos treated with saline or AI. Each datum point is mean ± SEM (n = 3).

days 5 and 8 of incubation. In the control males there was no AMH mRNA signal in the gonads at day 4 (data not shown) but signals were first detectable at day 5 and these increased markedly at day 6 of incubation and thereafter. There was no specific mRNA signal of AMH in the gonads of the control females at days 4 (data not shown) and 5, but weak signals were detected at days 6 and 7. In the AI-treated females, there were no AMH mRNA signals at days 4 and 5 but distinct signals were observed after day 6 of incubation. Specific mRNA signals of AMH were observed in the medullary cords of the gonad.

The relative expression of AMH mRNA in the gonads of the three groups are shown in Figure 7. The expression increased rapidly from the low levels of expression at day 5 of incubation to the plateau level at day 6 of incubation both in the left and right gonad of the control males. In contrast, in the control females in the expression remained low throughout the examined period of incubation. In the AI-treated females the expression at days 5 and 6 was low in both left and right gonads but significantly ($P < 0.05$) increased by days 7 and 8 of incubation. Levels were significantly higher in the right gonad than those in the left gonad at the corresponding days, respectively ($P < 0.05$).

Since an inverse relationship was noted in levels of P450arom (Fig. 4) and AMH mRNA (Fig. 7) between AI-treated and control females, as well as between gonads in AI-treated females, levels of these mRNAs were further investigated on day 10 of incubation. The result is shown in Figure 8. Notably, the left gonad of the AI-treated female, the AMH mRNA was located in the dorsal regions, which reside close to the kidney (Fig 8B), whereas more P450arom mRNA was mainly localized to the ventral region (Fig. 8C). The right gonad of the AI-treated females showed AMH mRNA signals throughout the medullary cord (Fig. 8B), whereas levels of P450arom mRNA were very low (Fig. 8C).

In situ hybridization results of the group treated with E2 are shown in Figure 9, whereas data from the PD-treated group are not shown since the results were

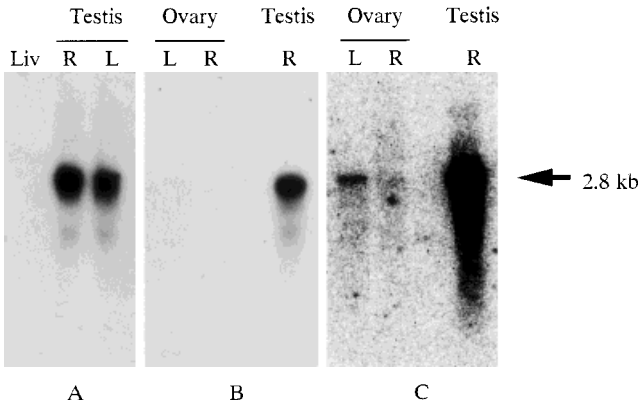

Fig. 5. Northern blot analysis of AMH mRNA in gonads of chicken embryos on day 10 of incubation. (**A**) and (**B**) Exposed for 20 hr. (**C**) BAS-2000 imaging analysis from the B membrane. L, left; R, right; Liv, liver.

not different from saline-treated groups (Figs. 1–4, 6–7). The E2-treated males displayed weak signals of P450 c17 mRNA at days 6 and 8 of incubation. The control male showed no appreciable mRNA signals of P450arom at 6 or 8 days (Fig. 3); the E2-injecteed males showed no signal of P450arom mRNA at day 6 in either gonad but marked signals were observed in the left gonad at day 8 of incubation. The control males showed intensive signals in both left and right gonads for AMH mRNA at days 6 and 8 of incubation (Fig. 6) In the E2-treated males, however, moderate signals were detected at day 6 but almost none were detected at day 8 of incubation.

Figure 10 demonstrates quantitative results of the experiment to reveal effects of the E2 treatment at day 3 of incubation on mRNA expression of P450c17, P450arom and AMH in the gonads at days 6 and 8. Levels of expression of each mRNA in the PD-injected group was observed as in the saline-injected females and males (Figs. 2, 4, and 7), respectively. In the control males, AMH and P450c17 mRNA were expressed at high levels by day 6 but injection of E2 resulted in the

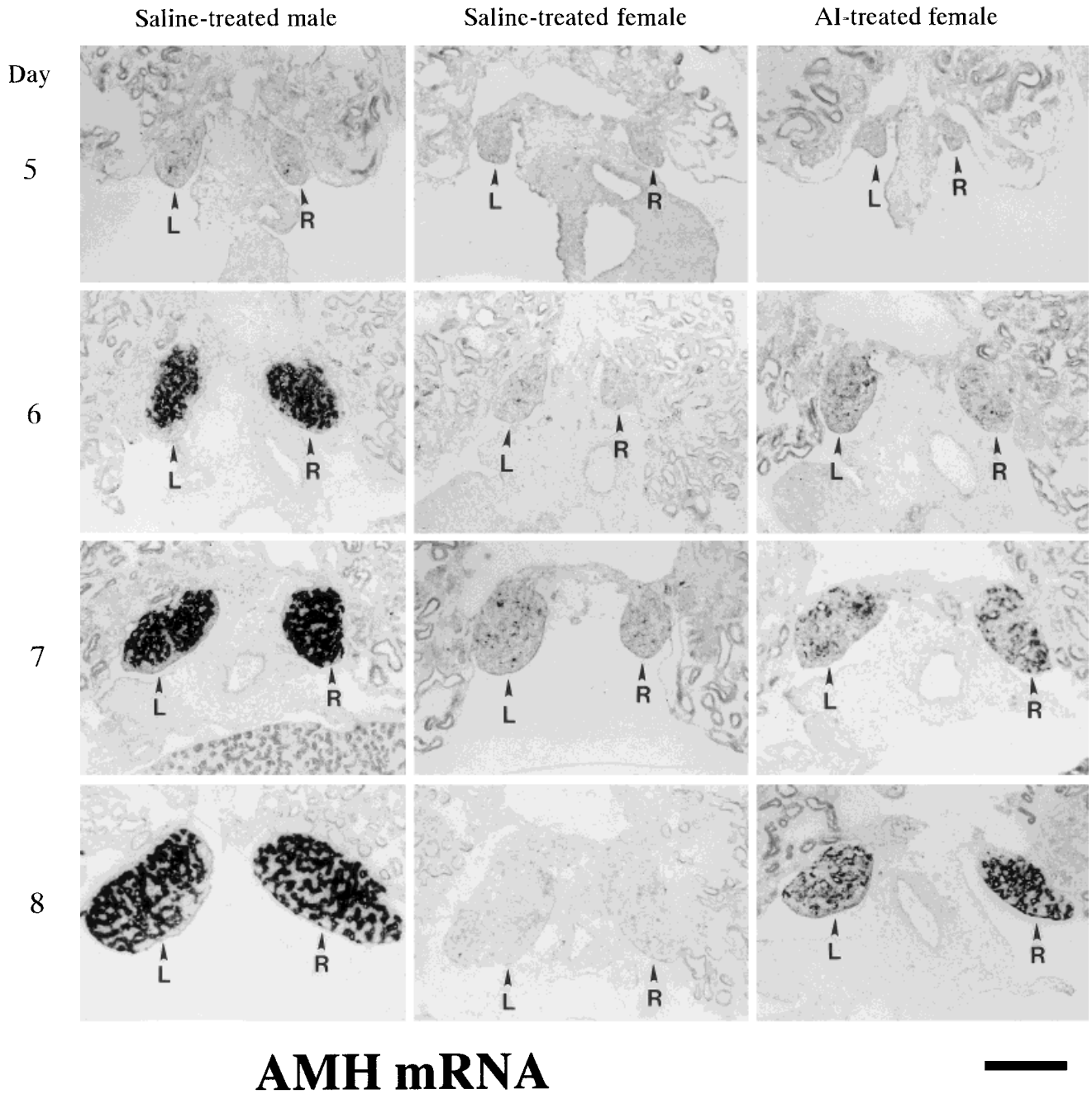

Fig. 6. In situ hybridization of AMH mRNA in the section of gonads of chicken embryos on days of 5–8 of incubation. Remainder of legend is the same as Fig. 1.

suppression of the mRNAs ($P < 0.05$). Conversely, levels of P450arom mRNA increased significantly over the control levels, in response to the injection of E2.

DISCUSSION

The present study clearly demonstrates tissue-specific and sex-specific expression of P450c17, P450arom, and AMH mRNA in the gonads of the chicken during the early phase of sexual differentiation. The localization of P450c17 and P450arom mRNA signals in the embryonic gonads are consistent with our previous results (Yoshida et al., 1996). Namely, both mRNA signals are expressed in the medullary cords but not in the germinal epithelium or in the cortical cords. Yoshida et al. (1996) first detected P450c17 mRNA at days 5–6 of incubation in some of the male and female embryos and P450arom mRNA at day 6.5 of incubation only in the female gonad. The present study found expression of specific mRNA of P450arom as early as day 6 of incubation, which is consistent with the result of RT-

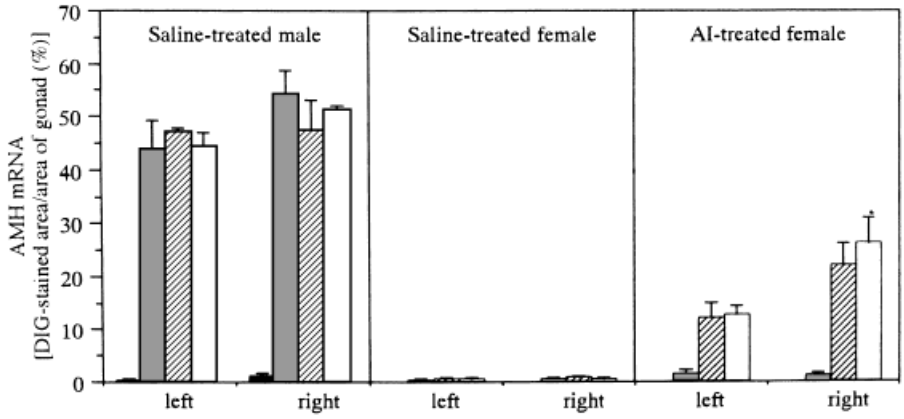

Fig. 7. Changes in AMH mRNA levels in gonads of embryos treated with saline or AI. Each datum point is mean ± SEM (n = 3).

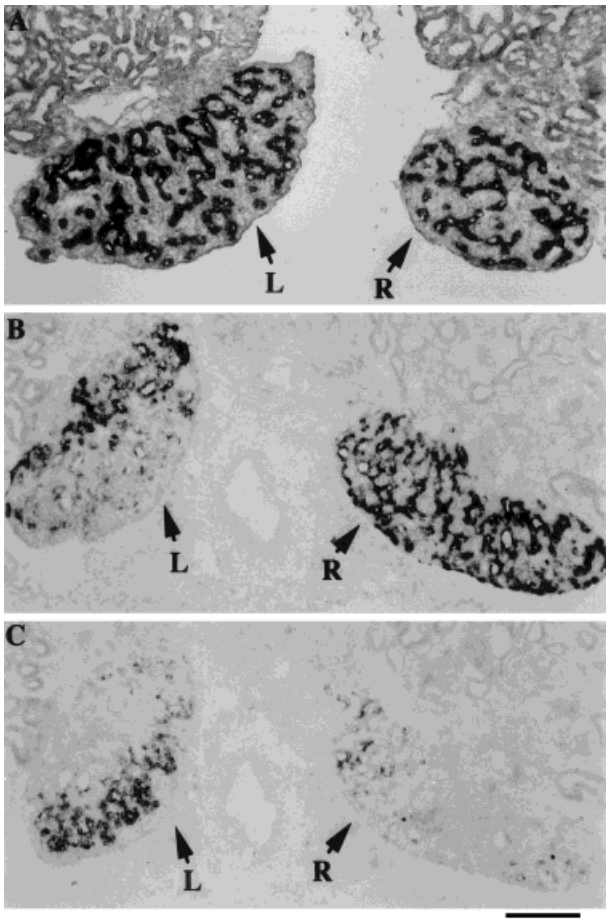

Fig. 8. In situ hybridization of AMH mRNA in gonads of chicken embryos on day 10 of incubation. (**A**) AMH mRNA in saline-treated male gonad. (**B**) AMH mRNA in AI-treated female gonad. (**C**) P450arom mRNA in AI-treated female gonad. Scale = 600 μm. Remainder of legend is the same as Fig. 1.

PCR analysis (Smith et al., 1997; Shimada, 1998). The present study also confirmed the observation of Carre-Eusebe et al. (1996) that expression in AMH mRNA was restricted to the medullary cords of the gonad.

Since the previous reports did not quantify the expression of mRNA for P450c17, P450arom, and AMH in the gonads of the chicken, it was difficult to demonstrate specific changes in expression of each of mRNA during the early incubation period. The quantitative analysis of the present in situ hybridization study revealed several new findings. First, in the normal females the gonadal expression of P450c17 and P450arom mRNA increases gradually during the period from days 5 to 8 of incubation, whereas in the normal males the expression of P450c17 increases sharply at day 6 of incubation but then decreases immediately to low levels by day 8 of incubation. Although we have not assessed de novo synthesis of E2 and testosterone, it is likely that these mRNAs are translated since aromatase activity increases (Imataka et al., 1988) and estrogen rises during this period. Hence, it is likely that this mRNA reflects levels of enzymic activity. Thus, the highest mRNA expression of P450c17 at day 6 of incubation in males may represent the highest production of androgen and thereafter it decreases by day 8 of incubation. However, such a marked but transient change in androgen production is not observed in male gonads, since a continuous increase in androgen concentration has been demonstrated (Woods and Poczaski, 1974). The significance of this transient expression of P450c17 mRNA therefore remains unclear but may represent one of the male-specific phenomena that may be associated with a male-determination mechanism. However, because P450c17 mRNA expression was assessed on the basis of the mRNA levels in a thin section of a portion of the gonad during the examined period, whether or not the mRNA expression increases as a whole throughout the gonad remains uncertain. Since the AI used in this study is a highly specific inhibitor of aromatase, no substantial effect of AI on P450c17 mRNA expression in the female gonad may be exerted (Fig. 2). Female-specific expression of P450arom mRNA is largely abolished by the AI treatment (Fig. 4). Abinawanto et al. (1996) quantified P450arom mRNA levels of the gonads of the chicken embryo at day 18 of

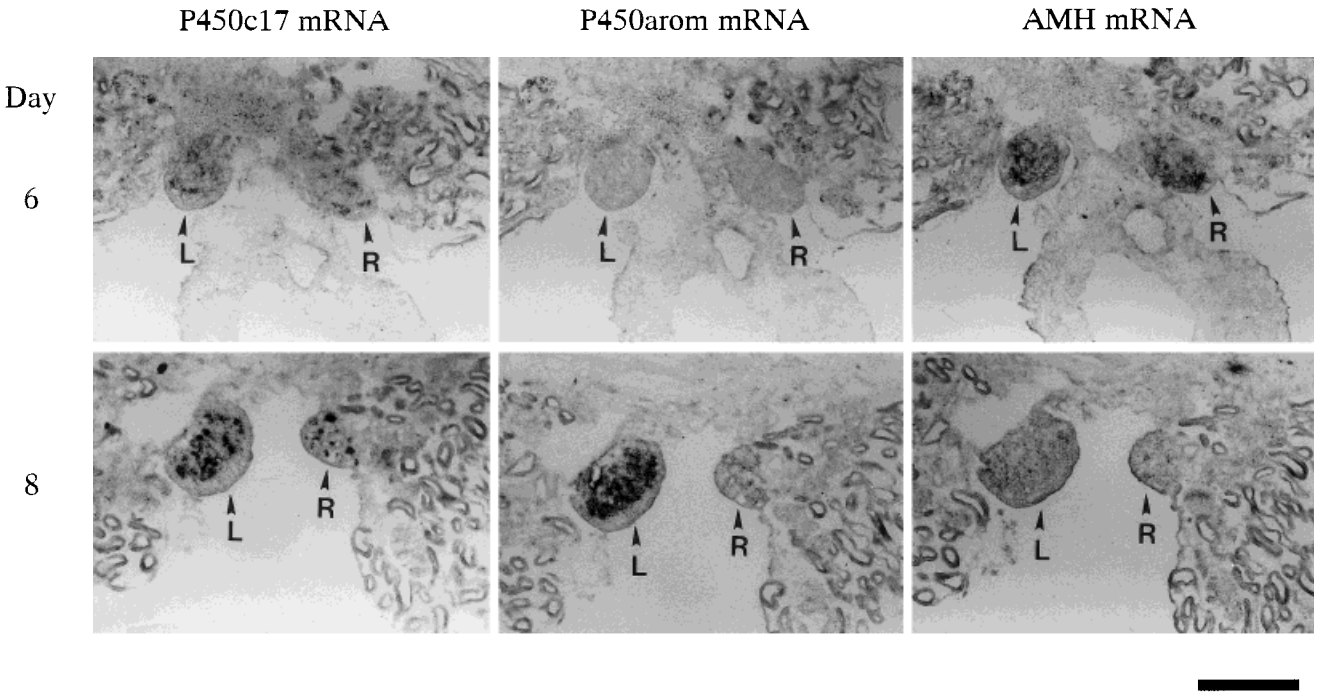

Fig. 9. In situ hybridization of P450c17, P450arom, and AMH mRNA in gonads of propanediol (PD)-treated female and male embryos and E2-treated male embryos on days 6 and 8 of incubation. Remainder of legend is the same as Fig. 1.

incubation after embryos were treated with AI at day 5 of incubation. They showed that levels of P450arom mRNA in the female gonads were lower after the AI treatment. The present study further demonstrated that this lowering effect of AI on P450arom mRNA expression was much more severe in the right gonad than in the left gonad, suggesting that the left gonad posseses a higher capability of P450arom mRNA expression than the right gonad. Scheib et al. (1983) also demonstrated that the right ovary produces more testosterone and less E2 than the left ovary. These observations are in accord with studies which indicate that left ovariectomy will induce masculinization (Etches and Kagami, 1997).

The most interesting result of the present study is the finding of first expression of AMH mRNA in the male gonad at day 5 of incubation followed by the striking increase in levels of mRNA expression at day 6 of incubation. The first expression of AMH mRNA at day 5 of incubation should be noted because AMH is the first signal for development of the male gonad and this gene itself can be the product of the male determining gene, or it can be one of the downstream genes for the male determination mechanism. Recently, DMRT-1, which resides on the Z chromosome, was proposed as a candidate for a testis-determining gene in the chicken (Nanda et al., 1999). Although the chicken AMH has promoter sequences that bind to Sox9, SF-1 and estrogen, binding to DMRT-1 protein has not been studied. The relationship between the expression of DMRT-1 and AMH genes may clarify a possible male-determining mechanism in birds.

The present study also provides another interesting finding that E2 stimulates P450arom mRNA expression but inhibits AMH mRNA expression, suggesting that these genes are expressed inversely in the undifferentiated gonad in the chicken. Whether or not an estrogen responsive element in the chicken AMH promoter is involved in the inhibitory effect of AMH on mRNA expression remains unknown. Estrogen is suggested to play a role in protecting organs from AMH action. If the gonad is sensitive to the action of AMH for only a brief time during the sex differentiation, as in the case of the Müllerian duct (Josso et al., 1977), it is likely that estrogen treatment may result in ovarian formation, although this effect may be transient since sex characteristics tend to return to normal by sexual maturity (Etches and Kagami, 1997).

Avian sexual differentiation clearly involves the complex interaction among a number of factors. In this study, the central role of estrogen in modulating differentiation is underscored. The injection of estrogen into males served to upregulate expression of P450 arom and downregulate both AMH and P450c17, whereas the suppression of estrogen in females increased the expression of AMH and decreased that of P450arom. This is in contrast to mammals, in which gonadal steroids appear to have no role in gonadal differentiation. In chickens, it is likely that estrogen or an estrogen-induced protein(s) has key roles in promoting gonadal differentiation once

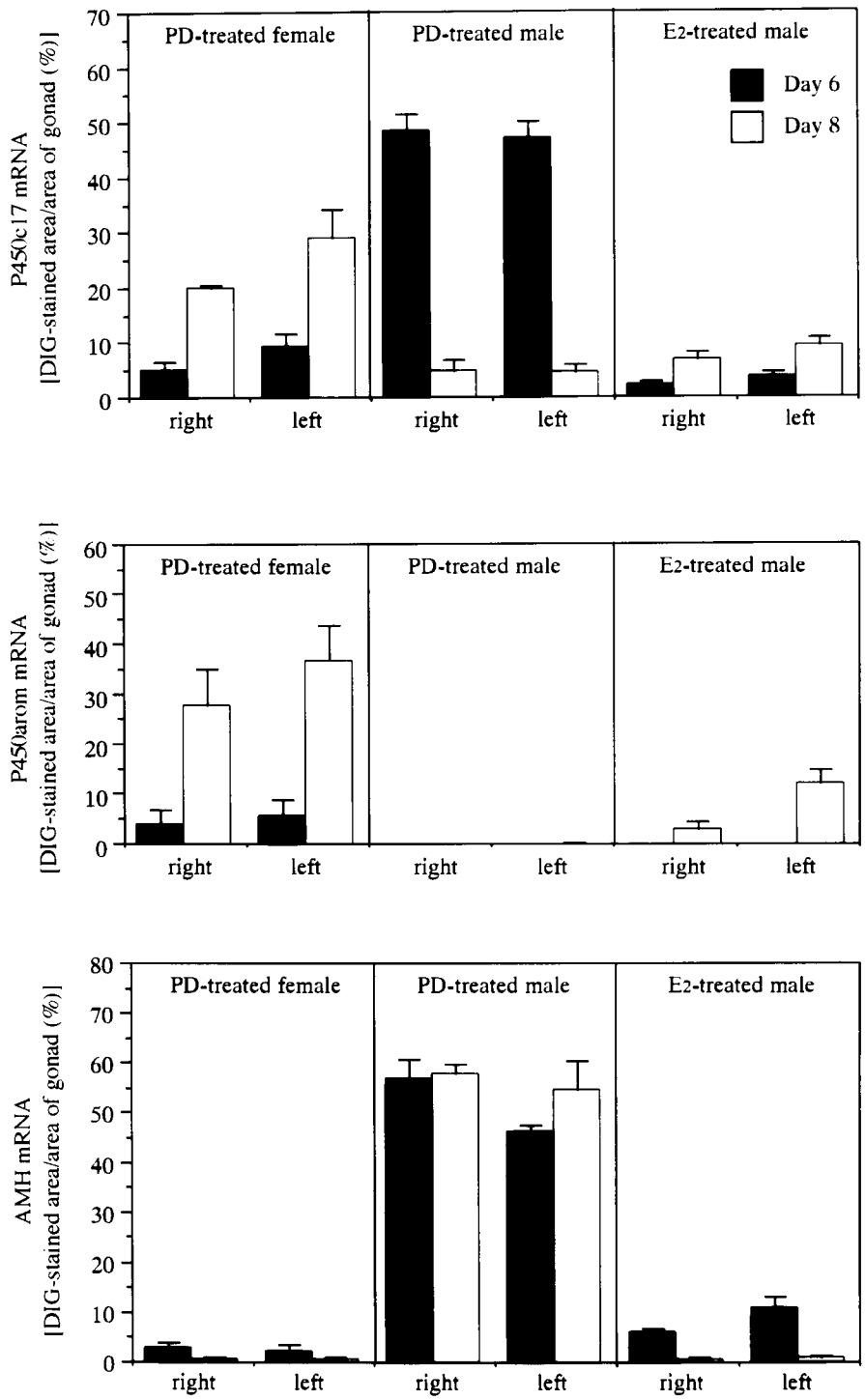

Fig. 10. Changes in P450c17, P450arom, and AMH mRNA levels in the section of gonads of propanediol (PD)-treated female and male embryos and E2-treated male embryo. Each datum point is mean ± SEM (n = 3).

the primary genetic signal has been expressed. The latter may involve the DM domain (DMRT1) which is present on the Z chromosome and is proposed to act in a dose dependent fashion to cause testicular differentiation (Nanda et al., 1999). Since males are ZZ, this may explain why estrogen treatment only causes transient sex reversal in males, whereas suppression of estrogen in females (ZW) causes permanent reversal. The rela-

tionship amongst DMRT1, other recognized genes in the gonadal differentiation pathway and estrogen warrants further investigation.

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