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The human SRY protein is present in fetal and adult Sertoli cells and germ cells

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ABSTRACT Sex determination in mammals is controlled by the Y chromosome located *SRY* gene. Despite recent advances towards understanding the mechanisms that regulate sex determination in mammals, the expression profile of the SRY protein in human tissues is unknown. To localize the SRY protein and determine its cellular distribution, we prepared monoclonal antibodies (mAb) against the recombinant SRY protein. One antibody, LSRY1.1, recognizes a SRY-specific epitope and was used to localize the protein in different cells and tissues. The mAb recognizes a protein of 27 kDa in total lysates of HeLa SRYB3 cells. Immunocytochemical staining showed a nuclear localization of the protein. Immunohistochemical studies performed on gonadal tissue of a fetus, a one month-old boy and an adult man, demonstrated the presence of SRY protein in the nucleus of Sertoli and germ cells. In addition two 46,XX SRY(+) males had the SRY protein in their gonadal tissues. All other samples were negative, including all female tissue studied and the testis of a 46,XX SRY(-) male. The presence of SRY protein in fetal and adult gonadal tissues including germ cells suggests that SRY may have other male-specific functions in addition to sex determinism.

KEY WORDS: *SRY protein, monoclonal antibody, testis, germ cells, Sertoli cells*

Introduction

The mechanism regulating mammalian sex determination depends on a molecular switch that is genetically controlled by a gene on the Y chromosome. The factor controlling this molecular switch has been termed Testis Determining Factor (TDF) in humans and Tdy in mice. Using 46,XX males that carried a portion of the Y chromosome translocated to an X chromosome, the *SRY* (Sex determining region Y chromosome) gene was cloned (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). Several lines of evidence indicate that *Sry* corresponds to TDF: 1) individuals with a 46,XY karyotype but presenting a female phenotype with complete gonadal dysgenesis, carry mutations in the *SRY* open reading frame (Berta *et al.*, 1990; Jäger *et al.*, 1990). 2) In normal developing mouse embryos, expression of *Sry* occurs in the genital ridge around 10.5-12.5 days post coitum (dpc), just before the differentiation of the genital ridge into testis (Koopman *et al.*, 1990). 3) Mice with a female karyotype developed testis when a 14 kb fragment of DNA containing the *Sry* gene was introduced as a transgene, demonstrating that Sry is both necessary and sufficient to determine testis formation (Koopman *et al.*, 1991).

The SRY protein has a 79-amino acid HMG (High Mobility Group) domain, a conserved motif present in many DNA-binding proteins including several transcription factors (Ner, 1992). SRY binds to DNA and induces substantial bending in target DNA sequences, suggesting that SRY may act as a transcriptional regulator of other downstream sex-determining genes (Ferrari *et al.*, 1992; Giese *et al.*, 1992; Van der Wetering and Clevers, 1992).

In mice, fetal expression of *Sry* is limited to male gonadal somatic tissues and does not require the presence of germ cells (Koopman *et al.*, 1990). High levels of *Sry* mRNA are also found in adult mouse testis, but this transcript has a circular structure, is not

Abbreviations used in this paper: SRY, Sex-determining region Y chromosome; TDF, Testis Determining Factor; HMG, High Mobility Group; mAb, monoclonal antibody; GST, glutathione S-transferase; Sox protein, SRY-box containing protein; AMH, anti-Müllerian hormone.

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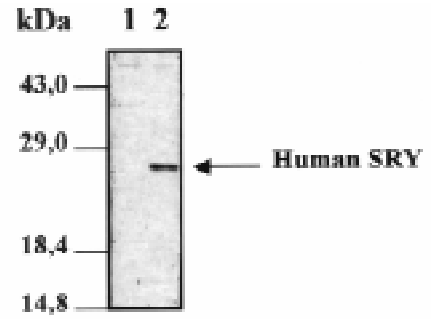

Fig. 1. Western blotting on Hela 7A and HeLa SRYB3 cells. *Total extracts were prepared as described in Material and Methods. Extracts were electrophoresed, transferred to nitrocellulose membranes and screened with the LSRY mAb. Lane 1, HeLa 7A total extract; lane 2, total extract of HeLa SRYB3. A black arrow shows the position of the SRY protein. Molecular weight of standards are given in kDa. The human SRY has a molecular weight of approximately 27 kDa.*

associated with polysomes and is therefore probably not translated (Sinclair *et al.*, 1990; Rossi *et al.*, 1993; Cao *et al.*, 1995). A high level of expression of mRNA has been observed in murine meiotic and post-meiotic germ cells (Rossi *et al.*, 1993). A similar pattern of mRNA expression has been described for the human *SRY* gene, and the protein has been demonstrated to be present in the male genital ridge before testis formation (Clépet *et al.*, 1993; Poulat *et al.*, 1995). In the present work, we describe the production of a monoclonal antibody directed against SRY and demonstrate the localization of this protein in cell lines and in human gonadal tissue. Our results show that SRY expression is restricted to the nucleus of gonadal tissue. Furthermore, the data demonstrate that SRY is expressed not only during fetal development, a time when it is postulated it exerts its regulatory action, but it is also present in adult gonadal tissue, suggesting that SRY may have additional functions.

Results

Production of monoclonal antibodies against the recombinant SRY protein

In order to produce monoclonal antibodies, female Balb/c mice were immunized with 50 μg of recombinant SRY protein, purified from bacterial extracts (Smith and Johnson, 1988). Fusion of spleen cells from the SRY immunized mouse with NSO/2 murine myeloma cells lines gave 12 SRY-positive hybridomas screened by ELISA using recombinant SRY protein as antigen. After two cycles of subcloning, a hybridoma which produced a monoclonal antibody specific for the SRY protein was selected (mAb LSRY 1.1). The mouse isotype immunoglobulin of LSRY 1.1 was IgG-2a.

According to the published sequence of the *SRY* gene, the deduced molecular weight of the SRY protein corresponds to 25 kDa (Sinclair *et al.*, 1990). This protein contains an HMG domain which is present in all the proteins of the *Sox* gene family. Since we used the complete sequence of the SRY protein as an immunogen, constructs containing GST-SRY, GST-HMG or the HMG domain alone were used to determine the specificity of the LSRY 1.1 mAb. The LSRY 1.1 monoclonal reacted with the complete GST-SRY protein only (data not shown) establishing that this antibody does not recognize the HMG domain.

Cellular localization of the SRY protein

To confirm the specificity of the monoclonal antibody, we performed western blots on total lysates of HeLa SRYB3 cells, a stable cell line transfected with the human *SRY* gene under the control of the SV-40 promoter (Poulat *et al.*, 1995). As control, we used total extracts of normal HeLa 7A, a cell line derived from a female human cervix carcinoma, which does not carry the Y chromosome and thus it does not express the SRY protein. Figure 1 shows that the LSRY 1.1 monoclonal antibody detects a protein of approximately 27 kDa from lysates prepared from HeLa SRYB3, (Fig. 1, lane 2), a size corresponding to the expected molecular weight for SRY (Desclozeaux *et al.*, 1998). No reaction was observed with total extracts from the HeLa 7A cell line (Fig. 1, lane 1). LSRY 1.1 monoclonal antibody detects a 25 kDa protein in Western blots using the recombinant SRY protein as a target (data not shown). The difference in size from 25 kDa and 27 kDa between the recombinant SRY protein and the protein obtained from the cell line, corresponds to unphosphorylated and phosphorylated SRY proteins respectively (Desclozeaux *et al.*, 1998).

Flow cytometry analysis of permeabilized and non-permeabilized HeLa B3SRY cells showed that LSRY 1.1 recognizes an intracellular protein (data not shown). To determine the intracellular localization of the SRY protein, fixed and permeabilized HeLa SRYB3 cells and control HeLa 7A cells were labeled by indirect immunofluorescence with the LSRY 1.1 mAb and observed by confocal fluorescent microscopy. Figure 2 shows that the LSRY1.1 antibody reacts with an antigen located in the nucleus of the HeLa B3SRY cells (Fig. 2d). No labeling can be observed in the control HeLa 7A cells (Fig. 2b) or in control experiments done with the second antibody (Fig. 2a and c). The heterogeneous level of staining observed in the HeLa SRYB3 cells could reflect cells at different stages of the cell cycle.

Expression of SRY in human tissues

The presence of *SRY* mRNA transcripts has been demonstrated in the human cell lines, DU145 and HepG2 as well as in different human tissues (Clepet *et al.*, 1993; Tricoli *et al.*, 1993). However, the presence of the SRY protein has not been clearly demonstrated. We used the LSRY 1.1 mAb to screen for the presence of SRY protein in the DU 145 and Hep G2 cell lines. In spite of the fact that the mRNA was clearly demonstrated by RT-PCR (data not shown), no protein could be detected with our antibody by immunoblot, confocal microscopy or flow cytometry (data not shown).

In order to investigate the presence of SRY protein in human tissues, immunohistochemical analysis was performed on different tissues with the LSRY 1.1 mAb. First we investigated the presence of SRY protein in testis tissue from a 26 week old fetus. LSRY 1.1 mAb presented a positive reaction (brown spot) in the nucleus of Sertoli cells and germ cells (Fig. 3b). No staining was observed in ovarian tissue (Fig. 3a) of the same age. A similar pattern of SRY expression was observed in the Sertoli and germ cells from a 1 month old boy (Fig. 3c). Furthermore, testis tissue from a normal adult male (32 years old), showed a positive labeling with the LSRY 1.1 mAb in the nuclei of Sertoli cells as well as in round spermatids (Fig. 3d). No staining was observed in liver, kidney or lung (data not shown).

Immunochemical studies were performed also in gonadal tissues of three sex reversal patients with a 46,XX chromosome

complement who had been previously investigated for the presence or absence of the *SRY* gene (Abbas *et al*., 1993; Vilain *et al*., 1994).

Patient 1 was a 46,XX *SRY*-negative male as demonstrated after PCR analysis for the presence of the *SRY* transcript in different tissue, including the gonads (Vilain *et al*., 1994). Figure 3e shows that in this case there was no reaction with the LSRY 1.1 monoclonal antibody.

Figure 3f shows the reaction of mAb LSRY1.1 with gonadal tissue of a second 46,XX true hermaphrodite that contains a 35 Kb Y-specific sequence including the *SRY* gene on a X chromosome. DNA from this individual was used to isolate the *SRY* gene (Sinclair *et al*., 1990). This patient presented both an ovotestis and a testis. In the ovotestis, we observed that the nuclei of granulosa-like cells and of the ovogonia were positive with this antibody.

Finally, the third patient was a 46,XX SRY(+) male. This patient had a portion of Y chromosome on the X chromosome and gonadal tissue that was identified as testis. The LSRY1.1 antibody recognized an antigen in the nuclei of Sertoli and germ cells as demonstrated for the normal male, (Fig. 3g).

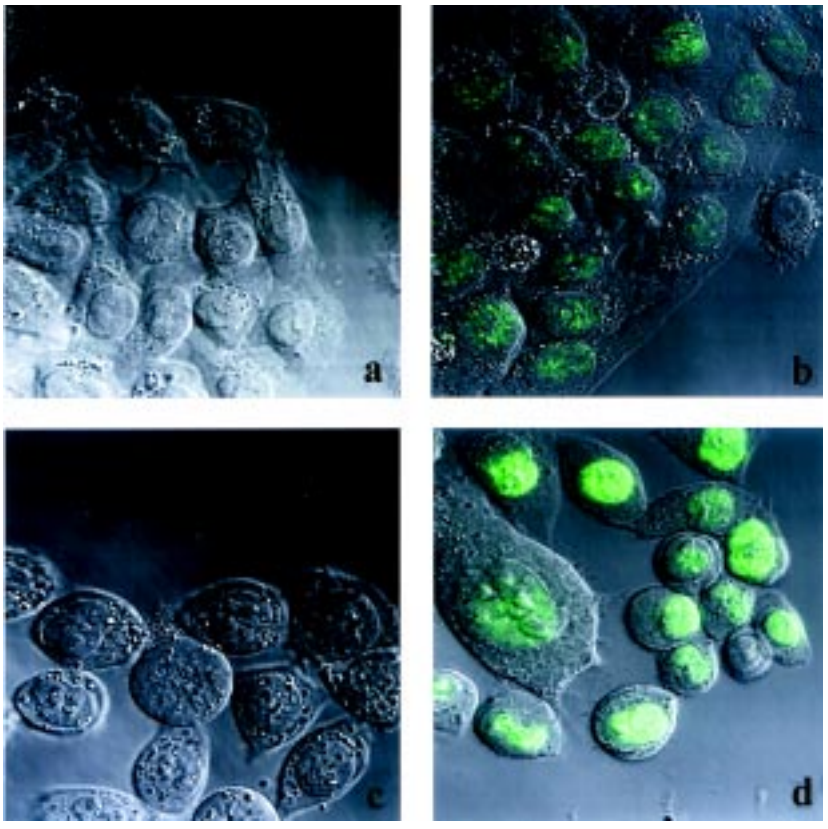

Fig. 2. Cellular localization of SRY protein. *Immunochemical analysis was done in HeLa 7A and HeLa SRYB3 cells fixed and permeabilized, and then stained with monoclonal antibody LSRY 1.1. (a and c) Controls of HeLa 7A and HeLa SRYB3 cells respectively stained with second antibody only. HeLa SRYB3 cells show a high level of expression of SRY protein in the nuclei.* **(a)** *HeLa 7A cells negative control.* **(b)** *HeLa 7A cells stained by monoclonal antibody.* **(c)** *HeLa SRYB3 cells negative control. Using a confocal microscope, only HeLa SRYB3 cells display green spots of fluorescence which correspond to a nuclear localization of SRY protein* **(d)**.

Discussion

The master gene in the mammalian sex determination pathway is *SRY*. There are a relatively large number of studies describing expression of the *SRY* transcript in a number species, however the presence of the SRY protein has not been well-defined. In the present study, the localization of the SRY protein was demonstrated, in both foetal and adult tissues, using a mAb prepared against SRY. Since monoclonal antibodies were prepared using the complete recombinant SRY protein as immunogen, it was possible that some of the selected hybridomas could produce antibodies directed against the HMG box, a domain that SRY shares with other proteins of the SOX family. One of the mAb obtained here (LSRY1.1), showed no reaction when tested against several proteins of this family or against an isolated HMG domain. Western blot analysis performed with this antibody against the isolated recombinant protein as well as lysates of HeLa SRYB3 cells (a cell line of female origin transfected with the *SRY* gene), showed that the antibody recognizes a 25 kDa and a 27 kDa protein, the expected sizes for the unphosphorylated and phosphorylated proteins respectively (Poulat *et al., 1995*; Desclozeaux *et al*., 1998).

The monoclonal antibody was used to analyze endogenous *SRY* expression in several human cell lines. In spite of the presence of *SRY* transcripts, no protein was detected in the DU145 cell line, a cerebral metastasis of a human prostate carcinoma, and in the HepG2 cell line, a human hepatocarcinoma (Clepet *et al*., 1993; Tricoli *et al*., 1993). The SRY protein was present in HeLa SRYB3 cells as demonstrated by both Western blotting and immunofluorescence. Consistent with the hypothesis that SRY may function as a transcriptional regulator, staining was observed in cell nuclei.

In normal human testis, the SRY protein was observed in gonads from a 26 week old fetus, testis from a 1 month old boy and in adult testis. This suggests that SRY protein is present from the period of testicular formation early in fetal life until adult testis. This contrasts with the profile of expression of murine *Sry* where transcripts are present from 10.5 dpc until 12 dpc. The expression profile in man is similar to SRY expression in sheep gonads, were expression of *SRY* mRNA is maintained from the moment of testis determination until adult life (Payen *et al*., 1996).

At all ages, the human SRY protein was present in the nuclei of both Sertoli and germ cells. In adult tissues, the protein was present in round spermatids. These observations are similar to the findings of Rossi *et al.* (1993), who described the expression of murine *Sry* in meiotic and post-meiotic germ cells. Since, 46,XY individuals who carry mutations in the *SRY* gene are sex-reversed females, with gonads that consist of a streak of fibrous tissue lacking germ cells, one cannot exclude the possibility that SRY may have an other male-specific functions in addition to sex determination.

The presence of SRY protein was investigated in gonads obtained from three sex-reversed individuals presenting a 46,XX chromosomal complement and a male phenotype or female with genital ambiguities (true hermaphrodite). Two individuals carried a

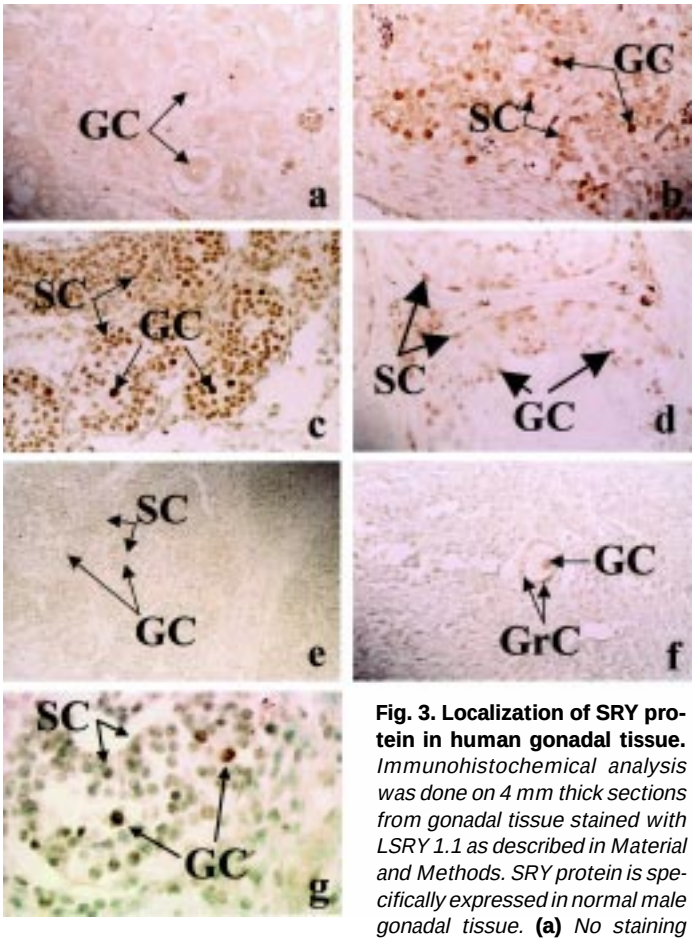

Fig. 3. Localization of SRY protein in human gonadal tissue. *Immunohistochemical analysis was done on 4 mm thick sections from gonadal tissue stained with LSRY 1.1 as described in Material and Methods. SRY protein is specifically expressed in normal male gonadal tissue.* **(a)** *No staining was observed in the ovary of a fetus of 26 weeks of embryonic life. Brown spots are observed in the nuclei of Sertoli cells (SC) and germ cells (GC) in testes from* **(b)** *a fetus of 26 weeks of embryonary life,* **(c)** *a 1 month-old boy,* **(d)** *a 32 year-old adult and* **(g)** *a 46, XX SRY(+) male.* **(f)** *SRY is also expressed in the ovotestis of a male 46, XX SRY(+) true hermaphrodite in the granulosa cells (GrC) and in the germ cell.* **(e)** *SRY expression was neither detected in the testis of male 46, XX SRY(-). Magnification was: a, b, c, d and f: x400; e: x200; g: x600.*

portion of the Y chromosome including the *SRY* gene on an X chromosome. One is a XX male and the other a XX true hermaphrodite. The third male was a 46,XX individual who did not carry the *SRY* gene (Vilain *et al.*, 1994). The immunochemical results clearly demonstrated the presence of the antigen in the gonad of the two *SRY*(+) individuals and the absence of SRY protein in the *SRY*(-) male. In the 46,XX *SRY*(+) true hermaphrodite with an ovotestes, positive staining was observed both in the testicular portion of the gonad and also in some regions of the ovarian tissue. Brown spots were observed in both the ovocyte and also in granulosa-like cells surrounding the ovocyte. This result is consistent with the hypothesis that Sertoli and granulosa cells share a common cell progenitor, the colleomic epithelium cells (Barber, 1988). In the presence of the testis determining gene, *SRY*, these progenitor cells differentiate to become Sertoli cells. The presence of SRY protein in granulosa-like cells may at first seem contradictory, however, there are a number of hypotheses that may explain why these cells have not differentiated into Sertoli cells even though the SRY protein is present. The true hermaphrodite analyzed in this study, carries the *SRY* gene on the short arm of her paternal X chromosome (Abbas *et al.*, 1993). This case and her 46,XX male brother carry only 35 kilobases of the Y chromosome including the *SRY* gene, translocated into the pseudoautosomal region (Sinclair *et al.*, 1990; Abbas *et al.*, 1993). Incomplete testis determination of these individuals is considered to be the result of inactivation of the translocated X chromosome. In a previous study, cells from a 46,XX *SRY*(+) male were analyzed, and the translocated X chromosome was observed to be late replicating in approximately 50% of cells (Fechner *et al.*, 1994). As the late-replicating chromosome is presumed to be the inactive X chromosome, selection of cells in which the Y-bearing X chromosome has been inactivated may play a role in the incomplete testis determination in 46,XX true hermaphroditism. This mechanism could result in insufficient SRY protein, necessary to induce completely Sertoli cell formation. Dosage effects appear to be an important cause of sex reversal (Bardoni *et al.*, 1994). X-inactivation may also result in the disruption of the timing of *SRY* expression. The timing of expression of the murine *Sry* gene appears to be critical for normal testis determination (Eicher *et al.*, 1995).

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), high glucose DMEM, trypsin, penicillin, streptomycin and glutamine were purchased from GIBCO-BRL (Life Tecnologies, France). Foetal Calf Serum (FCS), Glutathione agarose, reduced glutathione, IPTG, thrombin, Triton X-100, Tween-20 and complete Freund's adjuvant were purchased from Sigma (France). Goat anti mouse polyclonal antibody conjugated to FITC was obtained from DAKO (France). Goat anti-mouse polyclonal antibody conjugated to horseradish peroxidase was obtained from BIOSYS (Compiegne-France). ECL detection system was obtained from Amersham (Buckinghamshire, England).

Cell lines

The human cervix carcinoma, HeLa 7A, was obtained from the American Type Culture Collection. These cells were cultured in DMEM containing 10% FCS, 100 I.U. penicillin, 100 mg/ml of streptomycin, and 2 mM glutamine. HeLa SRYB3, a cell line that expresses *SRY* under the control of the SV-40 promoter was a kind gift from Dr. Philippe Berta (Centre de Recherche de Biochimie Macromoléculaire, Montpellier, France); (Poulat *et al.*, 1995). HeLa SRYB3 cells were cultured in high glucose DMEM, supplemented with 10% FCS, 100 I.U. penicillin, 100 mg/ml of streptomycin, and 2 mM glutamine.

Production of the recombinant SRY

The SRY open reading frame was cloned into the pGEX-2T expression vector (Smith and Johnson, 1988; Sinclair *et al.*, 1990). The protein was produced as a fusion protein with Glutathione-S-transferase (GST), that contains a site of hydrolysis for thrombin.

E. coli bacterial strain HP101 was transformed with the vector containing the fusion protein as previously described by Smith and Johnson, (1988). Cells were grown at 30°C and induced by IPTG (0,1 mM) for 2 h. The cells were pelleted at 2,500xg and then lysed by sonication in PBS 1% Triton X-100 (10 mM phosphate, 150 mM NaCl, pH 7.2).

Protein purification

SRY recombinant fusion protein was purified by affinity chromatography on a column of Glutathione-agarose beads. The lysates containing recombinant protein were run through the column and then the column was washed with PBS 1% Triton X-100. The fusion protein was eluted by competition with 50 mM TRIS containing 5 mM reduced glutathione pH 8.0.

Recombinant SRY protein was hydrolyzed from GST by treatment with thrombin at room temperature in 50 mM TRIS pH 8.0, 150 mM NaCl and 2.5 mM $CaCl_2$. The solution containing both proteins was passed again through the Glutathione-agarose column and the recombinant SRY was obtained as the eluate. Protein purity was assessed by 10% SDS-PAGE after staining with Coomassie Brilliant blue.

Antibody production

Balb/c female mice were immunized with three weekly injections of 50 µg each of recombinant SRY in complete Freund's adjuvant. Antibodies titers were checked four weeks after the first injection. The mouse presenting the highest antibody titer was sacrificed and spleen cells were fused with NSO/2 murine myeloma cells using standard procedures (Köhler and Milstein, 1976). SRY reacting hybridomas were screened by an ELISA using the recombinant SRY as antigen.

Immunofluorescence assay

Immunofluorescence was performed on adherent HeLa 7A and HeLa SRYB3 cells grown on coverslips. Cells were fixed with 3.2% paraformaldehyde in PBS and permeabilized in PBS 0.05% Triton X-100, and washed with PBS. Immunofluorescence was done by incubating the cells for 30 min at room temperature with an appropriate dilution of the antibody, then washed in PBS 2% FCS, and incubated for 30 additional min with a FITC-labeled goat anti mouse IgG antibody. The coverslips were mounted and observed on Carl Zeiss confocal microscope.

Immunohistochemistry

Gonadal tissues were obtained by extraction (in accordance with the Comité Consultatif National d'Ethique Pour les Sciences de la Vie et de la Santé, Ref AB/hd N° 90-293, July 1990). Formalin fixed tissues were embedded in paraffin and sections of 4 mm were cut on a microtome. After deparaffinization and rehydration, the selected sections were submitted to microwave oven retrieval (immersed in 0.1 mM citrate buffer pH 6.0), 2x5 min at 95°C.

Immunostaining was done in a DAKO hem MATE TM system using the anti-SRY mAb as a first antibody and a secondary antibody (mixture of anti-mouse and anti-rabbit biotinylated immunoglobulins, DAKO), followed by peroxidase conjugated streptavidin. Antigen localization was done after by reaction of diaminobenzidine and H_2O_2. Nuclei were detected after 1 min of green methyl coloration.

Western blotting

Cellular lysates were prepared as follows: cells washed with cold PBS were lysed directly in Laemmli sample buffer at 95°C (40 mM TRIS-HCl, pH: 6.8, 1% SDS, 50 mM DTT, 7.5% Glycerol, 0.3% bromophenol blue) and the lysate passed through a tuberculin needle by DNA binding protein extraction. The samples were then heating at 95°C for 5 min and then centrifuged at 12000xg for 5 min at 4°C. Proteins were separated on a 15% SDS-PAGE and transferred to nitrocellulose. Free sites on the nitrocellulose sheets were saturated in PBS-Tween (PBS with 0.1% Tween-20) for 1.5 h, and incubated overnight at 4°C with LSRY 1.1 monoclonal antibody in PBS-Tween. After 3 washes in PBS-Tween, the nitrocellulose sheets were incubated for 1 h with goat anti-mouse IgG peroxidase-labeled antibody diluted appropriately in PBS-Tween. Antibody detection was carried out by using the ECL detection system.

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