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Transcription of the sex-determining region genes *Sry* and *Zfy* in the mouse preimplantation embryo

(sexual dimorphism/testes/reverse-transcription polymerase chain reaction)

THERESA ZWINGMAN, ROBERT P. ERICKSON*, TIMOTHY BOYER, AND ASANGLA AO

Steele Memorial Children's Research Center, Departments of Pediatrics and Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85724

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ABSTRACT **We have confirmed the faster growth of male preimplantation mouse embryos. We have also studied the transcription of Y chromosomal genes postulated to have a role in sex determination, using the highly sensitive technique of reverse-transcription polymerase chain reaction at these early stages. We find that two sex-determining region genes, *Sry* and *Zfy*, are transcribed during mouse preimplantation development, while the *Zfy* homologs *Zfx* and *Zfa* and a sex-determining region gene originally called *A1s9* (now called *Ube1y-1*) are not. We also show that the anti-Müllerian hormone gene, which contains a *Sry* consensus binding element in its 5′ promoter region, is not transcribed at this time. Developmental curves show that *Sry* and *Zfy* are expressed commencing at the two-cell stage. These results suggest that mammalian sex determination starts prior to gonad differentiation.**

The recent cloning of a sex-related Y gene, *SRY* in humans (1) and *Sry* in mice (2), is an exciting advance for studies of sex determination. The finding that 40,XY female mice (3) had a deletion in this gene (2) and the identification of point mutations in this gene in some human 46,XY females (4) were highly supportive of the postulated role of this gene in sex determination. However, the most convincing proof has been the demonstration of sex reversal in some 40,XX mice transgenic for the mouse genomic clone (5). Studies on expression of *Sry* have focused on its transcription in presumptive Sertoli cells in the gonadal ridge at the time of gonadal differentiation—i.e., 11.5 days (6). These authors used the W^e mutation to show that the transcription at that time did not require germ cells. The earliest stage studied in this report was the embryonic stage at 7.5 days postcoitus, and no transcription was found until 11.5 days (6). We find much earlier transcription—as early as the two-cell stage.

There had been previous evidence of differences in gene expression between male and female embryos. Although it was somewhat controversial at first, it is now well established that the male-specific antigen (MSA, serological H-Y) is detectable on preimplantation embryos (7, 8). It is clear that a "growth factor" on the Y chromosome is expressed early, since embryos separated as to early blastulation (fast developers) versus late blastulation (slow developers) are predominantly male in the first group (9). We have now confirmed this effect for the preimplantation mouse embryo by a method not requiring embryo transfer, whereas others have shown that it persists to midgestation (10, 11) and also occurs in Bovidae (12, 13). *Sry* or *Zfy* (Y chromosome-linked zinc finger protein) transcription may be related to these differences.

MATERIALS AND METHODS

Chemicals. Pregnant mare serum gonadotropin, human chorionic gonadotropin (hCG), and most chemicals were from Sigma. The Micro-FastTrack mRNA isolation kit was from Invitrogen (San Diego), the reverse-transcription system and "fmol" DNA sequencing systems were from Promega, and the PCR purification spin kit was from Quiagen (Studio City, CA).

Animals. Animals were from The Jackson Laboratory or were bred from their stock in our colony. Twenty-one-day-old (C57BL/6J × SJL/J)F_1 female mice were superovulated by interaperitoneal injections of 5 units of pregnant mare serum gonadotropin followed by 5 units of human chorionic gonadotropin 48 hr later and then were mated with F_1 males of the same cross. The time of coitus and fertilization was assumed to be at midnight after mating.

Rate of Development of Male Versus Female Embryos. Embryos were flushed from the oviducts on day 1 and cultured *in vitro*. They were classified as to early, intermediate, and late development by the time of the appearance of the blastocoele cavity. Single embryos were then lysed by boiling in water, and polymerase chain reactions (PCRs) were performed to detect the *Zfy* gene as described below. Single embryos could readily be detected as positive or negative.

Reverse-Transcription PCR (RT-PCR). Poly(A)$^+$ mRNA was isolated from 280 late two-cell-stage, 155 and 95 four- to eight-cell-stage (separate preparations), 83 and 115 morula-stage, and 180, 135, and 260 blastocyst-stage embryos by using the Micro-FastTrack mRNA isolation kit. The RNA was treated with RNase-free DNase for 20 min at 37°C. RNA (1 μg) was added to a reverse-transcription reaction (reverse-transcription system). Subsequent PCR contained ≈0.1 μg of reverse-transcribed cDNA in a 100-μl reaction mixture containing 50 mM KCl, 10 mM Tris·HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% gelatin (wt/vol), 1% Triton X-100, 200 μmol of each dNTP, 2.5 units of *Taq* polymerase, and 0.5 μM of each primer. Each reaction was topped with 50 μl of light mineral oil. Amplification for *Zfy*, *Sry*, and *Hprt* consisted of 33–38 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min in a Techne PHC-1 thermocycler. An extension time of 10 min was added at the end of the final cycle. Nine microliters of the final sample was electrophoresed on an ethidium bromide-stained 3% NuSieve (FMC BioProducts) 1% agarose gel. Primers for *Zfy* were 5′-CTCCTGATGGACAAACTTTACGTCTC and 3′-GCTGAGCCTCTTTGGTATCTGAGAAA. Primers for *Sry* (Fig. 1) were 5′-GAGAGCATGGAGGGCCATG and 3′-GAGTACAGGTGTGCAGCTC. Additional *Sry* primers (Fig. 2) were 5′-CTCTGAAGAAGAGACAAGTT and 3′-CTGTGTAGGATCTTCAATC.

Blastocyst samples tested with the gene encoding anti-Müllerian hormone (AMH) were amplified for 38 cycles at 95°C for 1.3 min, 56°C for 1.3 min, and 72°C for 1.3 min. The primers for the AMH gene were 5′-GGCTCTGATTCCCGCTGTT and 3′-GCCAGTTGCGTGTTCGAAG. They pro-

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Abbreviations: AMH, anti-Müllerian hormone; RT-PCR, reverse-transcription polymerase chain reaction.

*To whom reprint requests should be addressed.

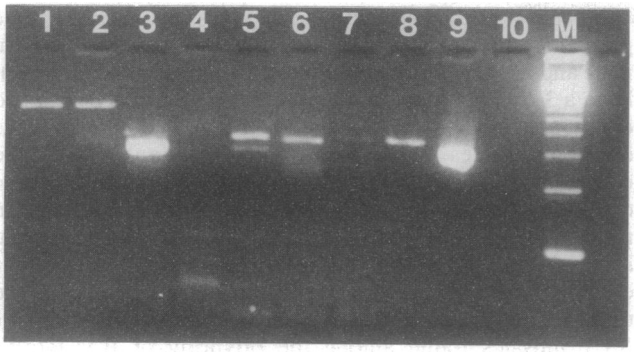

FIG. 1. Analysis of *Sry* and *Zfy* transcription in mouse blastocysts with RT–PCR. RT was performed on RNA from a pooled sample of female and male embryos with and without reverse transcriptase. Oligonucleotide primers for hypoxanthine phosphoribosyl transferase (*Hprt* PCR product of 352 bp) were included in each analysis as a control for the presence of cDNA (14). Lanes: 1 and 2, replicate PCR samples of blastocysts with reverse transcriptase and *Zfy* primers; 3 and 9, samples with reverse transcriptase and *Hprt* primers; 4 and 10, samples without reverse transcriptase but with *Zfy* and *Sry* primers, respectively, as negative controls for DNA contamination of the reverse transcriptase sample; 5–8, serial dilution PCR samples of blastocyst with reverse transcriptase and *Sry* primers (first set); M, DNA markers (615, 492, 369, 246, 123 bp; Boehringer Mannheim). *Zfy* PCR product was 617 bp; *Sry* PCR product was 380 bp. The band fainter in 4 than in 8 reflects PCR variability.

duce a fragment 235 base pairs (bp) long (335 bp if amplified from genomic DNA; ref. 15). *Zfx* and *Zfa* samples were amplified for 30 cycles at 94°C for 30 sec, 53°C for 1.0 min, and 72°C for 1.0 min. The primers for *Zfa* were 5′-CTCACTGCTGTGAGCACTGCA and 3′-TACCTTGATGGACCAGCTAAC (16). The primers for Zfx were 5′-CTCACCGCTGTGAGTACTGCA and 3′-TACCTTGATGGTACTCTTGTA (17). The primers bracket a target sequence 421 and 419 bp long, respectively. The primers for the mouse gene originally called *A1s9* [now called *Ube1y-1*; analogous to human *UBE1* encoding the ubiquitin-activating enzyme E1 (A1s9T temperature-sensitivity complementing)] were 5′-TGATGCCCTTGAATGTCTCC and 3′-CTTCTCTTGTAGGTCTGATCC and produced a fragment 123 bp long

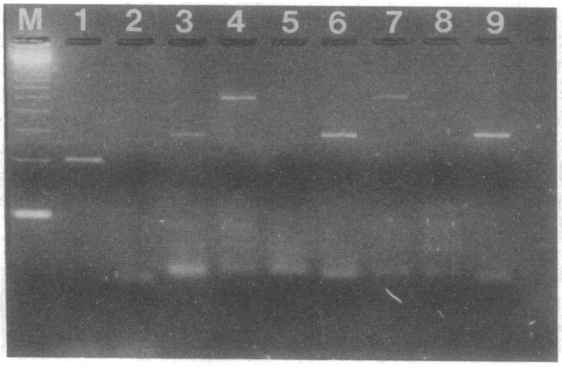

FIG. 2. Analysis of late two-cell and four- to eight-cell embryos for *Zfy* and *Sry* transcription. Abbreviations are as in Fig. 1. Lanes: 1, two-cell embryos with reverse transcriptase and *Sry* primers; 2, sample without reverse transcriptase but with *Sry* primers; 3 and 6, two-cell embryos with reverse transcriptase and *Hprt* primers (14); 4, two-cell embryos with reverse transcriptase and *Zfy* primers; 5, two-cell embryos without reverse transcriptase but with *Zfy* primers; 7 and 8, four- to eight-cell embryos amplified with *Zfy* primers with and without reverse transcriptase, respectively; 9, four- to eight-cell embryos with reverse transcriptase and *Hprt* primers; M, DNA markers as in Fig. 1. *Zfy* PCR product was 617 bp; *Sry* PCR product was 246 bp. Faint bands at the electrophoretic front are free primers.

(18). Blastocyst samples tested with *A1s9* were amplified for 35 cycles at 95°C for 1.0 min, 55°C for 1.0 min, and 72°C for 1.0 min. All PCR reactions had a final extension at 72°C for 10 min. Nine microliters of the PCR product was run on a 10% nondenaturing polyacrylamide gel and subsequentially stained with a silver nitrate solution as described (19).

PCR Sequencing. *Sry* PCR product from the two-cell-stage embryo was reamplified by using the conditions described in Fig. 2 and purified with the PCR purification spin kit. Sequencing was completed with the "fmol" DNA sequencing system.

RESULTS

Confirmation of Growth Factor Y by PCR. When embryos were classified as early, intermediate, or slow developers on the basis of the time of appearance of the blastocoele cavity (Table 1), we found an effect that was statistically significant (but not as marked as previously reported) when superovulated embryos were studied in this manner. Thus, we extended this study with naturally ovulated embryos. In this circumstance, the effect was much more marked. We believe that the difference between the superovulated and naturally ovulated embryos probably has to do with slight changes in the condition of the embryos induced by superovulation. Nonetheless, these results clearly confirm earlier results (9) by a direct method not depending on embryo transfer to the oviducts of pseudopregnant females.

Transcription of *Sry* and *Zfy* in Blastocysts. Reverse transcription polymerase chain reaction was used initially to search for *Sry* and *Zfy* transcription at the blastocyst stage. Embryos were collected and pooled (only half were male). RNA was prepared as described, and cDNA was synthesized. The PCR results showed that both *Sry* and *Zfy* are transcribed in blastocysts (Fig. 1), which strongly implies expression. Inasmuch as *Sry* belongs to a conserved gene family (2) and there was a small possibility that some member of this family could amplify with our primers, we performed RT-PCR with two different primer pairs. In both cases, the product of the correct size was found (Figs. 1 and 2). Thus, it is very unlikely that a related gene rather than *Sry* was being detected. To further strengthen this conclusion, we performed PCR sequencing of the RT-PCR product. The results confirmed the identity of the transcribed product to *Sry* over an additional 163-bp region (bases 49–211; numbering of ref. 2).

Developmental Curves for *Sry* and *Zfy*. We studied earlier stages to determine the time of onset of transcription of these two sex-determining region genes. Because of variable results in detecting the PCR products with ethidium bromide staining, we also used silver staining as a more sensitive technique to detect the PCR products (Table 2). *Sry* and *Zfy* transcription was detected as early as the two-cell stage (Fig.

Table 1. Growth factor Y by *Zfy* PCR

	Embryo development			Statistical analysis P by χ^2	
Embryo ovulation	Early	Intermediate	Slow	Early/slow	Male/female
Superovulated					
Ratio ♂/♀	13/10	10/12	7/16	<0.01	
% male	57	46	30		NS
Naturally ovulated					
Ratio ♂/♀	19/4	11/7	6/17	<<0.01	
% male	83	61	26		NS

Embryos were classified as to early, intermediate, or slow developers on the basis of the time of appearance of the blastocoele cavity and then were sexed by PCR for *Zfy*.

Table 2. Summary of transcription of *Sry, Zfy, AMH, Zfx, Zfa,* and *A1s9* at various developmental stages in early mouse embryos

Gene	Mode of product detection	Positive RT-PCR reactions at different embryonic developmental stages, no.			
		2 cells	4–8 cells	Morula	Blastocyst
Zfy	EthBr	2 (3)	4 (8)	0 (3)	3 (9)
	SS			1 (1)	1 (2)
Sry	EthBr	1 (3)	0 (6)	0 (5)	5 (13)
	SS			1 (2)	2 (2)
AMH	EthBr	ND	ND	ND	0 (3)
Zfx	EthBr	ND	ND	ND	0 (2)
Zfa	EthBr	ND	ND	ND	0 (2)
A1s9	EthBr	ND	ND	ND	0 (5)
	SS	ND	ND	ND	0 (4)

The number of positive RT-PCR reactions that were observed on ethidium bromide (EthBr) gels or by using silver-staining techniques (SS) is indicated; the total number of determinations from more than one RNA preparation except in the case of two-cell embryos is shown in parentheses. SS techniques were used to examine samples that were negative after EthBr staining. *Zfx, Zfa, A1s9,* and the gene for AMH were tested only at the blastocyst developmental stage (ND, not determined).

2, Table 2) and during stages intermediate between the two-cell and blastocyst stages.

Search for Preimplantation Transcription of Related Genes. We also looked for transcription of other genes to be sure that we were not detecting artifactual transcripts or, because of the sensitivity of the technique, were not detecting some very low level of generalized transcription. Thus, both *Zfx* and *Zfa* were assayed at the blastocyst stage. No evidence for transcription of these two zinc finger protein-encoding genes, both related to *Zfy*, one X-linked (*Zfx*) and one autosomal (*Zfa*), was seen in the blastocyst (Table 2) with ethidium bromide staining; silver staining was not used in these cases because the contrast to the positive controls was very marked. Another gene from the sex-determining region of the mouse Y chromosome (*A1s9*) was recently cloned by two groups (18, 20). This gene, whose product sequence is related to the ubiquitin-activating enzyme (E1), was isolated as cDNA that could complement a temperature-sensitive cell-cycle mutation in a mutagenized mouse cell line. Thus, it is a candidate for "growth factor Y." We carefully searched for the expression of *A1s9* in blastocysts using the silver-staining technique. However, no evidence for the transcription of *A1s9* at this stage was found (Table 2). We were also interested in whether the autosomal gene for AMH was transcribed in the preimplantation embryo since the AMH gene is located "downstream" from the *Sry* binding element (CCTTTGA; refs. 21 and 22) in the AMH gene 5′ promoter region at positions −148 to −142 in the mouse sequence (15). AMH gene transcription was not detected at the blastocyst stage (Table 2).

DISCUSSION

We are impressed that "growth factor Y" activity may be responsible for fetal gonad growth differences. Ursula Mittwoch demonstrated that at the earliest time male and female gonads can be distinguished, the testis is already larger in rats (23) and man (24). In contrast, she found that in birds the ovary—i.e., the gonad of the heterogametic sex—was faster growing than the testis (25). The transcription of sex-determining region genes in the preimplantation embryo, which strongly implies expression since we know of no example of a gene that is transcribed but not expressed in the preimplantation embryo, may provide a molecular mechanism for this phenomenon.

It is possible that "growth factor Y" is related to the male preponderance seen in intersex chimeras. Chimeras between male and female embryos (sex unknown at the time of fusion) have a 3-to-1 preponderance of males in relatively balanced chimeras, which is explained because XX ↔ XY chimeras develop as males (26). Previous interpretations of the male excess have focused on analyses of the gonads, where ovotestes were sometimes found with the meiotic cells in testes undergoing degeneration, presumably due to the usual lack of survival of XX germ cells when in a testicular environment (27). The same loss of female germ cells and the usual development of testes is seen in XO/XY, XO/XY/XYY mosaics found among the offspring of BALB/cWt males (28, 29). Thus, while the fate of germ cells in gonads has been well studied, the question of the possible role of "growth factor Y" in effecting gonadal growth and subsequent differentiation to testes has not been investigated. Studies of growth regulation in chimeras had focused on the adult weights of chimeras between strains selected for size, small and large (30). Given the well-known regulative properties of the embryo (the ability of half embryos, or of four embryos when fused, to give rise to normal-size fetuses; refs. 31 and 32), there are intriguing questions to be asked about the time and degree of embryonic regulation in XY ↔ XX chimeras and the possible relationship to gonadal growth and sex determination.

Our findings can be related to regulation of sex determination by temperature in reptiles. Elevated egg incubation temperatures increase the frequency of male lizards (33) and alligators (34), which would fit with the mammalian, testis-imposed model and faster growth caused by increasing temperature. The decreased frequency of males with elevated egg incubation temperatures among turtles (35) is explicable if they are in the avian evolutionary line (i.e., ovary-imposed mechanism). The presence or absence of distinct sex chromosomes does not help us among these reptiles as they are variably present, and males or females may be heterogametic in not distantly related species (36). The finding that snapping turtles show a high frequency of males at an intermediate temperature with much lower frequencies at higher and lower temperatures (37) may reflect the possibility that too high temperatures are detrimental to growth. However, if crocodilians are more closely related to birds than to other reptiles (38), the hypothesis is weakened.

Finally, our results suggest that sexual determination in placental mammals may be more similar to that in marsupials than previously recognized. Tammar wallabies show extensive sexual dimorphism before any morphological differentiation of the gonads (39). These hormone-independent, sexual differentiation events affect multiple tissues but not Wolffian duct derivatives. We postulate that preimplantation expression of sex-determining region genes may be related to such differentiative events in mammals.

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