Journal of Herpetology, Vol. 33, No. 1, pp. 149–152, 1999

Müllerian Duct Development and Regression in a Turtle with Temperature-dependent Sex Determination

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The development and regression of the Müllerian ducts represent major events in the sexual development of vertebrates (van Tienhoven, 1983). Müllerian ducts develop in both male and female embryos. In females the Müllerian ducts become the oviducts, whereas in male vertebrates they regress. Studies in mammals and in the chicken indicate that the regression is stimulated by Müllerian inhibiting substance (i.e., MIS, also called anti-Müllerian hormone or AMH) produced by the embryonic testis (Tran and Josso, 1977; Josso, 1986; Donahoe et al., 1987; Josso et al., 1993; Lee and Donahoe, 1993; Behringer et al., 1994).

In reptiles, Müllerian duct development and regression has been described in a number of species (reviewed by Fox, 1977; Raynaud and Pieau, 1985; also see Austin, 1988, 1989, 1995). The Müllerian duct consists of a ring of epithelial cells surrounded by a stroma of mesenchymal cells. Some variation has been reported in the regression of the Müllerian ducts among reptiles. In the alligator, Müllerian duct regression appears similar to that in the chicken and in mammals, in which a distinct "epithelial cuff" forms, and consists of a swirl of condensed mesenchymal cells surrounding the epithelial cells (Austin, 1989; 1995). In contrast, the mesenchymal cells of the stroma in other reptiles appear to shrink in size without forming a cuff (Austin, 1988).

Although the MIS gene has not been cloned in reptiles, data from a number of past studies are consistent with the hypothesis that a testis-specific hormone (presumably MIS) stimulates Müllerian duct regression: (1) Müllerian duct regression occurs soon after testicular differentiation (reviewed by Fox, 1977; Raynaud and Pieau, 1985), (2) testicular grafts in alligators stimulate Müllerian duct regression (Austin, 1989) and (3) MIS cDNA has been cloned from embryonic testes of a turtle (Wibbels and LeBoeuf, 1997; Wibbels et al., 1998).

In the current study, Müllerian duct development and regression is histologically examined in the red-eared slider, *Trachemys scripta*. The study is specifically designed to provide a detailed morphology and chronology of Müllerian duct regression that can be used to effectively design future studies addressing the physiology underlying this event.

The red-eared slider turtle, *T. scripta*, was chosen for this study because it represents a useful system for examining sex determination and sex differentiation in reptiles (Wibbels et al., 1994, 1998). In particular, its sex determination and gonadal differentiation have been well-documented in previous studies (Crews et al., 1991; Wibbels et al., 1991a, b, 1993, 1994, 1998; Wibbels and Crews, 1992, 1995) and its eggs are commercially available in large numbers. Freshly laid eggs were obtained commercially (R. Kliebert, Hammond, Louisiana). After transport to the laboratory, they were placed in containers with moistened vermiculite (vermiculite : water, 1:2). Previous studies indicated that a continuous incubation temperature of 31 C produces all female hatchlings whereas an incubation temperature of 26 C produces all male hatchlings (Bull et al., 1982; Crews et al., 1991; Wibbels et al., 1991a). Eggs were assigned randomly to either a female producing (31 C) or a male-producing (26 C) temperature. Embryonic development was monitored by candling eggs and by dissecting two to four eggs approximately twice a week to verify specific developmental stages, based on criteria described for the snapping turtle, *Chelydra serpentina* (Yntema, 1968).

To evaluate Müllerian duct development and regression, eggs were dissected at stages 18, 19, 20, 21, 22, 23, and 26 at both male- and female-producing temperatures (a minimum of five eggs per stage per temperature). A complex of tissue which included the kidneys (both mesonephros and metanephros), gonads, and adrenals was dissected from each embryo and was fixed in Bouin's preservative. Fixed tissues were infiltrated with paraffin, sectioned at 6–8 μm, and stained with hematoxylin and eosin (Humason, 1972).

The portion of the Müllerian duct adjacent to the gonad (i.e., in the same cross-sections as the gonad) was used for the histological analysis. This region was chosen since it represents an optimal region for use in future studies utilizing Müllerian duct regression as a bioassay for MIS activity. Specifically, it is closest portion of the Müllerian ducts to the gonads (i.e., the source of MIS) and it is easily removable along with the mesonephros for in vitro studies. Additionally, the gonads provide a reference point in histological cross sections when attempting to compare Müllerian ducts from different embryos.

Consistent with previous studies of reptiles, the Müllerian ducts in *T. scripta* formed in a cranial to caudal direction with Müllerian duct formation reaching the level of the gonad at approximately stages 18 to 19. Figure 1 shows cross sections of typical Müllerian ducts (in the region adjacent to the gonads) from various stages of embryonic development at male-producing and female-producing temperatures. Once formed, the Müllerian ducts consisted of a ring of epithelial cells surrounded by a stroma containing mesenchymal cells (see Fig. 1E, F). The ring of epithelial tissue formed from within an accumulation of coelomic epithelium (Fig. 1A, B) referred to as the Müllerian crest (Raynaud and Pieau, 1985) which was near the Wolffian duct. In the region adjacent to the gonad, this ring of epithelial cells formed between stage 18 and 19 (Fig. 1C, D) and was then surrounded by a stroma containing mesenchymal cells (Fig. 1E, F). The formation and development of the Müllerian duct was similar at both the male- and the female-producing

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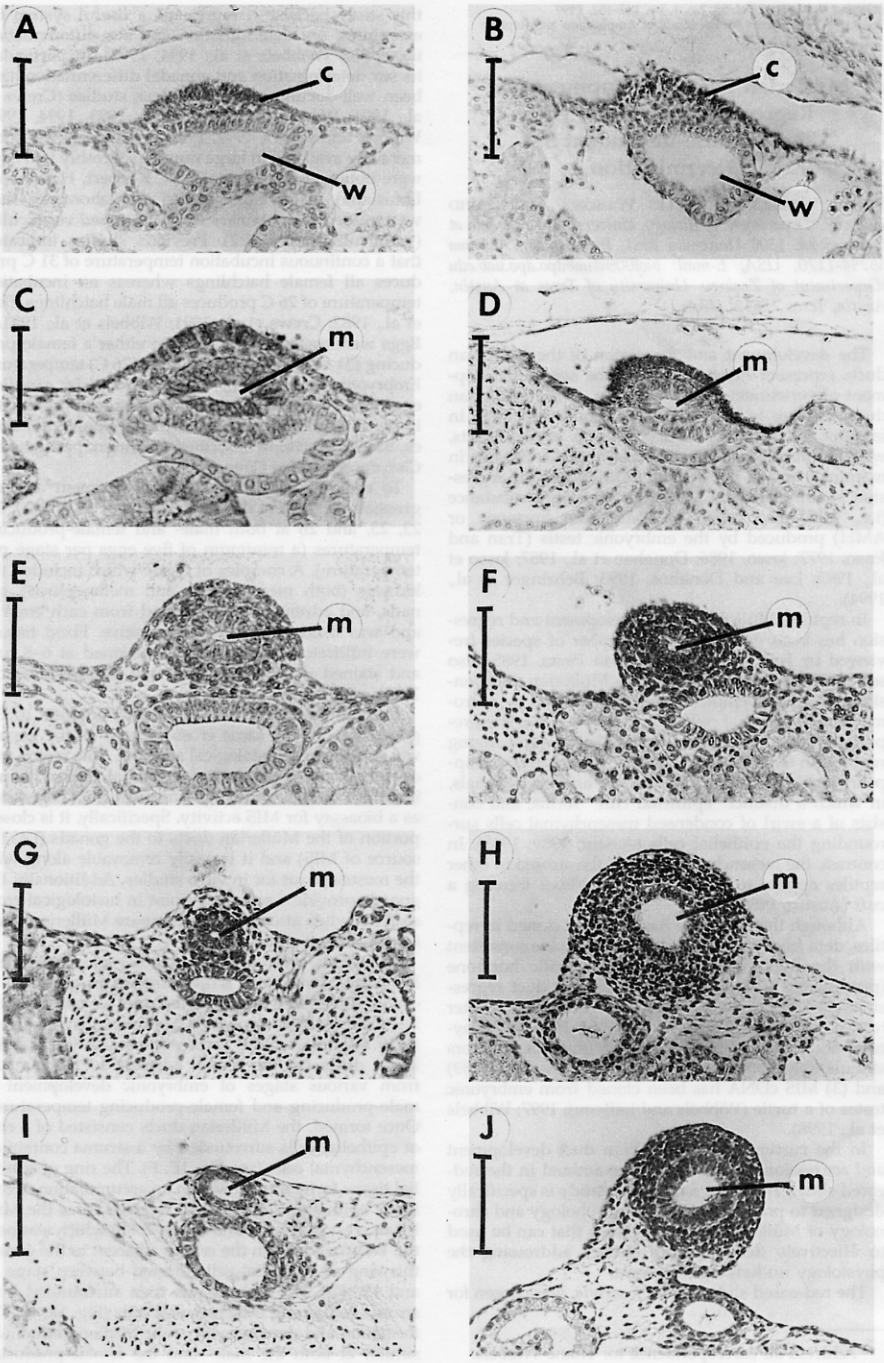
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temperatures through approximately stage 20–21 (Fig. 1A–F). In males, Müllerian duct regression was noticeable in some embryos at stage 22 and was obvious in all embryos examined at stage 23 (Fig. 1G, I). During Müllerian duct regression there was a loss of mesenchymal cells in the stroma, and the ring of epithelial cells shrank (Fig. 1G, I). During this same time period in the female, both the stroma of mesenchymal cells and the ring of epithelial cells continue to proliferate (Fig. 1H, J). By hatching (i.e., stage 26) the Müllerian ducts were absent in males, whereas in females the oviducts were distinct.

During the current study, testicular differentiation (based on histology) was detectable by approximately stage 19 to 20. This is consistent with a previous study of gonadal differentiation in *T. scripta* using the same incubation temperatures (for 1991a). Thus, Müllerian duct regression occurred shortly after the sexual differentiation of the testes.

The results indicate that the morphology of Müllerian duct development in *T. scripta* is similar to that described for other reptiles (Raynaud and Pieau, 1985) with the duct arising within a zone of coelomic epithelium covering the embryonic kidney (Fig. 1). The region of the Müllerian duct adjacent to the gonad begins to form at approximately stage 18 to 19, a time period during which the gonads are just beginning to sexually differentiate (Wibbels et al., 1991a). In male embryos, the Müllerian ducts begin to regress at approximately stage 22 and regression is obvious by stage 23. During regression, there is a gradual loss of mesenchymal cells in the stroma surrounding the ring of epithelial cells. However, an "epithelial cuff" such as that described for the alligator (Austin, 1989, 1995) does not form in *T. scripta*.

The chronology of Müllerian duct regression indicates that it occurs within several embryonic stages after testicular differentiation becomes noticeable. These results are similar to those reported in previous studies of reptiles showing a temporal sequence in which Müllerian duct regression occurs soon after testicular differentiation (reviewed by Fox, 1977; Raynaud and Pieau, 1985; also see Austin, 1988, 1989). These findings are consistent with the hypothesis that the reptilian testis produces a regressor hormone (presumably MIS) soon after it differentiates.

The results of current study provide a foundation for effectively designing studies addressing the physiology underlying Müllerian duct regression in *T. scripta*. For example, these data provide the basis for developing an organ culture bioassay system in *T. scripta* for investigating the physiology of Müllerian duct regression. Based on the current results, the mesonephros/Müllerian duct complex can be dissected from stage 19 to 21 embryos (i.e. after the Müllerian ducts have formed but before they have regressed) and placed into short term culture in a similar fashion to studies of Müllerian duct regression in the mammals (Picon, 1969; Donahoe et al., 1977). The development of such a bioassay system is a prerequisite for investigating the putative role of MIS during Müllerian duct regression.

Acknowledgments.—This work is a result of research sponsored in part by the National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under Grant NA56RG0129 from the Mississippi-Alabama Sea Grant Consortium. We would like to acknowledge the technical assistance of David Lee.

Literature Cited

Austin, H. B. 1988. Differentiation and development of the reproductive system in the iguanid lizard, *Sceloporus undulatus*. Gen. Comp. Endocrinol. 72: 351–363.

———. 1989. Müllerian-duct regression in the American alligator (*Alligator mississippiensis*): Its morphology and testicular reduction. J. Exp. Zool. 251: 329–338.

———. 1995. Dil Analysis of cell migration during Müllerian duct regression. Dev. Biol. 169:29–36.

Bull, J. J., R. C. Vogt, and C. J. McCoy. 1982. Sex determining temperatures in turtles: a geographic comparison. Evolution 36:326–332.

Behringher, R. R., M. J. Finegold, and R. Cate. 1994. Müllerian inhibiting substance function during mammalian sexual development. Cell 79:415–425.

Crews, D., J. J. Bull, and T. Wibbels. 1991. Estrogen and sex reversal in turtles: a dose-dependent phenomenon. Gen. Comp. Endocrinol. 81:357–364.

Donahoe, P. K., R. L. Cate, D. T. MacLaughlin, J. Epstein, A. F. Fuller, M. Takahashi, J. P. Coughlin, E. G. Ninfa, and L. A. Taylor. 1987. Müllerian inhibiting substance: Gene structure and mechanism of action in a fetal regressor. Recent Prog. Horm. Res. 43:431–467.

Donahoe, P. K., Y. Ito, S. Marfatia, and W. H. Hendren. 1977. A graded organ culture assay for the detection of Müllerian inhibiting substance. J. Surg. Res. 23:141–148.

Fox, H. 1977. The urogenital system of reptiles. *In* C. Gans and T. S. Parsons (eds.), Biology of the Reptilia, Vol. 6, pp. 1–157. Academic Press, New York.

Humason, G. L. 1972. Animal Tissue Techniques. W. H. Freeman, San Francisco.

Josso, N. 1986. Anti-Müllerian hormone: new perspectives for a sexist hormone. Endocrine Rev. 7: 421–433.

Josso, N., R. L. Cate, J.-Y. Picard, B. Vigier, N. Di Clemente, C. Wilson, S. Imbeaud, R. B. Pepinsky, D. Buerrier, L. Boussin, L. Legeai, and D. Car-

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Fig. 1. Müllerian duct differentiation and regression in the red-eared slider, *Trachemys scripta*. Cross sections of Müllerian ducts and Wolffian ducts from the region of the mesonephros near the gonad are shown from male-producing temperature (26 C) in A, C, E, G, and I, and female-producing temperature (31 C) in B, D, F, H, J. Embryonic stage 18 (A and B), stage 19 (C and D), stage 21 (E and F), stage 22 (G and H), and stage 23 (I and J) are shown. Embryonic staging was based on criteria from Yntema, 1968. c = Müllerian crest, m = müllerian duct, w = Wolffian duct, scale bar on left of each photo represents 50 μm.

RE'-EUSEBE. 1993. Anti-Müllerian hormone: the Jost factor. Recent. Prog. Horm. Res. 8:379–413.

LEE, M. M., AND P. K. DONAHOE. 1993. Müllerian inhibiting substance: a gonadal hormone with multiple functions. Endocrine Rev. 14:152–164.

PICON, R. 1969. Action du testicule foetal sur le development in vitro des canaux de Müller ches let rat. Arch. Anat. Micros. Morphol. Exp. 58:1–19.

RAYNAUD, A., AND C. PIEAU. 1985. Embryonic development of the genital system. *In* C. Gans and F. Billet (eds.), Biology of the Reptilia, Vol. 15, pp. 149–300. Wiley, New York.

TRAN, D., AND N. JOSSO. 1977. Relationship between avian and mammalian anti-Müllerian hormone. Biol. Reprod. 16:267–273.

VAN TIENHOVEN, A. 1983. Reproductive Physiology of Vertebrates. Cornell Univ. Press, Ithaca, New York.

WIBBELS, T., AND D. CREWS. 1992. Specificity of steroid hormone-induced sex determination in a turtle. J. Endocrinol. 133:121–129.

———, AND ———. 1995. Steroid-induced sex determination at incubation temperatures producing mixed sex ratios in a turtle with TSD. Gen. Comp. Endocrinol. 100:53–63.

———, J. J. BULL, AND D. CREWS. 1991a. Chronology and morphology of temperature-dependent sex determination. J. Exp. Zool. 260:71–381.

———, ———, AND ———. 1991b. Synergism of temperature and estradiol: A common pathway in sex determination? J. Exp. Zool. 260:130–134.

———, P. GIDEON, J. J. BULL, AND D. CREWS. 1993. Estrogen- and temperature-induced medullary cord regression during gonadal differentiation in a turtle. Differentiation 53:149–154.

———, J. J. BULL, AND D. CREWS. 1994. Temperature-dependent sex determination: A mechanistic approach. J. Exp. Zool. 270:71–78.

———, J. COWAN, AND R. LEBOEUF. 1998. Temperature-dependent sex determination in the red-eared slider turtle, *Trachemys scripta*. J. Exp. Zool. 281: 409–416.

———, AND R. D. LEBOEUF. 1997. Cloning of a cDNA for Müllerian inhibiting substance in a turtle. Amer. Zool. 177A.

YNTEMA, C. L. 1968. A series of stages in the embryonic development of *Chelydra serpentina*. J. Morphol. 125:219–252.

Accepted: 14 October 1998.