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# Sex Determination in Reptiles: An Update[1]

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SYNOPSIS. **Sex determination and sex differentiation are two separate but related phenomena. Sex differentiation is a programmed cascade of events in which the indifferent gonad develops as a testis or an ovary with the appropriate urogenital and secondary sex characters. Sex determination is the event that sets this cascade in motion. In placental mammals, there is good evidence that sex is determined by a gene on the Y chromosome (SRY) that initiates testis formation. In the absence of SRY an ovary develops. There are, however, examples of placental mammals that develop as normal males with no detectable SRY. In reptiles, sex differentiation appears to be similar to mammals (*i.e.*, the same genes and hormones act in a similar manner), but sex determination is clearly very different. Ovarian differentiation in placental mammals can occur in the absence of estrogen or an estrogen receptor. Ovarian differentiation in reptiles requires the presence of estrogen. In the absence of estrogen a testis develops. In TSD reptiles, embryos will develop as females when treated with estrogen even if eggs are incubated at male-inducing temperatures, and conversely, will develop as males when estrogen synthesis is blocked in eggs incubated at female-inducing temperatures. A number of other genes have also been shown to be important in mammalian sex determination. One of these genes, Sox9, which is expressed in differentiating mouse testis, has recently been found to be expressed in embryonic reptile testis. Other genes that appear to be common to both mammals and reptiles in the sex determining cascade are SF-1, MIH, and possibly DAX-1. Current research is now focused on how the gene that produces the enzyme necessary for estrogen synthesis (aromatase) is regulated in the embryos of reptiles with genetic or environmental sex determination. Controversial issues in reptilian sex determination are 1) the role of the brain in gonadal sex determination, and 2) the role of steroid hormones in the yolk prior to sex determination.**

## INTRODUCTION

*Sex determination* and *sex differentiation* are two separate but related phenomena. Sex differentiation is a programmed cascade of genetic and hormonal events in which the indifferent gonad develops as a testis or an ovary with the appropriate urogenital and secondary sex characters. Sex determination is the event that sets this cascade in motion. It has been assumed that the mammalian model of sex determination in which a male phenotype is imposed on the neutral or default female phenotype applied to all vertebrates. However, there is now evidence strongly suggesting that sex determination in reptiles and other egg-laying vertebrates may be fundamentally different from the mammalian system in that a female phenotype is imposed on the neutral, or default male phenotype.

## MAMMALIAN SEX DETERMINATION

In placental mammals sex is determined by the action of a gene on the Y chromosome that initiates testis formation. This gene, SRY in humans, Sry in mice has been shown to be present on the short arm of the Y chromosome in most placental mammals examined, including marsupials (Foster *et al.*, 1992), but, despite considerable effort, it has not been detected in the DNA of the egg-laying mammals, echidna and platypus (J. M. Graves, personal communication). What is more confusing is that it is also

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absent from two species of vole, *Ellobius lutescens* and *E. tancrei*, but it is present in *E. fuscocapillus* and all other closely related rodents tested (Just *et al.*, 1995). In the six years since the publication of the structure of SRY (Gubbay et al., 1990; Sinclair *et al.*, 1990) there has been an intense research effort on the function of this gene, but as yet it is still unclear as to what it does (Capel, 1996; Ramkissoon and Goodfellow, 1996). In the absence of SRY an ovary develops. The unusual variation in structure of SRY among mammals, and the differing pattern of its expression in different species (see Harry *et al.*, 1995, for example) have compounded the problem. In egg-laying vertebrates there is no evidence for a sex-specific SRY. However, attempts to find SRY in egg-laying vertebrates (Coriat *et al.*, 1993; Spotila *et al.*, 1994) have uncovered an entire family of genes known as SOX genes (SRY-related HMG box containing genes) that code for protein with a DNA binding motif (Laudet *et al.*, 1993). There are probably more than 20 genes in this family with wide tissue expression during development (Prior and Walter, 1996). It has been suggested that the mammalian SRY evolved from a SOX3 gene present on the marsupial X chromosome, and presumably on the primitive Y chromosome (Foster and Graves, 1994). The role of SOX3 in sex determination, however, is still obscure. Another member of this family, SOX9, is more firmly established as having a role in sex determination. Mutation in SOX9 results in campomelic displasia and XY sex reversal in humans (Muscatelli *et al.* 1994: Zanaria *et al.*, 1994; Foster *et al.*, 1994). Although Sox9 has been shown to be principally involved in chondrogenesis in mice (Wright *et al.*, 1995) it was recently shown to be expressed in embryonic gonadal tissue of both chicken and mouse and appears to be involved in testis differentiation in both species (Morais da Silva et al., 1996). It is also expressed in the gonadal tissue of male turtle embryos (L. Spotila, personal communication). The role of Sox9 in testis determination thus appears to be strongly conserved as it has now been shown to be expressed in the embryonic testes of all amniotes studied.

Work from the laboratories of Keith Parker and Ken-ichiri Morohashi has shown that steroidogenic factor 1 (SF-1), also known as Ad4BP, an orphan nuclear receptor with a zinc finger DNA binding domain, the mammalian homologue of fushi tarazu factor 1 (FTZ-F1) from the fruit fly (Lala *et al.*, 1992), is a key regulator of steroidogenic enzyme gene expression (Lynch *et al.* 1993; Morohashi *et al.*, 1993). This nuclear protein of 52,000 mw binds to the promoter region of all steroidogenic P450 genes including aromatase and has been shown to act as a transcriptional activator. SF-1 has also been shown to be important in MIS expression (Shen, W-H. *et al.*, 1994), and pituitary expression of LHβ, FSHβ, and the receptor for gonadotropin releasing hormone (Ingraham *et al*, 1994).

In rats SF-1 was first detected in the primordial adrenal glands and testes of the 13.5 day pc fetus, but only trace amounts were detected in the fetal ovaries (Hatano *et al.*, 1994). When the gene for this factor was disrupted in mice, the embryos developed without gonads or adrenal glands and died shortly after birth (Luo *et al.*, 1994). Clearly this factor is a key determinant of gonadal development. However, given the widespread tissue distribution of SF-1 and its multiple roles in steroidogenesis and pituitary function it is obvious that other factors must also be involved.

Another recently discovered gene, DAX-1, a member of the nuclear hormone receptor superfamily, is also involved in gonadal development (Zanaria *et al.*, 1994; Muscatelli *et al.*, 1994). This gene has been mapped to the short arm of the human X chromosome, and when this region is duplicated results in XY sex reversal. Mutations in DAX-1 result in X-linked adrenal hypoplasia congenita (Muscatelli et al., 1994). DAX-1 shows a similar distribution to SF-1 and is transcribed in fetal gonads and in adult testis, ovary and adrenal (Zanaria *et al.*, 1994; Swain *et al.*, 1996; Tamai *et al.*, 1996). Swain *et al.* (1996) suggest that DAX-1 is required for ovarian but not testis differentiation. It is very likely that these two transcription factor genes (SF-1 and DAX-1) are also important in bird and reptile gonad differentiation. There is a

TABLE 1. *Phylogenetic occurrence of genes known to be involved in mammalian sex determination.*

| Gene | Animal |
|---|---|
| SRY | Placental mammals |
| SOX-9 | Mammals, birds, reptiles |
| SF-1 | Mammals, reptiles, fish |
| DAX-1 | Mammals, reptiles |
| MIH | Mammals, birds, reptiles |

DAX-like gene expressed in alligator ovary (Lance, unpublished). A list of these genes and their occurrence is given in Table 1. While genes similar to these mammalian genes have now been identified in reptiles, tissue-specific expression during gonadal differentiation remains to be demonstrated.

Although some mammalian fetal ovaries are capable of synthesizing estrogen, female sex differentiation in mammals occurs normally in the absence of estrogen. A human female with a mutation in the aromatase gene and thus unable to synthesize estrogen had normal ovarian development at 17 months, but exhibited infantile genitalia and polycystic ovaries at age 18 (Ito *et al.*, 1993). A gene knockout experiment in the mouse in which a non-functioning estrogen receptor was introduced into the germline resulted in female mice that were totally unresponsive to estrogen, yet the gonad apparently differentiated normally as an ovary, but as in the human aromatase deficiency case, exhibited cystic follicles (Lubhan *et al.*, 1993). Further evidence comes from a recent study on the ontogeny of steroidogenic enzyme gene expression in the mouse ovary. This research showed that three key steroidogenic enzymes, including aromatase were not expressed until after birth, again suggesting that estrogen is not necessary for ovarian differentiation or development (Greco and Payne, 1994). In reptiles on the other hand there is now abundant evidence that ovarian differentiation requires the presence of estrogen.

## REPTILE SEX DETERMINATION

The small amount of information available on the endocrinology of sex determination and sex differentiation in reptiles comes almost entirely from studies on species that exhibit temperature dependent sex determination (TSD), and of these, only one or two species of turtles and the American alligator have been studied in any detail. This review will therefore focus mainly on sex determination in reptiles with TSD. In 1992 a symposium entitled "Environmental Sex Determination in Reptiles" was held at the annual meeting of the American Society of Zoologists in Vancouver (Lance, 1994). For detailed reviews the interested reader is directed to the twelve papers covering the process of sex determination in squamates, chelonia and crocodylia (Lance, 1994). Other recent reviews may found in Crews, 1994, 1996; Crews *et al.*, 1994; Johnston *et al.*, 1995; Pieau, 1996; Pieau *et al.*, 1994). It was apparent at the time that the mechanism of TSD was far from clear. One common theme however, was that estrogen played a significant role in sex determination in reptiles with TSD. Today we are still unclear as to how temperature ultimately effects the sex of the embryo, but the argument for the role of estrogen appears even stronger.

The model of Jost (1953) in which maleness is imposed on the neutral, or default, female phenotype probably does not apply to egg-laying vertebrates. There is evidence in birds, for example, that femaleness is imposed on the default or neutral, male phenotype (Graves and Foster, 1994). Sex determination in birds and reptiles may depend upon the initiation of estrogen synthesis in the indifferent gonad which inhibits male differentiation and stimulates ovarian development. In the absence of this estrogenic signal a testis develops.

## ROLE OF ESTROGEN IN REPTILIAN SEX DETERMINATION

Steroid hormones have been thought to play a role in vertebrate sex determination since the 1930s (see Lance and Bogart, 1992; McCarrey and Abbot, 1979; Merchant-Larios, 1978 for references), but it is only within the last 20 years that steroids have again been implicated in sex determination, at least in the egg-laying vertebrates. It has been demonstrated many times over in a number of different reptile species that application of estrogens, or even estrogenic xenobiotics, to reptile em-

bryos will cause the embryo to develop as a female, whatever the incubation temperature (Crews, 1994, 1996; Crews *et al.*, 1994; Lance and Bogart, 1992, 1994; Pieau, 1996; Wibbels and Crews, 1992; Wibbels *et al.*, 1994). In turtles, estrogen was effective only when applied during the period of gonadal differentiation, stages 10 to 19 (Gutzke and Chymiy, 1988), whereas in alligators estrogen was effective in inducing ovarian development at male incubation temperatures when applied as early as day seven of development (Lance and Bogart, 1992). If the temperature at which turtle eggs are incubated is brought one or two degrees closer to the pivotal temperature (*i.e.,* an incubation temperature that would result in 50% males and 50% females), but still a temperature which would produce mostly males, less estrogen is required to induce all the embryos to develop as females than at a more extreme temperature (Wibbels *et al.*, 1991). This result has been interpreted as showing synergism between temperature and hormone, or that temperature and estrogen are physiologically equivalent (Crews, 1996). Similarly, turtle eggs at pivotal temperatures produced a preponderance of males when treated with a non-aromatizable androgen, dihydrotestosterone (Wibbels and Crews, 1995). Dihydrotestosterone however, has no effect when given at a temperature leading to 100% female hatchlings in both turtles and alligators. While there is generally good agreement among studies regarding the application of estrogen to reptile embryos, the use of antiestrogens has produced some unusual results. When turtle embryos (*Emys orbicularis*) at a female producing incubation temperatures were injected with the antiestrogen, tamoxifen, a partial masculinization of the ovary was noted (Dorizzi *et al.*, 1991). Similar results were reported in bird embryos (Scheib and Baulieau, 1981). This result was interpreted as the inability of endogenous estrogen to induce ovarian differentiation when the estrogen receptors were blocked by tamoxifen. However, similar experiments in embryos of the turtle, *Trachemys scripta* failed to demonstrate any masculinizing effect of tamoxifen (Wibbels and Crews, 1992). In a series of experiments with alligator embryos tamoxifen showed no antiestrogenic activity, but, paradoxically, was as potent as estradiol in inducing ovarian development in embryos at male inducing temperatures (Lance and Bogart, 1991, 1992, 1994).

In reptiles (and birds) sex *differentiation* appears to be similar to mammals (*i.e.,* the same genes and hormones probably act in a similar manner), but sex *determination* may be very different. Ovarian differentiation in mammals can proceed in the absence of estrogen (see above), whereas in egg-laying vertebrates ovarian differentiation cannot proceed in the absence of estrogen. In both birds and reptiles the synthesis of estrogen in the developing embryo appears to play an central role in sex differentiation, whereas in mammals it is clearly unimportant. Simply blocking estrogen synthesis in female chick embryos by injecting an aromatase inhibitor resulted in phenotypically male hatchlings that were capable of spermatogenesis when sexually mature (Elbrecht and Smith, 1992). Similar results have been found in turtles with temperature-dependent sex determination (TSD). Eggs incubated at a temperature known to produce female hatchlings produced male hatchlings when treated with aromatase inhibitors (Dorizzi *et al.*, 1994; Rhen and Lang, 1994; Richard-Mercier *et al.*, 1995). An even more remarkable finding was that it is possible to produce male lizards in a normally all-female parthenogenetic species simply by treating the eggs with an aromatase inhibitor (Wibbels and Crews, 1994).

The source of estrogen in the reptile embryo however, is still controversial. Attempts to measure estrogen in reptile embryos have produced mixed results. Dorizzi *et al.* (1991) have shown higher estrogen levels in gonadal tissue of female European pond turtle (*Emys orbicularis*) embryos than in male turtle embryos, whereas White and Thomas (1992) were unable to show any differences in plasma or whole-body estrogen levels in male and female red-eared slider turtle (*Trachemys scripta*) embryos. Lance and Bogart (1994) were unable to show any difference in plasma estradiol levels in male and female alligator em-

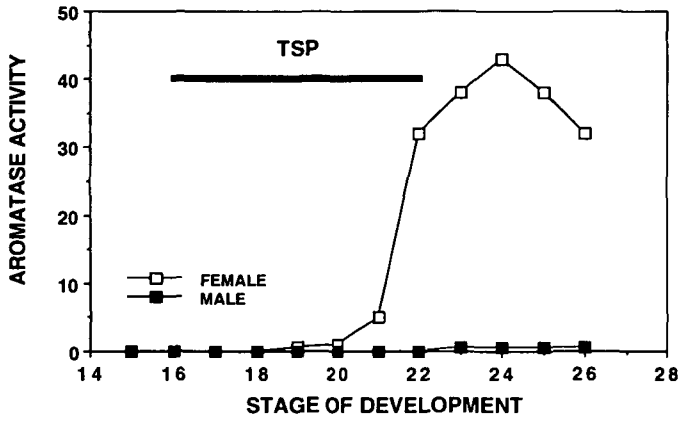


FIG. 1. Aromatase activity in the gonads of male and female turtles during the temperature sensitive period (TSP) of incubation. Units are arbitrarily derived from Desvages and Pieau (1992) and Desvages *et al.* (1993). Aromatase activity is first detectable only in female embryos during the period of gonadal differentiation.

bryos, and there was no clear elevation in estrogen during the period of sex determination.

If estrogen is capable of inducing a female phenotype in an embryo at a male-inducing incubation temperature it is more than likely that estrogen receptors are expressed. Crews (1996) has reported cloning the genes for estrogen and androgen receptors from the turtle *Trachemys scripta*, but as yet the stage and tissue specific expression of these receptors in embryonic gonads remains speculative.

## STUDIES ON AROMATASE

The gene for aromatase has been cloned from several mammalian species (see Simpson *et al.*, 1994), chicken (McPhaul *et al.*, 1988), zebra finch (Shen *et al.*, 1994), alligator and turtle (Pancharatnam *et al.*, 1996; Crews, 1996) and three species of teleost fish (Tanaka *et al.*, 1992; Trant, 1994; Chang *et al.*, 1997), and the regulation of its tissue specific expression in mammals has been the subject of considerable research (Simpson *et al.*, 1994). At least four promoters have been identified in the human aromatase gene, including SF-1, some of which are responsible for tissue-specific expression of the gene (Simpson *et al.*, 1994).

It has been proposed that it is a female-inducing incubation temperature during the TSP that initiates aromatase activity in the indifferent gonad and causes ovarian differentiation of reptile embryos (Pieau *et al.*, 1994; Pieau, 1996). Whether it is simply the activation of the aromatase gene or a gene or genes acting upstream from aromatase that is the initial trigger of the sex determining cascade remains to be determined. Aromatase activity in the gonads of two species of turtle, *Emys orbicularis*, and *Dermochelys coriacea* peaks in female embryos at the time of sex differentiation (Desvages and Pieau, 1992; Desvages *et al.*, 1993), see Figure 1, whereas in alligator and crocodile embryonic gonads (Smith and Joss, 1994; Smith *et al.*, 1995) aromatase activity is not detected until after gonadal differentiation (Fig. 2). Molecular studies on aromatase have confirmed the turtle pattern. In diamondback terrapin (*Malaclemys terrapin*) embryos there is significantly more aromatase mRNA in the adrenal-kidney-gonadal complex (AKG) of females than of males during the period of gonadal differentiation (Pancharatnam and Place, 1997).

## SOURCE OF SUBSTRATE FOR AROMATASE

Desvages and Pieau (1991) incubated embryonic turtle gonadal tissue from eggs incubated at male or female producing temperatures with radioactive pregnenolone, progesterone, DHEA and androstenedione. A large number of metabolites were isolated, but only trace amounts of estradiol and

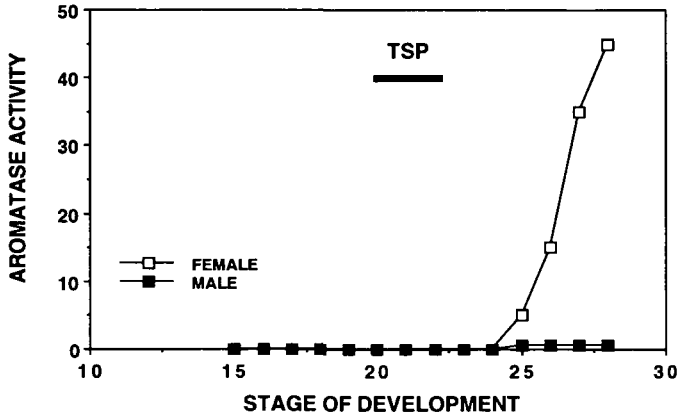


FIG. 2. Aromatase activity in the gonad/mesonephros/kidney complex of alligator and crocodile during the TSP. Note that aromatase activity is undetectable until *after* the period of gonadal differentiation, and only in female embryos, and that activity continues to increase until hatching. Figure derived from Smith and Joss (1994) and Smith *et al.* (1995)

estrone. Similar results were obtained by White and Thomas (1992) using radioactive pregnenolone and turtle AKG. They suggested that the kidney-adrenal and not the gonad is the source of steroids during sex differentiation. Histochemical studies have also failed to detect any activity of the enzyme 3β-hydroxysteroid dehydrogenease (3βHSD) in embryonic turtle gonads, but positive results were seen in embryonic adrenal tissue (Merchant-Larios *et al.*, 1989; Thomas *et al.*, 1992). Joss (1989) reported positive 3βHSD activity in gonads of male alligator embryos, but negative or weak activity in gonads of female embryos. Smith and Joss (1994), however, were able to detect 3βHSD activity only in the adrenal tissue of crocodile embryos.

## THE PROBLEM OF STEROIDS IN THE YOLK

Conley *et al.* (1997) have shown that alligator yolk has very high concentrations of sex steroids. Androstenedione, testosterone and estradiol were estimated as high as 20 ng per gram of yolk prior to the period of gonadal differentiation. During the TSP, when gonadal differentiation takes place these steroids virtually disappear from the yolk. What is confusing however, is that a similar drop in steroid concentration occurs at both male and female temperatures, and at pivotal temperatures (Fig. 3). The ultimate sex of the embryo bears no relation to the steroid concentration in the yolk. A similar pattern is seen in the yolk of turtles (J. Lang, personal communication). The authors did note that there was a significant clutch effect, *e.g.*, concentrations of estradiol in the yolk from one clutch could be four times higher than in another clutch. They suggested these differences could be the cause of the different sex ratios between clutches seen at pivotal temperatures. They further suggest that estrogen from the yolk initiates aromatase in the indifferent gonad and that the androstendione and testosterone are a source of substrate for the gonadal aromatase. However, a mechanism to account for this controlled movement of steroids from the yolk without swamping the effect of temperature, and the rapid elimination of large amounts of steroids from the yolk during the period of gonadogenesis needs to be addressed.

## IS THE BRAIN THE SENSOR OF TEMPERATURE

In an ultrastructural study of the embryonic gonad of the sea turtle, *Lepidochelys olivacea* Merchant-Larios *et al.* (1989) found that there were nerve terminals in the undifferentiated gonad. Nerve terminals are not present in early, undifferentiated mammalian gonads. The question is does the brain sense temperature and send signals via neurons to the gonad? Work from the same laboratory (Merchant-Larios, personal

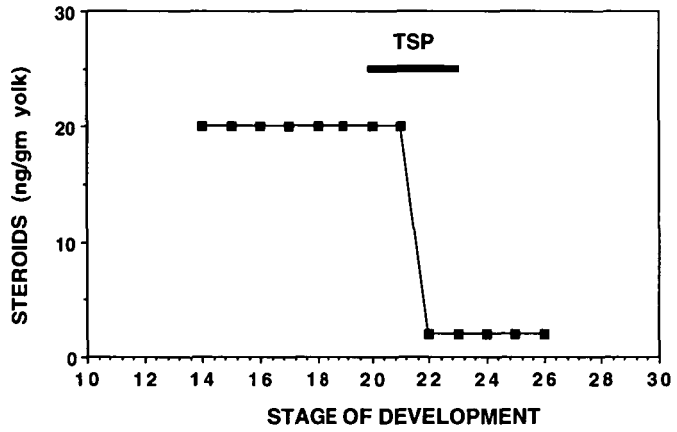


FIG. 3. Disappearance of steroids from the yolk of alligator embryos during the period of gonadal differentiation. Data taken from Conley *et al.* (1997).

communication) has shown that there is measurable estrogen in the brain of embryos at female-inducing incubation temperature just prior to gonadal sex differentiation, and very little estrogen in brains of embryos from male-inducing temperatures. A somewhat similar picture has been shown by Pancharatnam and Place (in press) who were able to measure aromatase mRNA in the brain of embryos prior to the period of sex determination in the turtle (*Malaclemys terrapin*). These results suggest that unlike the mammalian system where sex determination is independent of the brain and pituitary, the reptilian system may have input from the CNS. However, in studies on lizards with genetic sex determination, embryos continued to grow and gonads differentiated normally after decapitation of the embryos (Raynaud and Pieau, 1985). The authors interpreted these results as showing that sex differentiation is independent of the hypothalamus/pituitary. However, input from the nervous system in TSD reptiles cannot at present be excluded. An intriguing possibility first suggested by George and Ojeda (1987) is that VIP (vasoactive intestinal polypeptide) containing nerve fibers stimulate aromatase activity in immature rat ovaries. Their conclusion however, was based on *in vitro* experiments in which VIP was added to cultures of immature rat ovary. The nature of the fibers innervating embryonic turtle gonads remains to be demonstrated.

## CONCLUSIONS

Temperature-dependent sex determination in reptiles remains an enigma. An explanation for how both low and high incubation temperatures can produce females, and an intermediate temperature produce males is still beyond testable models. We know that a number of the genes (with the exception of SRY) involved in mammalian sex determination occur in reptiles, but we do not know how and where they act. What we do know is that estrogen is essential for an ovary to develop, and that in the absence of an estrogenic signal the indifferent gonad will develop as a testis. Regulation of the aromatase gene is clearly involved, but how and when is still not known. The presence of large amounts of steroid hormones in the yolk prior to sex differentiation and the subsequent disappearance of these steroids at the time of sex differentiation also needs further investigation. There are some interesting new data suggesting that the CNS senses incubation temperature and thus directs the indifferent gonad to develop as a testis or an ovary, but proof of this contention is a long way off.

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