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Transcription of paternal Y-linked genes in the human zygote as early as the pronucleate stage

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Summary

Global activation of the embryonic genome occurs at the 4- to 8-cell stage in human embryos and is marked by continuation of early cleavage divisions in the presence of transcriptional inhibitors. Here we demonstrate, using reverse transcriptase-polymerase chain reaction (RT-PCR), the presence of transcripts for two paternal Y chromosomal genes, *ZFY* and *SRY* in human preimplantation embryos. *ZFY* transcripts were detected as early as the pronucleate stage, 20–24 h post-insemination *in vitro* and at intermediate stages up to the blastocyst stage. *SRY* transcripts were also detected at 2-cell to blastocyst stages. The expression of *SRY* and *ZFY* at these early stages and the faster cleavage rate of male embryos observed in many mammalian species focuses attention on the role of events in sex determination prior to gonad differentiation.

Key words: Preimplantation human embryo, RT-PCR, sex determination, *SRY*, *ZFY*

Introduction

Mammalian embryos, like those of lower vertebrates and invertebrates, are dependent on maternally inherited products from the oocyte for an initial period following fertilization prior to activation of the embryonic genome. The timing of the onset of embryonic genome expression differs in different species. In the mouse, embryonic expression is initiated at the mid 2-cell stage (Braude *et al.*, 1979; Flach *et al.*, 1982; Clegg & Piko, 1982; Clegg *et al.*, 1983*a*, *b*; Bolton *et al.*, 1984). RNA synthesis (Clegg *et al.*, 1983*a*) and transcription of major histocompatibility complex (Mhc) class I genes, however, have been detected as early as the 1-cell stage (Sprinks *et al.*, 1993). In other species, embryonic gene transcription starts later at the 4-cell (pig, Tomanek *et al.*, 1989) or 8-cell stages (cow, Camouse *et al.*, 1986; sheep, Crosby *et al.*, 1988). In the human preimplantation embryo, major qualitative changes in polypeptide synthesis observed between the 4- and 8-cell stages are dependent on transcription, suggesting the onset of global expression of the embryonic genome at this stage (Braude *et al.*, 1988). Compared with the mouse, however, there is very limited information concerning RNA synthesis and the molecular mechanisms that regulate the onset of transcription. Tesarik *et al.* (1988) showed [^{3}H]uridine incorporation into nuclei and nucleoli at the 4-cell stage while mRNA for a specific embryonic product, chorionic gonadotrophin β (hCG-β), is first detected by *in situ* hybridisation at the 6- to 8-cell stage (Bonduelle *et al.*, 1988). However, recently Tesarik & Kopecny (1989) have shown [^{3}H]adenine incorporation at the 1-cell zygote stage.

The timing of paternal gene transcription provides a marker for the activation of the embyronic genome in male embryos. Here we report the use of reverse transcriptase-polymerase chain reaction (RT-PCR) to search for Y-linked gene transcription in human preimplantation embryos. In placental mammals, sex determination is mediated by the presence of testes determining gene(s) encoded by the Y chromosome. We have analysed the transcription of *ZFY*, a Y-coded

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zinc finger protein located adjacent to the sex-determining region (Page *et al.*, 1987) and *SRY* (*Sry* in mouse), the testes-determining factor (TDF: Sinclair *et al.*, 1990; Gubbay *et al.*, 1990). We demonstrate the presence of *ZFY* transcripts as early as the proncleate stage and *SRY* at the 2-cell stage (the earliest stage tested). The possible relationship between transcription of Y-linked genes at these early stages and the faster cleavage and development rate of male embryos observed in many mammalian species is discussed.

Materials and methods

Embryo collection and culture

Surplus human embryos were obtained with approval from patients undergoing *in vitro* fertilisation (IVF) or gamete intra-fallopian transfer (GIFT) procedures at the Wolfson Family Clinic, Hammersmith Hospital. Superovulation and oocyte retrieval were as described earlier (Rutherford *et al.*, 1988). Oocytes were collected by vaginal ultrasound-guided aspiration and inseminated with prepared sperm (day 0). Oocytes were examined for pronuclei 19–20 h post-insemination and embryos were classified as 'normal' or 'polyspermic' depending upon the presence of two or more pronuclei, respectively. Fertilised zygotes were either harvested immediately or transferred to culture medium for further development as described earlier (Hardy *et al.*, 1989).

This work has been approved by the Human Fertilization and Embryology Authority and the Research Ethics Committee of the Royal Postgraduate Medical School.

Preparation of RNA from tissue samples

Total RNA was prepared from human tissues using RNAzol™ B (Biogenesis Ltd) and poly A^+ RNA isolated using the 'Quick prep mRNA isolation kit' (Pharmacia Biotech). RNA was treated with RNase-free DNase for 20 min at 37°C. From 0.1 to 0.05 μg of RNA was added to reverse transcriptase reaction to synthesise cDNA.

Reverse transcription-polymerase chain reaction (RT-PCR)

The zona pellucida was removed from oocytes and embryos with a brief acid Tyrodes' treatment and the embryos washed thoroughly in phosphate-buffered saline (PBS). Oocytes and embryos of the same stage were pooled in an Eppendorf tube containing 5–10 μl lysis buffer (0.5% NP40, 10 mM Tris (pH 8.0), 10 mM NaCl and 3 mM $MgCl_2$) (Gilliland *et al.*, 1990). The tubes were spun for 1–2 min and the supernatant containing the RNA used for cDNA synthesis in the presence (RT +) or absence (RT −) of reverse transcriptase. The final reaction mixture containing 3–8 μl of crude RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 0.5 μg of oligo(dT) $_{12-18}$primer, 1 unit of RNase H, 100 units of M-MLV reverse transcriptase (BRL) which was incubated at 37°C for 1 h. The reaction was terminated by heating at 95°C for 5 min. Subsequent 30 μl PCR reactions contained 5–10 μl reverse-transcribed cDNA, 10 mM Trs-HCl, pH 8.3, 50 mM of each dNTP, 2.5 units of Amplitaq polymerase and 0.4 μM of each primer. Each reaction was overlayed with 50 μl silicone oil and heated to 95°C for 2 min. PCR was carried out essentially as previously described (Zwingman *et al.*, 1993) for 25–29 cycles with the outer primers, and 30–35 cycles with the inner, nested primers at the appropriate annealing temperatures for each set (see Table 1). For the nested PCR, 1 μl of primary product was added to 29 μl of freshly prepared mix as above. The amplified product was electrophoresed on 2% Nusieve (FMC Bioproducts), 1% agarose or 9% polyacrylamide gels and stained with ethidium bromide.

Table 1 Oligonucleotide primers used for RT-PCR assays

Gene	Primer type	Forward primer	Reverse primer	Anneal. temp. (°C)	Product length (bp)
ZFX/ZFY	Outer[a]	5'CATGATAGTGTAGTGGAAGCAGAAA-3'	5'-CCTCTCCTACGATCACTTCCATATA-3'	60	499
	Nested	5'-TTGGTAGCAGACTGTGCC(CorT)CT-3'	5'-AACCATCTTTTCCCTGGGAACA-3'	60	357
SRY	Outer	5'-GCGACCCATGAACGCATTCA-3'	5'-GTACCGAAGCGGGATCTGCG-3'	58	275
	Nested	5'-TCAGAGGCGCAAGATGGCTC-3'	5'-TTCTTCGGCAGCATCTTCGC-3'	60	204
HPRT	Outer	5'-CTCCGCCTCCTCCTCTGCT-3'	5'-GCCTGACCAAGGAAAGCAAAG-3'	50	528
	Nested	5'-GCCGGCTCCGTTATGGCG-3'	5'-AGCCCCCCTTGAGCACACAGA-3'	55	226

[a] Previously published sequence (Palmer *et al.*, 1990).

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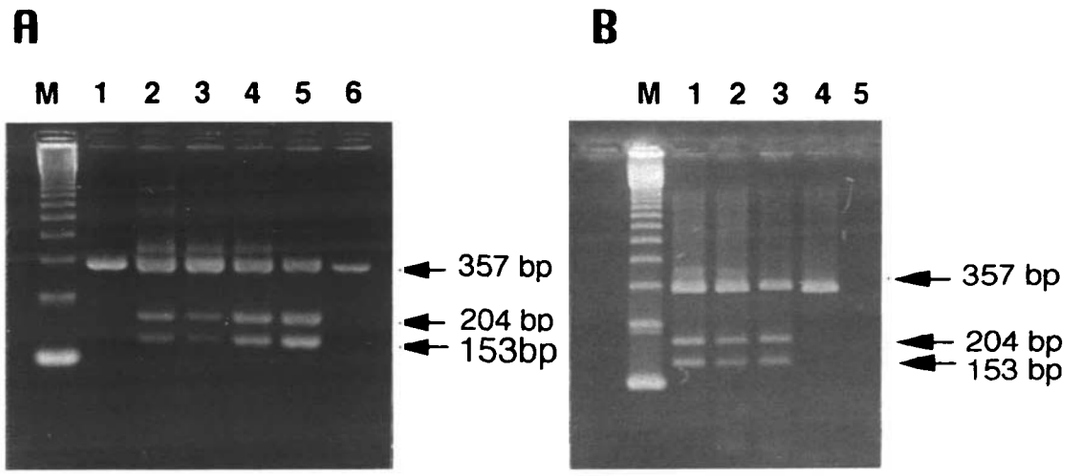

Figure 1 RT-PCR analysis of oocyte and preimplantation stage embryos for *ZFX* and *ZFY* transcription. RT-PCR was performed on RNAs from pooled oocytes, polyspermic zygotes and normal embryo samples (16 oocytes, 18 1-cell polyspermic zygotes, 15 2-cell, 20 4 to 8-cell, 8 morulae, 5 blastocysts). PCR products were digested with *Cla*I and resolved on a 2% Nusieve 1% agarose gel. (*A*) Lanes: 1, oocyte; 2, 4 to 8-cell; 3, morula; 4, blastocyst; 5, adult testis; 6, adult female spleen. (*B*) Lanes: 1, 2-cell; 2, 1-cell zygote; 3, adult testis; 4, oocyte; 5, H_2O control. M, 123 bp DNA marker. The *ZFX* product was 357 bp and *ZFY* products were 204 bp and 153 bp.

PCR Sequencing

ZFX/ZFY and *SRY* PCR products from oocytes and embryos were reamplified using the conditions described above and purified with the PCR purification spin kit (Quiagen). Sequencing was completed with the 'fmol' DNA sequencing system (Promega) using forward and reverse nested primers.

Results

cDNA samples were prepared from pooled oocytes and embryos, as only half were expected to be male, for analysis of the transcription of *ZFY* and *SRY* genes. The oligonucleotide primers used to analyse *ZFY* transcripts also amplify the homologue, *ZFX*, on the X chromosome (Palmer *et al.*, 1990). Nested primers were designed to anneal at the same temperature as the outer primer to minimise non-specific bands. On digestion with restriction enzyme *Cla*I, the two gene transcripts can be distinguished such that the expression of X or Y transcripts in different samples can be determined. The nested primers for *ZFX/ZFY* give a 357 bp band, and on digestion with *Cla*I *ZFY* transcripts give two bands corresponding to 204 bp and 153 bp. This is due to the presence of a *Cla*I site in the *ZFY* sequence which is absent in *ZFX*. In addition, the outer primers are known to span an intron (Palmer *et al.*, 1990), thus eliminating the possibility of genomic DNA contamination. Fig 1*A* shows the pattern of *ZFX/ZFY* expression at different stages from unfertilised oocytes and 4- to 8-cell to the blastocyst stages. A 357 bp product was obtained after nested PCR at all stages. On digestion with *Cla*I (Fig. 1*A*), the product from cleavage and blastocyst stage embryos yielded two fragments of 204 bp and 153 bp corresponding to *ZFY* transcripts in addition to *ZFX* bands. Similarly, the adult testis control yielded all three bands for *ZFX* and *ZFY* genes, whereas the female adult spleen yielded only the single *ZFX* band. The *ZFX* transcripts in unfertilised oocytes must be maternally inherited. However, our RT-PCR analysis is not quantitative; therefore the maternal or embryonic origin of *ZFX* transcripts detected at later stages cannot be distinguished.

We also studied the time of onset of *ZFY* transcription at earlier stages. Fig. 1*B* shows the presence of *ZFY* transcripts as early as the pronuclear stage (20–24 h post-insemination) and later at the 2-cell stage, whereas the unfertilised oocyte control (Fig. 1*B*, lane 4) again showed only *ZFX* transcsripts. Previous studies have shown that both *ZFX* and *ZFY* are expressed in a wide range of fetal and adult tissues (Palmer *et al.*, 1990). We tested the expression of both genes in fetal male and female intestine, liver, lung, muscle, brain and kidney, and in adult male lung and female spleen. The results confirmed the previous findings that *ZFX* was expressed in both male and female tissues whereas *ZFY* is expressed only in male tissues (results not shown). To further strengthen the conclusion, the RT-PCR products from oocytes and embryos were sequenced. Sequence analysis on oocyte (Fig. 2*A*, *B*) and embryo products (Fig. 2*C*, *D*) were performed with forward and reverse nested primers, respectively. With oocyte product, the sequence was identical to *ZFX*. The embryo product

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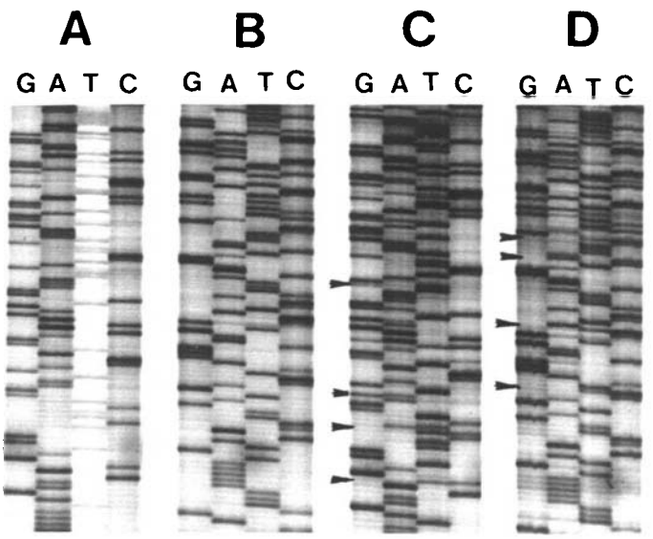

Figure 2 Sequence analysis on oocyte and embryo *ZFX/ZFY* RT-PCR products. Lanes *A* and *B*, the oocyte product, and lanes *C* and *D*, the embryo product, were sequenced with forward and reverse nested primers. Some sites are indicated at which two bases are found in embryos but not in oocytes.

sequence was a mixture of *ZFX* and *ZFY* products, i.e. at the 17 positions at which the two differ in this span, bases for both sequences were found. Eight of the 17 positions at which *ZFX* and *ZFY* differ (Palmer *et al.*, 1990) are indicated in the embryo sequencing lanes.

We also sought *SRY* transcription in oocytes, preimplantation embryos and human adult testis. *SRY* transcripts (204 bp) were present in adults testis and embryo samples, but not in oocytes. Amplification of HPRT transcripts from each sample confirmed that cDNA was present (Fig. 3*A*). *SRY* transcripts were present at the earliest preimplantation stage tested, the 2-cell stage, and also at the blastocyst stage (Fig. 3*B*). Sequence analysis of the product confirmed the identity as *SRY* (results not shown). Sequences corresponding to positions 234 to 344 from the forward nested primer and sequences corresponding to positions 264 to 160 from reverse nested primer were identical to the *SRY* published sequence (using the numbering of Gubbay *et al.*, 1990).

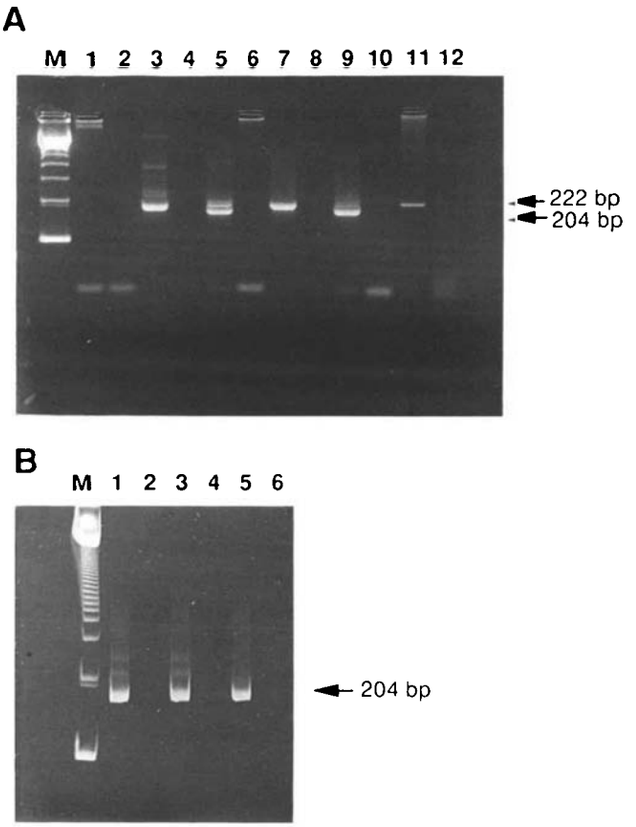

Figure 3 Transcriptional analysis of *SRY* expression in preimplantation embryos. RT-PCR was performed on RNAs from pooled oocytes and normal embryos. (16 oocytes, 8 2-cell, 5 blastocysts and 18 polyspermic mixed stage embryos). (*A*) Lanes 1, 5 and 9 were RT^+ samples with *SRY* primers and lanes 3, 7 and 11 were RT^+ samples with HPRT primers for oocytes, adult testis and polyspermic (mixed stages) embryos respectively. Lanes 2, 6 and 10 were RT^- samples with *SRY* primers and lanes 4, 8, 12 were RT^- samples with HPRT primers for oocytes, adult testis and polyspermic embryos respectively. (*B*) Lanes 1, 3 and 5 were RT^+ samples with *SRY* primers for 2-cell, blastocyst and adult testis and lanes 2, 4 and 6 were corresponding RT^- samples with *SRY* primers. M, 123 bp DNA molecular weight marker.

Discussion

Global activation of the human embryonic genome is thought to occur between the 4- and 8-cell stage on day 2 post-insemination. Evidence for this includes qualitative changes in polypeptide synthesis during this period which are sensitive to transcriptional inhibition with α-amanitin (Braude *et al.*, 1989). Also, cleavage is initially insensitive to transcriptional inhibition but is blocked at later stages, suggesting that early development is controlled by maternally inherited products (Braude *et al.*, 1988). In the mouse, numerous studies have documented the onset of embryonic gene expression at the 2-cell stage (Flach *et al.*, 1982; Bensaude *et al.*, 1983; Bolton *et al.*, 1984) accompanied by the degradation of maternal mRNA (Bachvarova & De Leon, 1980; Giebelhaus *et al.*, 1983; Graves *et al.*, 1985. Paynton *et al.*, 1988). In the human, similar studies are difficult mainly due to the limited availability of material. Transcription of hCG-β has been reported as early as the 6- to 8-cell stage (Bonduelle *et al.*, 1988), however, using *in situ* hybridisation.

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The recent development of sensitive RT-PCR methods has enabled us to examine transcripts in small numbers of human oocytes and embryos including, for example, transcription of epidermal growth factor and its receptor (M. Chia and A.H. Handyside, unpublished data). While it could be argued that these sensitive techniques might detect 'ectopic' transcripts (Chelly *et al.*, 1989; Roberts *et al.*, 1992), their sensitivity is not excessive considering the minute amount of starting material. The presence of paternal Y-linked transcripts before global activation of the embryonic genome at the 4- to 8-cell stage, *ZFY* as early as the pronucleate stage (Fig. 1*B*) and *SRY* at the 2-cell stage (Fig. 3*B*), is unexpected and suggests that at least some genes are actively transcribed earlier than previously thought. Furthermore, analysis of *SRY* transcripts at the 1-cell stage, which was not attempted by us, has been reported independently (E. Pergament and D.A. Rappolee, personal communication). Both Y-linked transcripts, therefore, are present during the first cycle after fertilisation. Similar observations indicating early transcription were recently reported for maternal and paternal Mhc class I genes transcripts in the mouse zygote, also using RT-PCR (Sprinks *et al.*, 1993).

The possibility that these transcripts are derived from the fertilising sperm seems unlikely but cannot be ruled out. c-*myc* transcripts have been detected in mature ejaculated sperm cells in the human (Kumar *et al.*, 1993). However, *SRY* transcripts were not detectable in washed sperm prepared in the same way as the other samples (data not shown). As antibodies to the *ZFY* and *SRY* products are not available, we were not able to confirm expression at the protein level. However, it seems likely that both genes are expressed, raising interesting questions concerning their functions at such an early stage of development.

Several recent findings have demonstrated that there is a differential growth rate between male and female, XX and XY embryos in several mammalian species. At preimplantation stages, male embryos have a faster cleavage rate than female embryos in both mice (Tsunoda *et al.*, 1985; Zwingman *et al.*, 1993; Bourgoyne, 1993) and cattle (Avery *et al.*, 1992; Xu *et al.*, 1992). Recently Pergament *et al.* (1994) reported that significantly more males are born when the mean number of cells per embryo is 4 or greater at the time of embryo transfer on day 2, 40–48 h post-insemination. Differences have also been observed at post-implantation stages, with male fetuses showing increased weight in rats (Scott & Holsen, 1977), somite numbers in mice (Seller & Perkins-Cole, 1987) and in first trimester human fetuses (Pedersen, 1980).

Mittwoch (1969, 1993; Mittwoch & Mahadevaiah, 1980) has suggested that a faster growth rate results in the development of a testis and that slow growth leads to the formation of ovary. It is possible that differences in the growth of fetal gonads may be attributed to the presence of a growth factor activity encoded by the Y chromosome. Alternatively, differences could result from the presence of a paternally imprinted X chromosome in female XX embryos. In mouse, Bourgoyne (1993) has used genetic crosses to confirm that the growth rate difference in preimplantation embryos is Y-linked.

The cloning of *SRY/Sry* in man and mouse (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990) and sex reversal of 40,XX mice transgenic for the *Sry* gene (Koopman *et al.*, 1991) provide evidence that *Sry* is the Tdy. Transcription of *Sry* was first reported in developing gonadal ridges at 10.5 days *post coitum*, which was considered appropriate for Tdy (Koopman *et al.*, 1991). We have shown transcription of *Sry* and *Zfy* at a much earlier stage, the 2-cell stage, with persistence of mRNA to the blastocyst stage (Zwingman *et al.*, 1993). Our finding of similar results in the human embryo, i.e. transcription of *SRY* and *ZFY* in early preimplantation stages (and confirmed for *SRY* by E. Pergament & D.A. Rappolee, personal communication) and differences in rate of development of male and female embryos (Pergament *et al.*, 1994) focuses attention on the role of events in sex determination prior to gonadal differentiation.

Previously male and female preimplantation embryos have been shown to differ for expression of the male specific antigen (MSA, serological H-Y). This has been found in mice (Krco & Goldberg, 1976; Epstein *et al.*, 1980), cattle (White *et al.*, 1987*a*), sheep (White *et al.*, 1987*b*), pigs (White *et al.*, 1987*c*), and horse blastocysts (Wood *et al.*, 1988). In all cases, half the embryos are reactive and, when karyotypes have been performed, only Y-bearing embryos are antigen positive. Although once a candidate sex determination factor, it now seems that the gene may be located on an autosome (Lau *et al.*, 1989), but its expression establishes the fact that not only transcription but also translation differs between mammalian male and female preimplantation embryos.

The mechanism by which *SRY* or *ZFY* expression could lead to more rapid growth of the Y-bearing preimplantation embryo has yet to be determined. Similarly, the relationship between more rapid growth and sexual differentiation (if any, since the two phenomenona may not be causally related) is unclear. However, the relationship may not depend on testosterone and müllerian inhibiting substance as second messengers.

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