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Original Article

Wilms' tumor suppressor gene (*WT1*) as a target gene of SRY function in a mouse ES cell line transfected with SRY

YAYOI TOYOOKA[1,5], SATOMI S. TANAKA[1], OSAMU HIROTA[2], SATOSHI TANAKA[3], NOBUO TAKAGI[4], KEITARO YAMANOUCHI[5], HIDEAKI TOJO[5] and CHIKASHI TACHI[5,6]*

[1]*Developmental Biology Group, Mitsubishi Kasei Institute of Life Sciences, Minamiooya, Machida-shi, Tokyo, Japan,* [2]*Nippon Boehringer-Ingelheim Co. Ltd., Yato, Kawanishi-shi, Japan* [3]*Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada,* [4]*Molecular Genetic Research Laboratory, University of Hokkaido, Sapporo, Hokkaido, Japan,* [5]*Laboratory of Applied Genetics, Department of Animal Resource Sciences, School of Agriculture and Life Sciences, University of Tokyo, Tokyo, Japan and* [6]*Laboratory of Developmental and Reproductive Biotechnology, Department of Animal Resource Sciences, School of Veterinary Medicine and Life Sciences, Azabu University, Fuchinobe, Sagamihara-shi, Kanagawa-ken, Japan*

ABSTRACT With the aim of identifying the gene(s) located downstream from *SRY*, we transfected an ES cell line with XX karyotype, TMA-18, with a *Sry* DNA construct and established cell lines, TS18-1 and TS18-2, where the transfected *Sry* was expressed in the functional linear mRNA form. Among the five potential SRY-target genes examined, i.e., *MIS*, *SF1*, *P450arom*, *Sox9* and *WT1*, only the expression of *WT1* was induced *de novo* by the unscheduled expression of *Sry* in the transfected cell lines. No clear indication of *Sry*-induced enhancement of *Sox9* expression was obtained in the present series of experiments. Function of a yet unidentified gene(s) located on the Y chromosome might be needed for the up-regulation of *Sox 9* expression which takes place during the development of male gonads. Quantitative RT-PCR analysis of the patterns of WT1 expression in developing fetal gonads revealed that although both male and female fetal gonads express *WT1*, male gonads invariably expressed *WT1* mRNA at higher levels than female ones after the *Sry* expression. Immunohistochemical analysis of the male fetal gonads between 10.5 and 13.5 dpc demonstrated the presence of strong *WT1* immunoreactivity in Sertoli cells of the primordial testes . Suggestions were made in the past indicating that both SF1 and *WT1* proteins might be active in a common pathway upstream from Sry. Our results showed that *WT1* is located downstream, rather than upstream from *Sry* and behaves independently from *SF1*. Analysis using an appropriate *in vitro* system will be essential to understand the molecular mechanisms of SRY action within cells.

KEY WORDS: *Sry, WT1, ES cells, transformation*

Introduction

The complete sequence of genomic SRY DNA was determined by Sinclair *et al.* (1990) in humans and by Gubbay *et al.* (1990) in mice. Subsequently, a 14 kb genomic DNA fragment containing the *Sry* locus was microinjected into XX mouse embryos and shown to direct the development of the male phenotype in chromosomally female mice (Koopman *et al.*, 1991). The phenotypically male XX transgenic mice were sterile (Koopman *et al.*, 1991), because the Y chromosome of the mouse contains genes responsible for spermatogenesis (Burgoyne *et al.*, 1992; Reijo *et al.*, 1996). The transgenic study demonstrated not only that *Sry* codes for the testis determining factor (Tdy) but also that, aside from those Y-linked genes responsible for spermatogenesis, all of the genes required for testicular differentiation are present in the XX genomes (Koopman *et al.*, 1991)

Although the *Sry* transgenic mice revealed many important facts about the function of SRY *in vivo*, relatively little has been learned about the cellular and molecular mechanisms through which *Sry* induces differentiation of the indifferent gonads into testes.

Since the SRY protein possesses a DNA-binding domain encoded by the HMG box of *SRY* (Sinclair *et al.*, 1990; Gubbay *et al.*, 1990; Payen and Cotinot, 1993,1994; Su and Lau, 1993; Coward *et al.*, 1994; Hacker *et al.*, 1995) and binds to specific DNA motifs *in vitro* (Harley *et al.*, 1994), it has generally been assumed to act as a transcription factor, triggering a cascade of gene expression that induces the bipotential indifferent gonad to develop into a testis (Dubin and Ostrer, 1994). So far, several genes have been put

***Address for reprints:** Laboratory of Developmental and Reproductive Biotechnology, Department of Animal Resource Sciences, School of Veterinary Medicine and Life Science, Azabu University, Fuchinobe, Kanagawa-ken 229, Japan. FAX: +81-427-86-7578. e-mail: tachi@azabu-u.ac.jp

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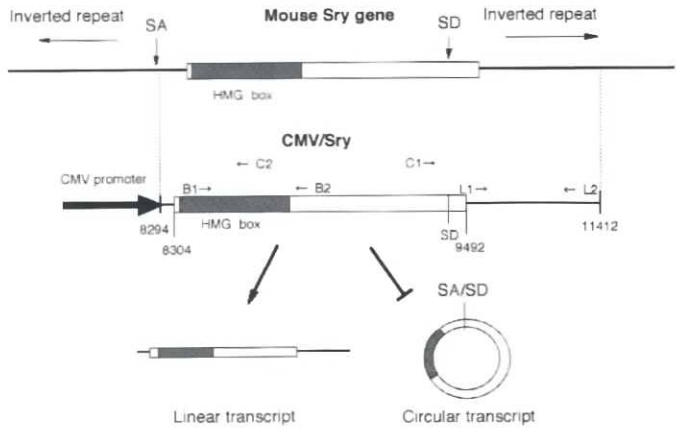

Fig. 1. Schematic representation of the Sry DNA construct used for the transfection experiments. *The structure of the construct is the same as the one described in our previous report (Hirota et al., 1996). The inverted repeat at the 5' end was deleted and fused with a CMV promoter. The construct yielded linear transcripts only; no circular transcripts were formed (the blunt-ended arrow). Nucleotide numbers are according to Gubbay et al. (1992). Approximate locations of the PCR primers are indicated by arrows. B1/B2, primer set designed to detect both the linear and the circular forms of the transcripts. C1/C2, primer set specific to the circular transcripts. L1/L2, primer set specifically detects the linear transcripts. Solid box represents the HMG box.*

forward as candidates for the genes comprising the hypothetical SRY cascade, including those coding for Müllerian inhibiting substance *(MIS)* (Haqq *et al.*, 1993, 1994), P450 aromatase *(P450 arom)* (Haqq *et al.*, 1993; Hirota *et al.*, 1996) and steroidogenic factor 1 (SF1) (or adrenal 4-binding protein, *Ad4BP*) (Ikeda *et al.*, 1994; Shen *et al.*, 1994), as well as *Sry*-like HMG-box gene 9 *(Sox9)* (Foster *et al.*, 1994; Wagner *et al.*, 1994) and the Wilms' Tumor suppressor gene *(WT1)* (Pelletier *et al.*, 1991; Denny *et al.*, 1992; Harley *et al.*, 1994). However, little information is available in the literature concerning the interaction within the cell between these genes and *SRY*/Sry.

It is known in the mouse that *Sry* mRNA is not only expressed in the somatic cells of the fetal gonad at the time of sex determination, but also in both germ cells and somatic cells of the adult testis at a high level (Rossi *et al.*, 1993; Zwingman *et al.*, 1994). The *Sry* transcripts in the adult testes, however, are circular and probably non-functional owing to their failure to properly interact with ribosomes (Capel *et al.*, 1993; Hacker *et al.*, 1995; Jeske *et al.*, 1995). The circular conformation of the *Sry* transcripts is due to the presence of inverted repeats on the 5' and 3' ends of the open reading frame (ORF). These inverted repeats are thought to lead to the formation of a loop structure that facilitates splicing to form the circle (Gubbay *et al.*, 1992; Dubin *et al.*, 1995).

To identify SRY target genes, we transfected cells from a cultured mouse Sertoli cell line, TM-4, which does not express the endogenous *Sry* gene with a mouse *Sry* DNA construct where the 5' inverted repeat flanking the ORF was removed, and replaced with the CMV promoter (Hirota *et al.*, 1996). In the established transfected cell lines (TMHm cell lines in our laboratory), the unscheduled expression of linear *Sry* transcripts induced the expression of the gene coding for P450 aromatase, *P450arom*, which is not expressed in the non-transfected TM-4 cells (Hirota *et al.*, 1996). Although this finding is of much interest, it may not be representative of the phenomena taking place during development *in vivo*, since TH-4 cells are already well differentiated cells. We wondered if different genes might be mobilized by the expression of *SRY* gene in undifferentiated pluripotent embryonic cells. To answer this question, we introduced the same *Sry* DNA construct that was employed to transfect TM-4 cells into cells of an embryonic stem (ES) cell line with an XX karyotype (TMA-18) and investigated

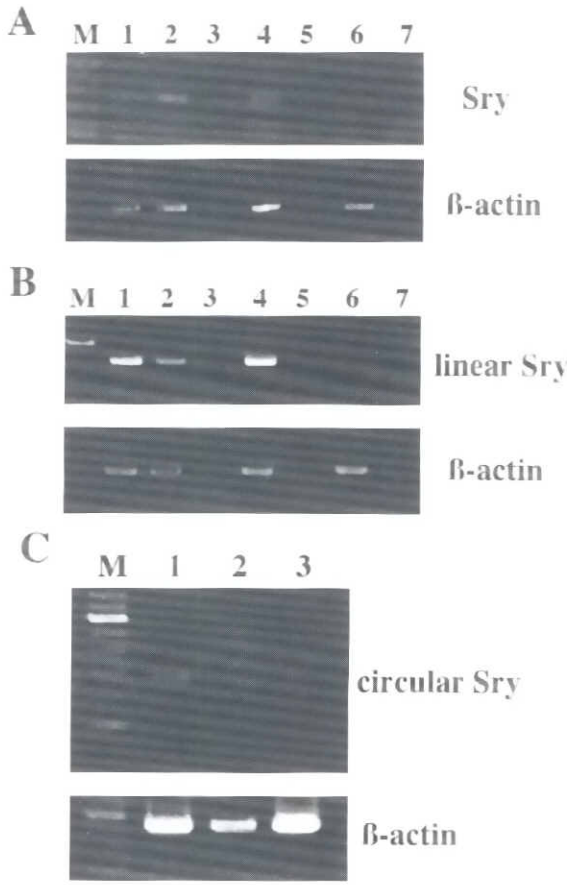

Fig. 2. RT-PCR analysis of *Sry* transgene expression using the primer sets specific for the HMG box (B1/B2) and for the linear transcripts (L1/L2). (A) *Expression of the Sry transgene as detected by the B1/B2 primer set. M, 100bp ladder; lane 1, mouse adult testis (positive control); lane 2-5, Sry-transfected cell lines, TS18-1 (lane 2,3) and TS18-2 (lane 4,5) with (lane 2, 4) or without (lane 3, 5) RTase. Lane 6 and 7, non-transfected cell line, TMA-18, with or without RTase, respectively. The transgene is expressed abundantly in the transfected cell lines.* **(B)** *Expression of the linear transcripts as detected by the L1/L2 primer set. Lane 1, undifferentiated gonads of 11.5 dpc (positive control); lane 2 and 3, Sry-transfected cell line TS18-1 in with (lane 2) or without (lane 3) RTase. Lane 4 and 5, Sry-transfected cell line TS18-2 with (lane 4) or without (lane 5) RTase. Lane 6 and 7, non-transfected cell line with or without RTase, respectively. The expression of the functional linear form was confirmed in the transfected cell lines.* **(C)** *RT-PCR analysis of Sry transgene expression using the primer set specific for the circular transcripts (C1 and C2). The adult testis of the mouse was used as positive control. Lane 1, the adult testis (positive control); lane 2 and 3, Sry-transfected cell lines, TS18-1 and TS18-2, respectively. No band corresponding in size to the circular transcripts was detected in the transfected cell lines.*

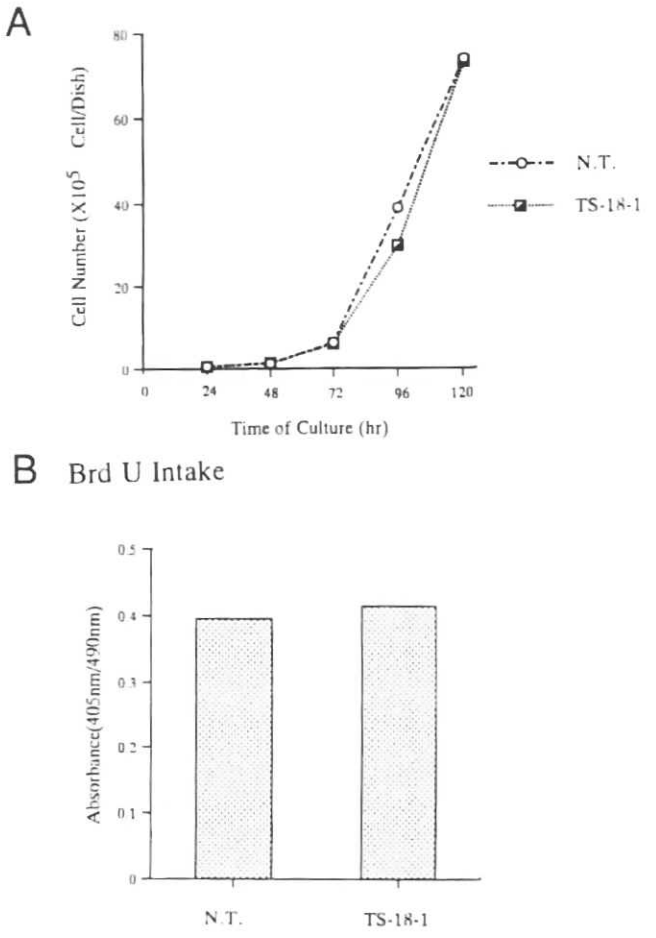

Fig. 3. Proliferative activities of the non-transfected TMA-18 and the *Sry*-transfected TS18-1 cells. (A) *Changes in cell numbers according to the time of culture. No significant difference was detected in the proliferative activity between the two lines.* **(B)** *Rates of BrdU uptake in the non-transfected TMA-18 (N.T.) cells and in the Sry-transfected TS-18-1 cells. Ordinate, relative rates of BrdU uptake as shown by absorbance at 405 nm.*

the effect of the foreign *Sry* gene expression upon hypothetical *SRY* cascade genes.

ES cells of the mouse maintain a high degree of pluripotency, and can differentiate *in vivo* into all cell lineages when injected into preimplantation embryos (Bradley *et al.*, 1984; Nagy *et al.*, 1990; Nagy and Rossant, 1993; Saburi *et al.*, 1997). They may furthermore be induced to differentiate *in vitro* into several cell lineages including skeletal muscle (Rohwedel *et al.*, 1995), cardiac muscle (Uno, 1982), hematopoietic (Miwa *et al.*, 1991; Schmitt *et al.*, 1991; Wiles and Keller, 1991) and neuron-like cells (Bain *et al.*, 1995; Fraichard *et al.*, 1995). We chose an ES cell line as our host for the transfection experiments, because most of the developmentally important genes in ES cells are expected to be in a state capable of expression when properly induced, and their differentiation pathways may be charted *de novo* under the influence of the transfected genes. ES cells that are karyotypically XX cells possess the gene set required for testicular differentiation (Koopman *et al.*, 1991), but the putative *Sry* cascade genes have never been under the influence of the endogenous *Sry* gene products prior to the transfection experiments.

The RT-PCR analysis of the transfected cell lines revealed *Sry* induced the expression of *WT1*, which is often considered to be situated upstream rather than downstream of *SRY* action.

Results

Establishment of XX ES cell lines expressing functional Sry transcripts

We established 17 lines of XX ES cells stably transfected with *Sry*. Throughout the subsequent series of experiments, 2 lines, i.e., TS18-1 and TS18-2, were used. RT-PCR analysis of the transfected cell lines was carried out using the two primer sets, i.e., B1/B2 which amplify the HMG box region, and L1/L2 specific to the linear *Sry* transcripts as described in Materials and Methods (Fig. 1). In the transfected cell lines screened by the RT-PCR method using B1/B2 primers (Fig. 2A), the expression of the linear transcripts was confirmed (Fig. 2B). On the other hand, RT-PCR analysis performed using the C1/C2 primer set specific to the circular transcripts yielded no visibly recognizable bands corresponding in size to the circular *Sry* mRNA in any transfected cell lines (Fig. 2C). From these results it was concluded that the *Sry* transgenes were expressed only in the functional linear form in the transfected lines.

Morphologically, the transfected ES cells were indistinguishable from the normal non-transfected TMA-18 cells. The transfected ES cells formed cystic embryoid bodies when they were cultured in non-adhesive dishes for 10 days, suggesting that they had maintained their pluripotency (data not shown).

Proliferative activities of the transfected and non-transfected cell lines were compared by either counting the number of cells or assaying the rate of BrdU incorporation during the log phase of growth. The expression of foreign *Sry* did not affect the rate of proliferation (Fig. 3A and B).

Effects of the expression of exogenously introduced Sry genes upon potential SRY-target genes in XX ES cell lines

We investigated the effects of expression of foreign *Sry* genes upon the expression of several possible *Sry*-target genes in the

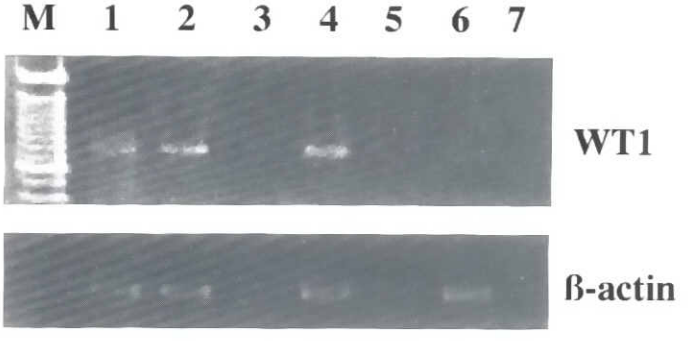

Fig. 4. RT-PCR analysis of the expression of *WT1* in the non-transfected (TMA-18) and the transfected cell lines (TS18-1 and -2). *Lane 1, the undifferentiated gonads on 11.5 dpc were used as positive control; lane 2-5, Sry-transfected cell lines, TS18-1 (lane 2,3) and TS18-2 (lane 4,5) with (lane 2,4) or without (lane 3,5) RTase. Lane 6 and 7, non-transfected cell line with or without RTase, respectively. WT1 expression was confirmed only in the transfected cell lines.*

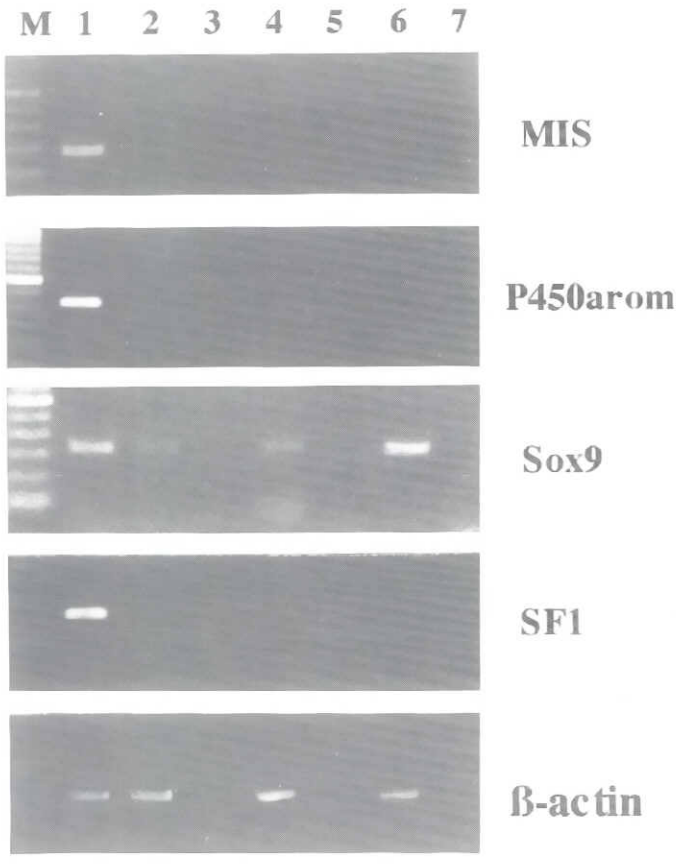

Fig. 5. RT-PCR analysis of the expression of *Sry*-related genes in the *Sry*-transfected cell lines. *Lane 1, Positive controls. The undifferentiated gonads of 11.5 dpc were used for MIS, SF1, and Sox9, and adult testis for P450 arom.; lane 2-5, Sry-transfected cell lines, TS18-1 (lane 2,3) and TS18-2 (lane 4,5) with (lane 2,4) or without (lane 3,5) RTase. Lane 6 and 7, non-transfected cell line in presence or absence of the RTase, respectively. No transcripts of MIS, SF1, and P450 arom were detected in either of the transfected cell lines (TS18-1 and TS18-2) or the non-transfected cell line (TMA-18). Sox9 was detected in both the transfected cell lines and the non-transfected cell line.*

transfected XX ES cell lines. The expression levels of five genes potentially under the direct or indirect control of *Sry*, i.e., *MIS*, *WT1*, *SF1*, *P450 arom*, and *Sox9* were examined in the transfected cell lines by the RT-PCR method.

To our surprise, it was found that the *WT1* was expressed at relatively high levels in the TS18-1 and TS18-2 cell lines (Fig. 4): no *WT1* expression was detected in the control non-transfected TMA-18 cells. On the other hand, no transcripts of *MIS*, *SF1* or *P450 arom* were detected in either the non-transfected cell line (TMA-18) or the transfected cell lines (TS18-1 and TS18-2) (Fig. 5).

Sox9, another putative target gene of *Sry* was expressed in both the TS18-1 and TS18-2 cell lines as well as in the non-transfected TMA-18 cell line (Fig. 5).

Expression patterns of WT1 in the developing gonad of the mouse

On the basis of the observation described in the previous section, that *WT1* expression was stimulated by the expression of exogenously-introduced *Sry* in XX ES cell lines, we hypothesized that *WT1* might be one of the direct target genes of *Sry*, up-regulated by SRY protein in the developing fetal testis.

Therefore we examined the patterns of *WT1* expression in the developing gonad during the period shortly after the expression of *Sry in vivo*. In the gonad of an XY fetus, *Sry* has a narrow window of expression: beginning on 10.5 dpc; peaking on 11.5 dpc; declining to a low level on 12.5 dpc; and being totally absent by 13.5 dpc (Koopman *et al.*, 1990; Hacker *et al.*, 1995).

We carried out quantitative RT-PCR analysis of the genital ridges of male and female gonads collected on 10.5-13.5 dpc (Fig. 6A,B). The results clearly demonstrated that the expression levels of *WT1* during these periods was invariably higher in the male fetal gonads than in the female gonads (Fig. 6B).

Western blot analysis of expression of WT1 protein in fetal gonads

Fetal gonads during the period of sexual differentiation were analyzed by Western blotting. The representative results are presented in Figure 7. Indifferent gonads and male fetal gonads exhibited strongly positive signals whereas the female gonads were only faintly positive, in agreement with the results of RT-PCR analysis.

Immunohistochemical localization of WT1 in the developing gonad

We examined the patterns of localization of *WT1* protein in 11.5-13.5 dpc fetal gonads by means of immunohistochemistry. In Figure 8 representative photomicrographs showing the localization of WT1 immunoreactivity in the male and female fetal gonads of 13.5 dpc are presented. Although the nuclei of the mesenchymal cells were generally stained both in the male and female fetal gonads, the nuclei of primordial Sertoli cells were stained more strongly than the mesenchymal cells in the male gonads, accounting for the high expression levels of *WT1* in the developing male gonad.

Discussion

In the present study, we succeeded in establishing XX ES cell lines, TS18-1 and TS18-2, where *Sry* was expressed in the functional linear mRNA form, and examined the expression of five potential target genes of SRY protein, i.e., *MIS*, *SF1*, *P450 arom*, *Sox9* and *WT1* in the transfected cell lines by the RT-PCR method. The most significant findings that emerged from the present series of experiments was that the unscheduled expression of *Sry* induced the expression of *WT1* in the transfected cell lines.

Capel and her co-workers (1993) transfected an epithelial cell line derived from mouse lungs with 13 kb genomic DNA fragment containing *Sry* ORF and the inverted repeat fused with the pBR vector. Although the transfected cell line SN18 expressed *Sry* transcripts at a high level, it was confirmed that they were circular and in non-functional form (Capel *et al.*, 1993). Since the formation of circular transcripts of *Sry* is due to the presence of inverted repeats at the 5' and 3' ends of ORF (Dubin *et al.*, 1995), we excised the Sry ORF so as to exclude the inverted repeat of the 5' end and joined it to the *CMV* promoter (Hirota *et al.*, 1996). The RT-PCR analysis done by using the primer set L1/L2 which is specific to the linear *Sry* transcripts established that the transfected ES cell lines indeed express functional *Sry* transcripts.

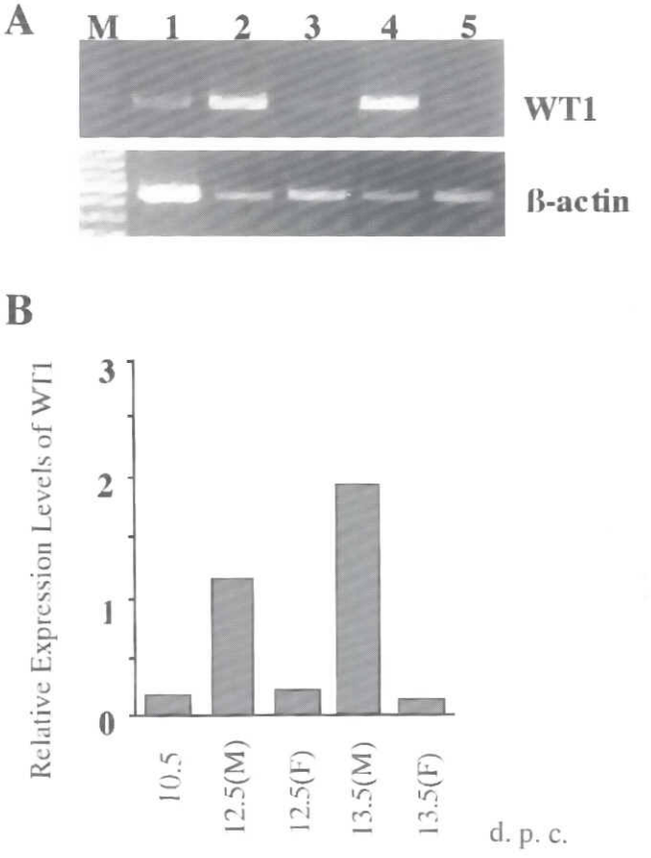

Fig. 6. *WT1* expression in fetal gonads. (A) *Electrophoretogram of the RT-PCR products of mRNA from the fetal gonads. Lane 1, an indifferent gonad (10.5 dpc). Lane 2 and 4, male gonad on 12.5 dpc and 13.5 dpc, respectively. Lane 3 and 5, female gonad on 12.5 dpc and 13.5 dpc, respectively.* **(B)** *Results of densitometric analysis of RT-PCR products of WT1 transcripts in male and female genital ridges collected on 10.5, 12.5 and 13.5 dpc. M, male; F, female.*

Cohen *et al.* (1994) also attempted to analyze the SRY action *in vitro*. They co-transfected CHO K1 cells with the plasmid construct which contains the 2.1-kb human SRY insert downstream of the cytomegalovirus early promoter, and another construct containing the CAT reporter gene driven by a 2.1-kb segment of the 5' regulatory region of the rat *fra-1* gene. Their results demonstrated that SRY enhanced the expression of the rat *fra-1* in the transiently-transfected cell lines (Cohen *et al.*, 1994). Since SRY proteins have been known to exhibit considerable species specific variations both structurally (e.g., Gubbay *et al.*, 1990; Su and Lau, 1993; Coward *et al.*, 1994; Payen and Cotinot, 1993,1994; Daneau *et al.*, 1995) and functionally (Koopman *et al.*, 1991; Hirota *et al.*, 1996), the system employed by Cohen *et al.* (1994), i.e., human SRY acting upon rat *fra-1* promoter sequence in Chinese hamster cells, is too complex to allow meaningful speculation about the function of SRY. In fact, it was found in a subsequent study that *fra1* is not expressed in fetal gonads at any stage at or around the critical period when *Sry* transcripts are present, excluding a role for *fra1* in sex determination and differentiation (Jeske *et al.*, 1996). Although the ES cells we used in the present series of experiments are not the cells in which *Sry* normally acts, our results are supported by our *in vivo* studies.

Curiously, *P450arom* expression which was induced by the exogenously introduced *Sry* construct in the Sertoli cell-derived TM-4 cells (Hirota *et al.*, 1996), was not induced in the *Sry*-transfected TS18-1 and TS18-2 cell lines. Similarly, MIS which is expressed in the TM-4 cells regardless of the expression of *Sry* (Hirota *et al.*, 1996) is not expressed in either the non-transfected TMA-18 nor the transfected cell lines.

Sox9 expression was observed in the non-transfected TMA-18 XX ES cell line as well as in the transfected cell lines. In mice, *Sox9* has been shown to be expressed at a low level in indifferent gonads of both XY and XX sex chromosome constitution. As gonadogenesis proceeds expression levels are gradually intensified in male gonads with the expression being localized to Sertoli cells. Conversely, in the female gonad, *Sox9* expression disappears after 12.5 dpc (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996). The sexually dimorphic patterns of *Sox9* expression suggested that *Sox9* might be involved in the hypothetical *Sry* cascade (Kent *et al.*, 1996; Morais da Silva *et al.*, 1996).

In TMA-18 cells, however, *Sox9* expression of a low level took place independently of *Sry* expression, in general agreement with the findings *in vivo*. It might be possible that function of a yet unidentified gene(s) located on the Y chromosome and activated in a temporally-regulated manner might be needed for the up-regulation of Sox 9 expression during the development of male gonads. Careful analysis of the patterns of *Sox9* expression in the *Sry*-transgenic XX mice will be of value to answer the question.

In our system, *WT1* expression was induced *de novo* by the action of the Sry transgene. In order to correlate the finding with the developmental events *in vivo*, we examined the patterns of *WT1* gene expression in fetal gonads during the period immediately after the expression of *Sry*. Quantitative RT-PCR analysis revealed that although both male and female fetal gonads express *WT1*, as reported earlier by Pelletier *et al.* (1991), a striking difference emerges in the amount of *WT1* transcripts between female and male gonads shortly after the window of *Sry* gene expression. Immunohistochemical analysis of the male and fetal gonads between 11.5 and 13.5 dpc revealed the presence of strong WT1 immunoreactivity in Sertoli cells, which become recog-

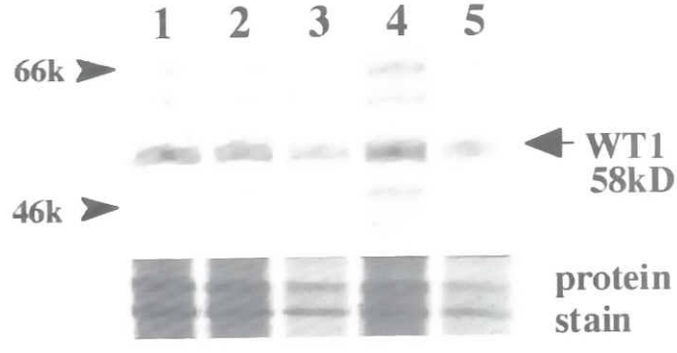

Fig. 7. Western blot analysis of *WT1* protein in developing male and female gonads. *Protein equivalent in amount to 3 fetal gonads was loaded in each lane. Lane 1, an indifferent gonad (10.5 dpc). Lane 2 and 4, male gonad on 12.5 dpc and 13.5 dpc, respectively. Lane 3 and 5, female gonad on 12.5 dpc and 13.5 dpc, respectively. Protein stain, two unidentified major bands stained with Sypro Orange are shown as the controls for the amount of protein loaded.*

nizable around 12.5 dpc in the primordial testes after the transient expression of *Sry*, probably accounting for the higher expression levels of *WT1* in the male fetal gonads.

Because *WT1* is expressed more in the lateral mesenchyme from 9.0 dpc before the onset of the surge of *Sry* expression, i.e., 10.5 dpc (Armstrong *et al.*, 1992), it was hypothesized that *WT1* is located upstream from *Sry* and essential for establishing the cellular environment for *Sry* expression (Koopman, 1995). Conversely, suggestions were made indicating that both SF1 and WT1 proteins might be active in a common pathway upstream of *Sry* (Ramkissoon and Goodfellow, 1996). In our system, however, *WT1* and *SF1* behaved independently of each other. Unfortunately, mice homozygous for null mutations at the *WT1* locus produced by gene targeting lack both kidneys and gonads and hence the effect of mutation in *WT1* upon *Sry* expression could not be determined (Kreidberg *et al.*, 1993).

Our results indicate that *WT1* is located downstream, rather than upstream, from *Sry*. *WT1* has been shown to be expressed in the developing kidney and in the genital ridge (Pritchard-Jones *et al.*, 1990; Pelletier *et al.*, 1991; Armstrong *et al.*, 1992). In the developing kidney, *WT1* is expressed in the condensed mesenchyme, renal vesicle and glomerular epithelium in cells undergoing the transition from mesenchymal cells to epithelial cells that were destined to form the tubular structures of the fetal kidney (Pritchard-Jones *et al.*, 1990; Armstrong *et al.*, 1992).

In the developing gonad, the formation of testicular cords is the first morphological sign of the differentiation of the testis, alignment of primordial Sertoli cells into cords around the germ cells being the first step of cord formation (reviewed in Hunter, 1995). Since the gonad and the kidney share a common mesodermal origin, it might be possible that *WT1* plays a critical role in both the tubular alignment of Sertoli cells in the testis and in the formation of ductal systems in the developing kidney.

It is not clear at present whether *WT1* is directly influenced by SRY protein in the *Sry*-transfected cell lines, or there are intermediary genes yet to be identified between *Sry* and *WT1*. There have been published reports, supporting the notion that *WT1* is directly regulated by *Sry*. For example, *WT1* promoter contains a consensus DNA binding sequence for *Sry*, i.e., 5'-AACAAT-3', and recombinant *Sry* protein was, in fact, found to bind to the AACAAT-containing oligonucleotide sequence of the WT1 promoter region (spanning from -241 to -214) by electrophoretic mobility shift assay (Denny *et al.*, 1992; Harley *et al.*, 1994).

In our previous study using the Sertoli-derived TM-4 cells the *Sry* transgene induced the expression of *P450arom*, whereas in the present series of experiments the same *Sry* gene construct induced the expression of *WT1* when transfected into the TMA-18 ES cells with an XX karyotype. Our results provide experimental proof that the cascade of genes mobilized by SRY may vary according to the differentiation status of the cells.

Careful studies using an appropriate combinations of *Sry* transgenes and cultured cell lines of different differentiation status and capabilities will give us important clues to understand the molecular mechanisms of SRY function within cells.

Materials and Methods

Animals and tissues

ICR mice were purchased from a local dealer (Sankyo Labo Service, Tokyo, Japan) and bred from the stock. The day when vaginal plugs were found was defined as 0.5 dpc *(days post coitum)*. The primordial gonads were collected from the fetuses between 10.5 and 15.5 dpc. Mesonephroi were carefully removed from the gonads on 12.5-15.5 dpc. When 10.5 and 11.5 dpc gonads were used, the mesonephroi could not be separated from the gonads and they were subjected as a whole to the RNA extraction procedures. The male fetal gonads were morphologically identified by the presence of testis cords under a binocular microscope at the time of dissection.

DNA constructs

Genomic *Sry* DNA was kindly provided by Prof. Robin Lovell-Badge (National Institute of Medical Research, London, U.K). *Sry* ORF was cut out from the genomic *Sry* DNA at *Xba* I sites excluding the inverted repeat located at the 5' end and fused to the *CMV* promoter sequence *(CMV/Sry)*, and digested with *NruI* to linearize the construct (Hirota *et al.*, 1996).

ES cell culture and electroporation

A mouse ES cell line with XX karyotype, TMA-18, was established from an embryo of 129 strain (Takagi, unpublished). Culture of the ES cells and transfection assays were performed as described by Wurst and Joyner (1993). Briefly, ES cells were maintained on Falcon tissue culture dishes (#3003) with embryonic fibroblast cells as feeder cells in Dulbecco's modified Eagle's medium with high glucose (DMEM high glucose; GIBCO/BRL, USA) containing 10 μM β-mercapto-ethanol, 1 mM non-essential amino acids, 1 mM sodium pyruvate (GIBCO/BRL), and 20% heat-inactivated fetal calf serum (JRH Biosciences, Australia). The cells were incubated at 37°C under a humidified atmosphere of 5% CO_2 in air. Approximately 2.0×10^7 ES cells in 800 μl of HEPES buffered saline (HBS) were electroporated in the presence of 50 μg of the DNA construct using a single pulse generated at the settings of 300 V and 800 μF in a model GTE-10 cell electroporation unit (Shimazu, Kyoto). Ten minutes after electroporation, the cells were distributed to three gelatin-coated Falcon tissue culture dishes (#3003). Selection of the successfully transfected cells relied on their ability to grow in a medium containing G418 (300 μg/ml; GIBCO/BRL) and LIF (10^3 Units/ml; GIBCO/BRL). The cells were cultured for 1 week; the resistant colonies were picked and subcultured.

Assay of cellular proliferation

Determination of cellular proliferation activities was performed using 5-Bromo-2'-deoxy-uridine (BrdU) labeling and a detection kit (Kit III, Boehringer Mannheim Biochemica), following the procedures provided by the manufacturer. Briefly, cells were placed in the wells of 96-well microtiter plates (#25860; Corning, USA) and incubated for 24 h at 37°C under a humidified atmosphere of 5% CO2 in air. Medium containing BrdU (110 μmol/l) was added and incubated at 37°C for additional 2 h. After incubation, the medium containing BrdU was removed and cells were washed twice with the fresh culture medium. After the last wash, cells were fixed with chilled 100% ethanol for 30 min at -20°C, washed 3 times with the culture medium, added with the nuclease solution, and incubated for 30 min at 37°C. Then the nuclease solution was removed and cells were washed 3 times with fresh culture medium. After removing the wash medium, anti-BrdU-POU Fab fragments (200 mU/ml) were added to the wells and incubated for 30 min at 37°C. After washing 3 times with the washing buffer, cells were incubated with peroxidase substrate solution for 2 min at room temperature. Absorbance of the samples was measured in a microtiter plate reader (model 700; Cambridge Biotech., U.K) at 405 nm with a reference wavelength at 490 nm.

RNA extraction and RT-PCR

Messenger RNA for the reverse transcriptase-mediated polymerase chain reaction (RT-PCR) was extracted from the cultures and fetal gonads of mice using Quickprep Micro mRNA Purification Kit (Pharmacia Biotech., Sweden) according to the manufacturer's instructions. The mRNA obtained was added to the reaction mixture for reverse transcription (RT) with or without reverse transcriptase (GIBCO/BRL). For the PCR analysis, 1-5 μg reverse transcribed cDNA was added to a 20 μl reaction mixture containing

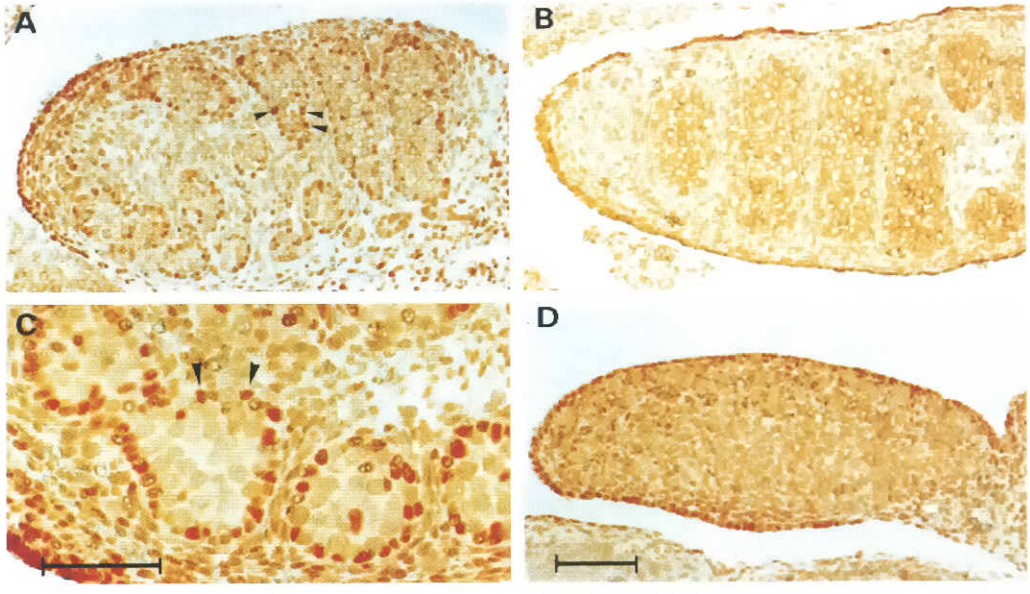

Fig. 8. Photomicrographs showing the localization of anti-*WT1* immunoreactivity in male and female fetal gonads. *Sections were stained with rabbit polyclonal antibodies against WT1 (0.1 μg/ml) or rabbit IgG (0.1 μg/ml) for the negative control.* **(A)** *A section of a male gonad on 13.5 dpc treated with the anti-WT1 antibody as the first antibody and horseradish peroxidase(HRP)-conjugated goat anti-rabbit IgG as the secondary antibody.* **(B)** *A section of a male gonad on 13.5 dpc treated with rabbit IgG and the HRP-conjugated goat anti-rabbit IgG as negative control.* **(C)** *A section of a male gonad on 13.5 dpc treated with anti-WT1 antibody as the first antibody and the HRP-conjugated goat anti-rabbit IgG seen at high magnification.* **(D)** *A section of a female gonad on 13.5 dpc treated with anti-WT1 antibody and the HRP-conjugated goat anti-rabbit IgG. Bar, 100 μm.*

250 μM dNTPs, 1 U Taq polymerase (rTaq; Takara, Kyoto), and 0.5 μM of each primer. PCR amplification was carried out for 26-35 cycles including a denaturation step for 1 min at 94°C, an annealing step for 1 min at a selected temperature between 50-65°C, and an elongation step for 1 min at 72°C in a thermocycler (Perkin Elmer Co. Ltd., USA). A 10 μl aliquot of the reaction mixture was electrophoresed on a 2% agarose gel. The procedure for the quantitative PCR was described elsewhere (Kawamata *et al.*, 1995).

Primers used to detect Sry transcripts

Sequences of the primers used to detect the *Sry* transcripts were as follows: 5'-GCCTTTATGCATAAGGAGTCAC-3' (5'-primer; L1) and 5'-GCTAACTGAAGGTCTGGGTATG-3' (3'-primer; L2) for the linear transcripts; 5'-CCTACTTACTAAACAGCTGAC-3' (5'-primer; C1) and 5'-TCTGTAAGGCTTTTCCACCT-3' (3'-primer; C2) (PrS and Pr4 designed by Cao *et al.* (1995)) for the circular form; 5'-AAGCGCCCCATGAATGCATTTATGGT-3' (5'-primer; B1) and 5'-ACACTTTAGCCCTCCGATGAGGCTGA-3' (3'-primer; B2) (Gubbay *et al.*, 1990) for the HMG box. The primer set specific to the linear transcripts was designed by the present authors.

Primers used to detect transcripts of potential Sry-target genes

The following primers were used to detect transcripts of the potential *Sry*-target genes: 5'-TCCTACATCTGGCTGAAGTGATATGGGAGC-3' (5'-primer) and 5'-CTCAGGGTGGCACCTTCTCTGCTTGGTTGA-3' (3'-primer) (Münsterberg and Lovell-Badge, 1991) for MIS; 5'-CCTGCCCAGCTGCCTGGAGAGCCAG-3' (5'-primer) and 5'-TTTTACCTGTATGAGTCCTGGTGTG-3' (3'-primer) for WT1; 5'-TGGTGTCCAGTGTCCACCCTTAT-3' (5'-primer) and 5'-TCCGTGCACGTGTAATGCTTGT-3' (3'-primer) (Stromstedt and Waterman, 1995) for SF1; 5'-TCGCTGAGAGACGTGGAGACCTGACGA-3' (5'-primer) and 5'-AGGCTGAAAGTACCTGTAGGGAACATTCT-3' (3'-primer) (Terashima *et al.*, 1991) for P450 arom; 5'-GTGGCAAGTATTGGTCAA-3' (5'-primer) and 5'-GAACAGACTCACATCTCT-3' (3'-primer) (9.5b and 9.5c of Kent *et al.*, 1996) for *Sox 9*.

Western blot analysis

Six fetal gonads were lysed in 20 μl loading buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 0.1% bromophenol blue) and boiled for 5 min. Aliquots of 10 μl were electrophoresed on a 7.5% polyacrylamide gel. Protein staining was carried out using SYPRO Orange (Molecular Probes, Oregon) as described by Steinberg *et al.* (1996). Observations were made using a model FLA-2000 image analyzer (Fuji Film, Tokyo) and Pictrography 1000 (Fujix, Tokyo). Then, the proteins were transferred onto a nitrocellulose filter (Immobilon Transfer Membrane, Millipore) in a Transblot Cell (Bio-Rad Laboratories, California) at 60V, 1.2 A. The filter was incubated with a blocking solution (PBS containing 5% skim milk) for 1 h at room temperature and treated with 0.1 μg/ml of the rabbit polyclonal antibodies against *WT1* (Santa Cruz Biotechnology, California) overnight at 4°C. After washing with PBS 3 times, the filters were incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, California) for 1 h at room temperature. The detection of AP activity was performed using 4-nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates (Sambrook *et al.*, 1989).

Immunohistochemistry

Fetal gonads were fixed with 4% paraformaldehyde in PBS for 30 min at 4°C, embedded in paraffin, and sectioned at 5 μm. After passing through a graded ethanol series, the sections were heated in a microwave oven below 95°C for 10 min in 0.01 M citric acid buffer to recover antigenicity. The specimens were then incubated with the blocking solution (PBS, containing 5% skim milk) for 1 h at room temperature and incubated with the rabbit polyclonal antibodies against *WT1* (0.1 μg/ml) or rabbit IgG dissolved in PBS (0.1 μg/ml) as a negative control overnight at 4°C. After washing with PBS 3 times, the specimens were incubated with a 1:1000 dilution of horseradish peroxidase(HRP)-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, California) in PBS for 1 h at room temperature. Detection was performed by an HRP reaction using diaminobenzidine [DAB, 0.2 mg/ml in 0.1 M Tris-HCl (pH 7.5)] and hydrogen peroxide (final concentration of 0.03%) as substrates (Sambrook *et al.*, 1989). For microscopic observations, a model AX-80T microscope (Olympus) was used.

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